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Volatile induction of infected and neighbouring uninfected plants potentially influence attraction/repellence of a cereal herbivore

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Abstract

Pathogen infection can induce plant volatile organic compounds (VOCs). We infected 'McNeal' wheat and 'Harrington' barley with a *Fusarium* spp. blend (*F. graminearum*, *F. avenaceum* and *F. culmorum*). Both cereals had the greatest VOC induction 14 days after pathogen innoculation, only slightly lower induction occurred at 7 days, but displayed no induction at 1 days. The induced VOC bouquet for both cereals included six green leaf volatiles (GLVs; e.g. (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate), four terpenes (linalool, linalool oxide, (*Z*)-*β*-ocimene and (*E*)-*β*-caryophyllene) and benzyl acetate. Neighbouring, uninfected individuals of both cereals had significant VOC induction when exposed to an infected, conspecific plant. The temporal pattern and VOC blend were qualitatively similar to infected plants but with quantitative reductions for all induced VOCs. The degree of neighbouring, uninfected plant induction was negatively related to distance from an infected plant. Plant VOC induction in response to pathogen infection potentially influences herbivore attraction or repellency. Y-tube tests showed that herbivorous female and male *Oulema cyanella* Voet. (Chrysomelidae: Coleoptera) were significantly attracted to (*Z*)-3-hexenal and (*Z*)-3-hexenyl acetate at 300 and 1500 ng/h but were repelled by both GLVs as well as (*Z*)-*β*-ocimene and linalool at 7500 ng/h. These *O. cyanella* behavioural responses were significantly at higher concentrations than those emitted by single plants with pathogen-induced VOCs, so adults might only be able to respond to a dense group of infected plants. Also, *O. cyanella* dose responses differ from the previously tested congeneric *O. melanopus* (cereal leaf beetle), which was attracted to three VOCs induced by *Fusarium* infection of maize, barley and wheat. Future behavioural tests may indicate whether different herbivore dose responses measured with each VOC singly can help to predict attraction or repellency to injured and uninjured VOC bouquets from different host plant species.

Introduction

Plant species are vulnerable to attack by a variety of organisms during their life. However, immobile plants are not merely passive victims to attack (Whittaker and Feeny 1971; Karban and Baldwin 1997; Rasmann et al. 2005; Dicke et al. 2009). To protect themselves, plants have evolved an arsenal of physical and

chemical defences (Kessler and Baldwin 2002; Arimura et al. 2005; Schoonhoven et al. 2005). The evolution of specific VOCs has been suggested to be associated with protection from or tolerance of abiotic stresses (Peňuelas and Llusià 2003; Holopainen and Gershenzon 2010) or as defences against pathogens and/or herbivores (Arimura et al. 2005; Halitschke et al. 2008; Dicke et al. 2009; Vickers et al. 2009). There are many types of VOCs: terpenes, fatty acid derivatives, benzenoids, phenyl propanoids and amino acid-derived metabolites (Baldwin et al. 2006; Dudareva and Pichersky 2008). Plant VOCs can be constitutively expressed, while those quantitatively or qualitatively induced after herbivory are often called herbivore induced plant volatiles (HIPVs) (Van Den Boom et al. 2004; Turlings and Ton 2006; Dicke et al. 2009; Piesik et al. 2010, 2011a). Plant chemical and VOC defence induction after mechanical injury, chewing insect herbivory and necrotrophic pathogen infection is usually regulated by jasmonic acid, while biotrophic pathogen infection and sucking arthropod herbivory are usually regulated by salicylic acid (Pieterse et al. 2009).

Plant-induced VOC defensive functions include directly deterring herbivores (De Moraes et al. 2001; Kessler and Baldwin 2001; Laothawornkitkul et al. 2008; Unsicker et al. 2009), indirectly attracting natural enemies of attackers (Turlings et al. 1990; McCall et al. 1993; De Moraes et al. 1998; Arimura et al. 2005; Rasmann et al. 2005; Mumm et al. 2008) and priming defences of uninjured organs on the same plant (Frost et al. 2007; Heil and Silva Bueno 2007; Rodriguez-Saona et al. 2009) or neighbouring uninjured plants (Engelberth et al. 2004; Baldwin et al. 2006; Heil and Karban 2010). GLVs can be responsible for defence preparation on uninjured regions of an injured plant (Farag and Paré 2002; Arimura et al. 2005; Shiojiri et al. 2006; Frost et al. 2007). Also, GLVs, terpenes and methyl salicylate can stimulate uninjured plant-defensive responses (Shulaev et al. 1997; Dicke and Bruin 2001; Engelberth et al. 2004; Baldwin et al. 2006; Ton et al. 2007; Arimura et al. 2009) and VOC induction (Piesik et al. 2010, 2011a, b). Thus, uninjured plants can prime their metabolic machinery without investment costs of actual defence induction, in preparation for more efficacious defence if attacked in the future (Baldwin et al. 2006; Kessler et al. 2006; Frost et al. 2007; Rodriguez-Saona et al. 2009; Unsicker et al. 2009). Yet, a caveat of caution needs to be pointed out concerning ecological and evolutionary aspects of VOC induction. For the evolution of VOC induction, whether natural selection acts on the VOC sender (emitting plant) and/or receiver (pathogen, parasite, herbivore natural enemies, herbivores themselves and uninjured conspecific and heterospecific plants) has rarely been experimentally demonstrated (Kessler and Heil 2011). Also, concerning the ecological significance of VOC induction, there is still a lack of studies that demonstrate clear fitness benefits and costs to VOC-inducing plants (Kessler and Heil 2011).

Besides herbivores, fungi are a major group of organisms that are often pathogenic to plants, although a single fungus species is not always pathogenic to all plant hosts (Redman et al. 2001). Fungal growth substantially damages food and animal feeds. These forms of food damage can be manifested as general spoilage, reduced nutritional quality, mycotoxin formation and production of potentially allergenic spores (Schnurer et al. 1999). Two fungal pathogen pests, *Fusarium graminearum* Schwabe Gr1 and *F. culmorum* (W. G. Smith) Sacc., are considered the primary causal agents of cortical (foot) rot disease of wheat (Cook 1980), while *F. avenaceum* (Fr.) Sacc. is parasitic on wheat (Burgess 1981). Plants infected by *Fusarium* spp. often develop severe foot or crown rot diseases (Dodman and Wildermuth 1987). Plants have a tremendous capacity to counteract pathogens by reprogramming their gene expression and metabolism (De Vos and Jander 2010). Pathogen infection can also result in qualitative or quantitative plant VOC induction (Piesik et al. 2007, 2011a,b; Leitner et al. 2008; Yi et al. 2009).

The purpose of the current research was to examine the VOC induction responses of two cereal grasses, wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) to a blend of three *Fusarium* spp. (*F. graminearum*, *F. culmorum* and *F. avenaceum*). We measured induction at three intervals after plant inoculation with *Fusarium* spp. to examine whether induction was rapid (1 day) and whether the degree of induction increased over time (7 and 14 days). Uninfected plant VOC induction was measured from plants 1 and 3 m from a conspecific-infected plant. This was conducted to examine how the degree of induction decreases as distance from an infected plant increases and whether this reflects changes in infected plant VOC induction over time. Finally, we conducted behavioural experiments to test the orientation responses of an herbivorous cereal beetle, *Oulema cyanella* Voet. (Chrysomelidae: Coleoptera). Specifically, we tested *O. cyanella* dose responses to each of six VOCs singly, which included three GLVs, two terpenes and benzyl acetate. These six VOCs were induced at the highest concentrations from both barley and wheat infected by *Fusarium spp.* in the current study. Thus, we sought

to explore how attack by one biotic agent (pathogen infection) affects cereal grass VOC induction and possibly influence responses by a second biotic agent (herbivore) by studying dose-dependent responses to some of the induced VOCs. A congener of our study beetle is the cereal leaf beetle, *O. melanopus* (Piesik et al. 2011b); this species is the most important wheat pest in many European countries (Dimitrijević et al. 1999) and now an important cereal pest in North America (Ihrig et al. 2001; Buntin et al. 2004). Three of the induced VOCs from *Fusarium* spp. infection of barley and wheat that were tested with *O. cyanella* in the current study were also induced by *Fusarium* spp. infection of maize and tested for *O. melanopus* dose responses (Piesik et al. 2011b).

Materials and Methods

Plant culture

The study was performed at the Plant Growth Centre at University of Technology and Life Sciences (Bydgoszcz, Poland) in the spring of both 2009 and 2010. Hard red spring wheat variety 'McNeal' (*T. aestivum*) and malting barley variety 'Harrington' (*H. vulgare*) plants were planted and grown together in the same greenhouse bay at the same time with supplemental light and ambient humidity. The photoperiod was 16:8 (day/night). Daytime temperature was 22 ± 2°C, and the overnight temperature was 18 ± 2°C. Two plants were grown per pot (ø20 cm, height 25 cm) in sterilized soil. The plants were watered four times weekly and fertilized with Peters® General Purpose Fertilizer (J.R. Peters Inc., Allentown, PA, USA) at 100 ppm in aqueous solution twice each week as part of the regular watering schedule. Fertilizing commenced when the plants reached the third leaf stage (BBCH 32).

Fusarium preparation

Cultures of *F. avenaceum* (Fr.) Sacc., *F. culmorum* (W. G. Smith) Sacc. and *F. graminearum* Schwabe were used to prepare a fungal pathogen blend to infect our study cereals. These species were found and isolated from the wheat roots and crown and deposited at Department of Phytopathology (University of Technology and Life Sciences) collection on a potato dextrose agar (PDA) slant. For *Fusarium* spp. identification, the following keys were used: Booth (1971), Nelson et al. (1983) and Leslie and Summerell (2006). For fungal inoculum preparation, the stored cultures were rejuvenated on the PDA plates at 21–24°C for 14 days. Equal parts of barley and wheat grain totalling 250 g were mixed and placed in water in 1000-ml glass jars for 16 h. Excess water was removed, and the grain was autoclaved for 30 min at 121°C. Grain was inoculated with three 1-cm-diameter mycelium plugs from a PDA culture and incubated for 21 days at 24°C (Dodman and Wildermuth 1987). Colonized grain was air-dried and ground in a laboratory mill to pass through a 1-mm sieve. 'McNeal' wheat and 'Harrington' barley seeds were planted 3 cm deep in pots (1210 cm^3) filled with sterilized soil and moistened to 37.5% (Wildermuth and McNamara 1994) during the duration of the experiments. Dry, ground inoculum was applied as a layer 1 cm above a planted seed at rate of 1.4 g in each pot.

Disease and rating

The *Fusarium* spp. inoculum was a mixture of cultures of *F. avenaceum*, *F. culmorum* and *F. graminearum*). This is similar to a blend used by Piesik et al. (2011b) on maize, except that *F. oxysporum* was not included in the current study because this pathogen was not available at this time. Fungal isolates originated from the collection of pathogenic fungi maintained at the Department of Phytopathology and Molecular Mycology, University of Technology and Life Sciences. The fungi were isolated from wheat leaves displaying disease symptoms. For the inoculum preparation, the stored cultures were rejuvenated on the Petri plates with PDA medium (Difco) at 20–22°C for 10 days. After incubation, the spores produced by mycelium were suspended in distilled water. The cfu (colony-forming units) concentration was estimated microscopically with a Thoma haemocytometer (Thoma, Hawksley, London, United Kingdom) and adjusted to 1.5×10^{-6} cfu/ml. Healthy infection treatment plants were artificially inoculated with *Fusarium* infection material by foliar spray (Kwazar, Merkury PRO + 1l; whole plant was sprayed). Control treatment plants were sprayed with distilled water only.

At 7 days post-inoculation, the degree of infestation was evaluated based on a five-degree scale: 0 – no symptoms, 1 – single spots, 1 – 10% leaf area with infection symptoms, 2 – from 11% to 30% of the leaf-area infection, mild wilting, 3 – from 31% to 60% of the area with infection symptoms, clearly evident wilting, 4 – above 60% of the area with disease symptoms, heavy wilting. Analyses were conducted in four replicates where a single pot constitutes one replicate. Sixty leaves (5 tillers × 3 leaves per tiller × 4 replicates) were scored. Degrees of infection were transformed into disease indexes (DI in%) according

to Townsend and Heuberger formula (Piesik et al. 2011b). Decreased values of DI indicate an increase in plant resistance to the pathogen. Pathogen isolates from disinfected leaves with lesions on PDA medium were made for confirmation of the identity of casual agents.

Undamaged neighbouring plants

The plants were elevated on the bench in the greenhouse. Uninfected cereal plants at BBCH 32 were placed as neighbours to infected ones. There were no other plants present in the greenhouse bay where infected plant exposure to uninfected plants occurred. Plants were exposed at one time (one infected plant, one uninfected 1 m plant and one uninfected 3 m plant). Moreover, only one group was exposed at one time. No tubing was used to direct air from infected to uninfected plants. Uninfected plants simply had open air exposure from an infected plant. Uninfected barley and wheat plants were exposed at the same time but in different greenhouse bays. Uninfected plants were kept outside of exposure times and VOC collection times, and 1 and 3 m plants were kept in different locations. Air mixing by cooling fans occurred in the greenhouse bay where uninfected plant exposure to infected plants took place. The distances from infected cereals to uninfected cereal plants were set-up in the following combinations:

- 1 m – 1 m from uninfected cereal plant to infected plant,
- 3 m – 3 m from uninfected cereal plant to infected plant.

Uninfected neighbour plant exposure to an infected plant was started at 10:00 h at 1, 7 and 14 days post-infection for the same uninfected plants that lasted 48 h and was followed by 4 h of VOC collection (starting at 10:00 h). At time-0 (10:00 h on day-0), infected and control treatment barley and wheat plants were sprayed, then VOC induction was measured at day-1 at 10:00 h from these plants. Then, neighbouring plants were exposed to infected plants starting at 14:00 h on day-1, and VOC induction was measured at 10:00 h on day-3. This cycle was repeated for VOC collection on days 7 and 14 for infection and control treatment plants and on days 9 and 16 for neighbouring plants.

Volatile collection system

Volatiles were collected separately and simultaneously from Nalophan enclosed 'McNeal' wheat and 'Harrington' barley plants. The apparatus allowed for VOC odour collection from four plants at the same time. A volatile collector trap (6.35 mm OD, 76-mm-long glass tube; Analytical Research Systems, Inc., Gainesville, FL, USA) containing 30 mg of Super-Q (Alltech Associates, Inc., Deerfield, IL, USA) adsorbent was inserted into each of four Tygon tubes (connection between airflow metre and collector trap). Purified, humidified air was delivered at a rate of 1.0 l/min over the plants, and a vacuum pump sucked 20% less (0.8 l/min) to avoid collecting odours from any gap of the system. Main stem VOCs for each cereal species were collected from plants in eight blocks, where each block contained an infection treatment plant, uninfected control plant (not exposed to an infected plant) and two neighbouring uninfected plants at 1 or 3 m, respectively, from an infected plant. Tillers were placed outside each volatile collection chamber. The volatile collection duration was 4 h that started at 9:00 h. Additionally, two blanks (odours collected from empty Nalophan bags only) were collected to verify a lack of background presence of reported VOCs.

Analytical methods

Volatiles were eluted from the Super-Q in each volatile collection trap with 225 μl of hexane, followed by adding 7 ng of decane as an internal standard. Previous experiments showed that this quantity of hexane was sufficient to extract all trapped volatiles (Piesik et al. 2010, 2011a,b). Individual samples (1 μl) were injected and analysed by coupled gas chromatography-mass spectrometry (GC/MS). The GC/MS Auto System XL/Turbomass (Perkin Elmer Shelton, Branford, CT, USA) was fitted with a 30-m Rtx-5MS capillary column (0.25 mm ID, 0.25 μm film thickness; Restek, Bellefonte, PA, USA). The temperature programme increased from 40 to 200°C at 5°C/min. VOC identification was initially determined by the best match from NIST mass spectral library (Rev. D.02.00) and then verified by matching each compound with its authentic standard (note that a blend of Z and E isomers occur for ocimene) purchased from commercial sources (Sigma-Aldrich) that had the same GC retention time and mass spectral pattern. The concentration (ng/h) of each barley or wheat VOC from each plant was determined by comparing a compound's peak area to the peak area of the internal standard (Piesik et al. 2011a,b). For all compounds of interest, the detector response was linear across the range of concentrations studied.

Y – tube behavioural choice tests

The six compounds with the highest induction concentrations from the uninfected and infected plant VOC induction study were chosen for Y – tube experiments: three GLVs ((*Z*)-3-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate), two monoterpenes ((*Z*)-ocimene, *β*-linalool) and benzyl acetate. Attraction or repellency of female and male *O. cyanella* were assayed using a Y-tube olfactometer system similar to that described by Piesik et al. (2008, 2011b). A charcoal-purified and humidified air stream was connected to each arm of the Y-tube by a threaded 24/410 (inner diameter 24 mm) cap with a Teflon liner coupled to a 0.64-cm Swagelock union delivering air via Teflon tubing (outer diameter 0.64 cm). The airflow was set at 0.8 l/min using a flowmeter. The synthetic test-stimulus was placed in either a synthetic lure (outer diameter 24 mm, length 8 cm) or a Corning tube (outer diameter 24 mm, length 46 cm). The Y-tube olfactometer was comprised of Corning glass tubing (outer diameter 28 mm, length 30 cm) that branched at 20 cm. The interior angle of the 'Y' was 120°, the diverging arms extended for 4 cm in each direction before becoming parallel for their final 10 cm, and then terminated in a female ground–glass joint at the end of each arm. The male ground–glass joint on the stimulus-delivery tube was inserted into the female receiving arm of the Y-tube for an airtight fit.

Bioassay subjects were placed in the unbranched section of the Y-tube ~ 2 cm from the outlet. A 6.3 V bulb (lamp type 46; Radio Shack, Fort Worth, Texas, USA) was placed between the apexes of the Y-tube arms; equality in illumination supplied to the two arms was verified using a pyranometer (model LI-200SA; LI-COR Biosciences, Inc., Lincoln, Nebraska, USA). To screen *O. cyanella*, dose-dependent responses to each of six tested VOCs, commercially available pure compound (Sigma-Aldrich Chemical Co. Inc.) was used at five concentrations (0, 60, 300, 1500 and 7500 ng/h) for each VOC. Adult beetles were tested with each VOC, starting first with the lowest concentration (0 ng/h), and increasing tested concentration of the compound so that the highest concentration (7500 ng/h) was tested last. A set quantity (50 *μ*l) of each VOC was put on one quarter (circle Ø 70 mm cut in four pieces) of filter paper folded inside a micro-centrifuge tube. Suitable release was previously verified by volatile collection system and gas chromatography–mass spectrometry. The microcentrifuge cap was left open for 1 min to allow for solvent and VOC evaporation. Insects were observed for 5 min or until they have chosen one of the arms of the Y-tube. Each insect made a choice within 3 min. Twenty insects of each sex were tested at each concentration of each tested VOC, where each individual was tested alone and only once. All bioassays were conducted with naïve adults the day after emergence from their pupal case.

Statistical analysis

The VOC induction experiment was carried out using a randomized block design. A one-way repeated measures mixed model analysis of variance (ANOVA) was carried out using Proc Mixed in SAS 9.2 (SAS Institute 2007) to determine the effects of infection source on the concentrations of each VOC separately: (Z)-3-hexenal, (E)-2-hexenal, (Z)-3-hexenol, (E)-2-hexenol, (Z)-3-hexenyl acetate, 1-hexenyl acetate and (Z)-ocimene, *β*-linalool, linalool oxide, benzyl acetate and *β*-caryophyllene. To maintain $\alpha = 0.05$ as multiple ANOVA were conducted separately for each of 11 VOCs, a Bonferroni correction was used with $\alpha = 0.0046$ to determine significance of terms for analysis of each VOC for each cereal species. Plant infection status was a fixed factor and time was a repeated measures factor. The infection status × time interaction tested whether there were different temporal patterns of induction across infection treatments for each VOC. The normality of data for each VOC was tested using Shapiro–Wilk's normality test (Shapiro and Wilk 1965). To distinguish significantly different infection treatments at each time interval, t-tests were conducted following significant ANOVA for each VOC; a Bonferroni correction was applied so that $\alpha = 0.0003$.

To examine *O. cyanella* male and female dose responses to each of the six selected VOCs ((Z)-3-hexenal, (Z)-3-hexenol and (Z)-3-hexenyl acetate) and (Z)-ocimene, *β*-linalool and benzyl acetate), an initial G-test of independence was performed to determine where a significant dose response existed. The G-test was run on pooled count data of individuals that went to the compound side or the hexane solvent alone side for both sexes and was analysed across the five test doses (0, 60, 300, 1500 and 7500 ng/h). Then separate G-tests were performed to examine whether female or male *O. cyanella* had significant dose responses to each VOC. Finally, individual chi-square goodness of fit tests for small sample sizes (Sokal and Rohlf 1995) were performed for each sex at each dose for each VOC against an expected 10 : 10 ratio. This was performed to test whether choosing an arm was independent at each dose of a specific VOC. All data analysis was performed using the statistical package SAS 9.2 (SAS Institute 2007).

Results

Tested cultivars of barley and wheat were susceptible to infection with *Fusarium* species. Mean DI were 40.8% and 31.4% for barley and wheat, respectively. Re-isolation of pathogens from infected leaves verified that the complex of *Fusarium* spp. were the casual disease agents.

Measurement day was a significant factor for all VOCs for both cereals (table 1), where the general pattern of mean concentrations was 14 days > 7 days >> 1 day post-inoculation (table 2; fig. 1). While most VOCs were barely detectable at 1 day, there was $\geq$ 10× induction of all reported VOCs by 7 days post-inoculation (fig. 1; table 2). All VOCs also had infection as a significant main effect and infection*day as a significant interaction term with both cereals (table 1). This reflected that infection treatments were not significantly different from each other at 1 day post-inoculation, but at 7 and 14 days, the significant pattern for mean concentrations for each VOC tended to be: infected plant > 1 m uninfected neighbour > 3 m uninfected neighbour > control (table 2; fig. 1). The degree of induction in neighbouring uninfected plants was negatively related to distance from an infected plant. The concentrations of 1 m uninfected neighbouring plant VOCs were usually 25–50%, and 3 m uninfected neighbouring plants were usually 10–25%, of infected plant mean concentrations (table 2; fig. 1). There were two exceptions, as E-2-HOL and 1-HAC had little or no significant induction in neighbouring uninfected wheat plants at 7 and 14 days (table 2).

Significant *O. cyanella* dose responses occurred for (*Z*)-3-HAL, (*Z*)-3-HAC, Z-OCI and LIN (table 3). Dose responses were statistically significant for females for all four VOCs, while for males dose responses were significant for Z-3-HAL and Z-3-HAC and marginally significant (P = 0.06) for Z-OCI and LIN; for males, the marginal significance of dose responses for these two VOCs was in part because of responses of individuals to the 0 dose (table 3). Female and male *O. cyanella* were significantly attracted to (*Z*)-3-HAL at a concentration of 300 ng/h and at 1500 ng/h for (*Z*)-3-HAL and (*Z*)-3-HAC (table 3). Yet, significant repellency was observed for both females and males to (*Z*)-3-HAL, (*Z*)-3-HAC, (*Z*)-OCI and LIN at the highest tested concentration of 7500 ng/h and at 1500 ng/h for LIN (table 3). The strongest repellency was observed for (*Z*)-3-HAL and LIN at the highest dose, where 90% of females chose the arm not delivering these VOCs. There were no significant *O. cyanella* dose responses to Z-3-HOL and BAC (table 3).

Table 1 *F*-Statistics are shown from mixed model repeated measures ANOVA for individual volatile organic compounds (VOCs) for (A) barley and (B) wheat. Bonferroni correction was applied (11 VOCs for each cereal), so $\alpha = 0.0046$

VOCs[1]	$Infection_{3,53d.f.}$	$Day_{2,24d.f.}$	Infection × $Day_{6,24d.f.}$	$Block_{7,53d.f.}$
(A) Barley				
Z-3-HAL	2550**	6099**	786**	ns
E-2-HAL	289**	827**	89**	ns
Z-3-HOL	933**	3249**	384**	ns
E-2-HOL	202**	425**	53**	ns
Z-3-HAC	3921**	8193**	1100**	ns
1-HAC	307**	651**	85**	ns
(Z)-OCI	1521**	3321**	413**	ns
LIN	2545**	6742**	788**	ns
LOX	257**	675**	79**	ns
BAC	1119**	2300**	296**	ns
β-CAR	259**	705**	83**	ns
(B) Wheat				
Z-3-HAL	2086**	3559**	722**	ns
E-2-HAL	578**	1140**	183**	ns
Z-3-HOL	1220**	2953**	333**	ns
E-2-HOL	237**	22**	8*	ns
Z-3-HAC	1878**	2657**	562**	3.6*
1-HAC	123**	100**	42**	ns
(Z)-OCI	2334**	3530**	661**	ns
LIN	4513**	5924**	1173**	ns
LOX	294**	366**	100**	ns
BAC	620**	1362**	233**	ns
β-CAR	434**	596**	117**	ns

(*Z*)-3-HAL, (*Z*)-3-hexenal; (*E*)-2-HAL, (*E*)-2-hexenal; (*Z*)-3-HOL, (*Z*)-3-hexenol; (*E*)-2-HOL, (*E*)-2-hexenol; (*Z*)-3-HAC, (*Z*)-3-hexenyl acetate; 1-HAC, 1-hexenyl acetate; (*Z*)-OCI, (*Z*)-*β*-ocimene; LIN, linalool; LOX, linalool oxide; BAC, benzyl acetate; *β*-CAR, (*E*)-*β*-caryophyllene.

ns, not significant (P > 0.0046); *significant after Bonferroni correction (P < 0.0046); **P < 0.00001.

Discussion

Pathogen infection and VOC induction

In the first part of our experiment, we confirmed that pathogen infection significantly induced 11 VOCs from barley and wheat when compared to control uninfected plants: six GLVs, four terpenes and a shikimic acid pathway derivative (BAC). Of interest, although the GLVs, terpenes (LIN, LOX, (*Z*)-OCI, *β*-CAR) and BAC in the current study are often considered HIPVs (e.g. Dicke et al. 2009), they apparently are not only induced by herbivory as they can be pathogen induced plant volatile after cereal spp. infection by pathogens (Piesik et al. 2011a,b; current study).

Table 2 Mean values (ng/h) (and ±1 SD) are presented for (A) barley and (B) wheat volatile organic compounds at three times after initial *Fusarium* inoculation. Treatments with the same letter are not significantly different based on t-tests with Bonferroni correction ($\alpha = 0.0003$)

	1 day				7 days				14 days			
	Infested		Undamaged		Infested		Undamaged		Infested		Undamaged	
	Control	*Fusarium*[1]	1 m to p	3 m to p	Control	*Fusarium*[1]	1 m to p	3 m to p	Control	*Fusarium*[1]	1 m to p	3 m to p
(A) Barley												
Z-3-HAL	11.3 ± 1.12A	10.6 ± 0.80A	6.9 ± 0.38B	6.9 ± 0.58B	12 ± 1.0D	112 ± 5.3A	99 ± 2.2B	61 ± 1.9C	10 ± 1.4D	127 ± 7.0A	104 ± 5.0B	62 ± 2.2C
LIN	bd	nd	bd	bd	bd D	103 ± 4.7A	81 ± 2.3B	50 ± 1.7C	bd D	109 ± 6.3A	85 ± 3.4B	51 ± 1.8C
Z-3-HOL	bd	bd	bd	bd	nd D	31 ± 3.0A	26 ± 0.9B	17 ± 0.7C	nd D	35 ± 2.6A	27 ± 0.9B	18 ± 0.8C
BAC	nd	bd	nd	bd	bd D	31 ± 2.7A	23 ± 2.3B	15 ± 0.7C	bd D	33 ± 2.3A	24 ± 2.7B	16 ± 0.7C
(Z)-OCI	bd	bd	bd	bd	bd D	30 ± 1.6A	23 ± 1.7B	15 ± 0.9C	bd D	32 ± 1.8A	24 ± 1.8B	16 ± 1.2C
E-2-HAL	nd	bd	bd	bd	bd D	8.6 ± 0.80A	7.1 ± 0.47B	4.8 ± 0.70C	bd C	10.5 ± 1.85A	7.7 ± 0.63AB	5.1 ± 0.87B
β-CAR	nd	bd	bd	bd	nd C	3.7 ± 0.74A	3.8 ± 0.27A	2.2 ± 0.51B	nd C	4.2 ± 0.50A	4.0 ± 0.31A	2.4 ± 0.57B
E-2-HOL	bd	bd	bd	bd	nd D	3.3 ± 0.55A	2.3 ± 0.44B	1.6 ± 0.41C	nd C	4.0 ± 0.63A	2.7 ± 0.56B	1.9 ± 0.41B
LOX	bd	bd	bd	bd	nd D	3.2 ± 0.42A	2.4 ± 0.26B	1.5 ± 0.24C	nd C	3.9 ± 0.75A	2.7 ± 0.19AB	1.7 ± 0.20B
1-HAC	nd	bd	bd	bd	nd D	3.1 ± 0.53A	2.1 ± 0.29B	1.3 ± 0.23C	nd D	3.7 ± 0.32A	2.4 ± 0.40B	1.6 ± 0.27C
Fusarium[1] – complex of three *Fusarium* species: *F. graminearum*, *F. culmorum*, *F. avenaceum*; nd- not detected in any samples; bd- barely detected (<0.10 ng/h)												
(B) Wheat												
Z-3-HAL	6.4 ± 0.71	7.1 ± 0.53	6.1 ± 1.39	6.9 ± 0.58	7 ± 0.5D	77 ± 2.9A	44 ± 1.1B	22 ± 1.1C	6 ± 0.6D	82 ± 5.5A	47 ± 1.3B	23 ± 1.7C
LIN	bd	bd	bd	bd	bd D	41 ± 1.5A	22 ± 1.2B	12 ± 0.9C	bd D	43 ± 1.4A	23 ± 1.6B	12 ± 1.0C
(Z)-OCI	bd	bd	bd	bd	bd D	18 ± 0.8A	11 ± 0.7B	5.5 ± 0.5C	bd D	22 ± 1.2A	12 ± 0.7B	6.0 ± 0.8C
Z-3-HOL	bd	bd	bd	bd	2 ± 0.3D	18 ± 0.8A	11 ± 1.0B	5.6 ± 0.4C	3 ± 0.5D	18 ± 1.2A	12 ± 1.1B	6.1 ± 0.5C
BAC	nd	bd	bd	bd	bd D	18 ± 1.4A	10 ± 0.7B	5.2 ± 0.3C	bd D	19 ± 2.4A	11 ± 0.8B	5.7 ± 0.5C
E-2-HAL	bd	bd	bd	bd	nd D	5.3 ± 0.58A	3.7 ± 0.34B	1.7 ± 0.16C	bd D	6.4 ± 0.68A	4.3 ± 0.47B	1.9 ± 0.20C
β-CAR	nd	bd	nd	bd	nd D	3.1 ± 0.31A	1.4 ± 0.27B	0.7 ± 0.14C	nd D	3.5 ± 0.44A	1.7 ± 0.31B	0.9 ± 0.21C
LOX	bd	nd	bd	bd	nd D	1.9 ± 0.31A	0.8 ± 0.16B	0.4 ± 0.09C	nd C	3.0 ± 0.46A	1.0 ± 0.25B	0.5 ± 0.11B
E-2-HOL	bd	nd	bd	bd	bd C	1.9 ± 0.27A	bd BC	0.4 ± 0.08B	bd C	2.9 ± 0.57A	bd C	0.6 ± 0.13BC
1-HAC	bd	bd	bd	bd	bd B	1.8 ± 0.20A	bd B	bd B	bd B	2.8 ± 0.70A	0.3 ± 0.52B	0.4 ± 0.16B
Fusarium[1] – complex of three *Fusarium* species: *F. graminearum*, *F. culmorum*, *F. avenaceum*; nd – not detected in any samples; bd – barely detected (<0.30 ng/h)												

(*Z*)-3-HAL, (*Z*)-3-hexenal; (*E*)-2-HAL, (*E*)-2-hexenal; (*Z*)-3-HOL, (*Z*)-3-hexenol; (*E*)-2-HOL, (*E*)-2-hexenol; (*Z*)-3-HAC, (*Z*)-3-hexenyl acetate; 1-HAC, 1-hexenyl acetate; (*Z*)-OCI, (*Z*)-*β*-ocimene; LIN, linalool; LOX, linalool oxide; BAC, benzyl acetate; *β*-CAR, (*E*)-*β*-caryophyllene.

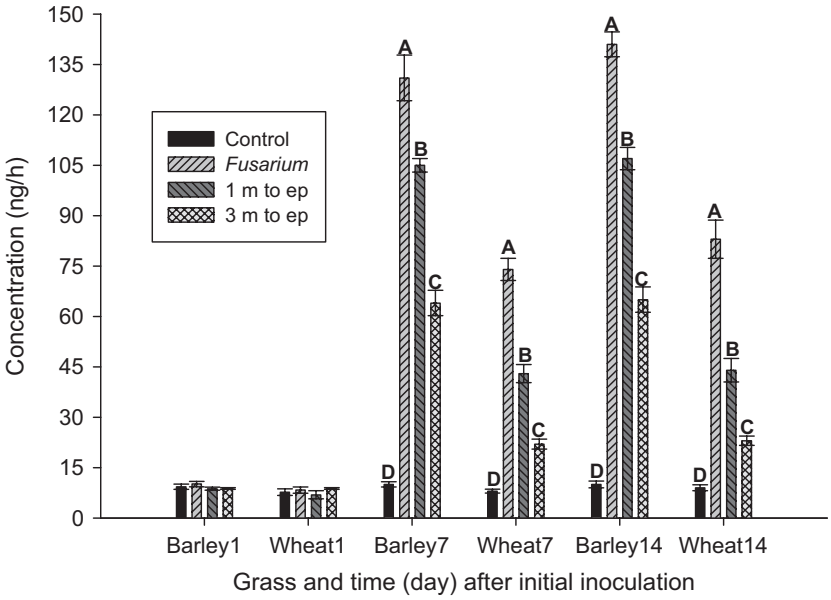

Fig. 1 Mean values (ng/h) (and ±1 SD) are presented for barley- and wheat-emitting (*Z*)-3-hexenyl acetate at three times after initial *Fusarium* inoculation. Treatments with the same letter are not significantly different at each time interval based on *t*-tests with Bonferroni correction ($\alpha = 0.0003$).

No significant infected plant VOC induction occurred at 1 day post-inoculation, but strong VOC induction was measured at 7 days and slightly stronger VOC induction at 14 days. It seems that the *Fusarium* spp. had not resulted in sufficient infection severity at 1 day post-inoculation to induce plant VOCs, but infection was sufficient to result in plant VOC induction at 7 days (and possibly earlier). With maize, significant VOC induction was measured at 3 days post *Fusarium* spp. blend inoculation where the degree of induction increased by 7 days (Piesik et al. 2011b). These results suggest that as *Fusarium* spp. infection severity increased over time, degree of plant VOC induction also increased over time. However, the current study suggests that strength of VOC induction (concentration of each induced VOC) may have reached a plateau by 14 days post-inoculation. This may become a large metabolic investment for infected plants, even if the induction is only diurnal; diurnal/nocturnal patterns of VOC induction in response to pathogen induction have not been reported to our knowledge. VOC induction occurs from several plants infected by pathogens besides cereal grasses, including potatoes (De Lacy Costello et al. 2001; Kushalappa et al. 2002), peanuts (Cardoza et al. 2002), onions (Vikram et al. 2005) and Monterey pine (ethylene; Bonello et al. 2001). Plant VOC induction in response to pathogen induction can benefit emitting plants as induced GLVs (Shiojiri et al. 2006; Arimura et al. 2009) and methyl salicylate (Shulaev et al. 1997) help inhibit pathogen growth or stimulate plant defence responses (Shulaev et al. 1997; Yi et al. 2009).

Uninfected neighbouring plant VOC induction

In the second part of our experiment, we demonstrated that uninfected barley and wheat plants neighbouring an infected conspecific plant had significant VOC induction that was qualitatively similar to, but quantitatively lower than, infected plants. Uninjured barley, oat and wheat VOC induction has been reported when near an injured (*O. melanopus* herbivory or mechanical injury) wheat plant (Piesik et al. 2010) or when uninfected maize was near an infected maize plant (Piesik et al. 2011b). This indicates that VOC emission by an infected plant stimulates VOC induction in nearby uninfected plants and was negatively related with the distance (1 or 3 m) that a plant was from an infected plant; this has been shown with barley, wheat and maize for these distances (Piesik et al. 2010, 2011b). Note also that when an injured wheat plant was partially surrounded by a nalophan bag, then uninjured barley, wheat and oat VOC induction was reduced relative to when the injured wheat plant was fully exposed (Piesik et al. 2010). When injured wheat plants were fully surrounded by a nalophan bag, there was no plant VOC induction in uninjured, neighbouring barley, wheat or oat (Piesik et al. 2010). This study suggested that exposure to injured wheat plant VOCs was the likely cause of uninjured, neighbouring barley, wheat and oat VOC induction (Piesik et al. 2010).

Induced VOCs like terpenes, and especially GLVs, have been shown to prime plant defences (Arimura et al. 2005) from uninjured parts of the same plant (Farag and Paré 2002; Frost et al. 2007; Rodriguez-Saona

Table 3 Effect of synthetic (*Z*)-3-HAL, (*Z*)-3-HAC, LIN, and *β*-CAR volatile organic compounds on adult *O. cyanella Voet.* behavior (females and males) at different doses (0, 60, 300, 1500, or 7500 ng/h)

Name of compound	Dose	ng/min	No. of females			No. of males		
			+	–	χ^2 (1)	+	–	χ^2 (1)
	Control	0	12	8	0.45 ns	9	11	0.05 ns
(*Z*)-3-HAL	1	60	11	9	0.05 ns	13	7	1.25 ns
	2	300	16	4	6.05* (a)	15	5	4.05* (a)
	3	1500	17	3	8.45** (a)	15	5	4.05* (a)
	4	7500	2	18	11.3*** (r)	3	17	8.45** (r)
$G_{4d.f.}$ = 52***			$G_{4d.f.}$ = 32***			$G_{4d.f.}$ = 22***		
	Control	0	11	9	0.05 ns	13	7	1.25 ns
(*Z*)-3-HOL	1	60	9	11	0.05 ns	13	7	1.25 ns
	2	300	11	9	0.05 ns	10	10	0.05 ns
	3	1500	10	10	0.05 ns	12	8	0.45 ns
	4	7500	7	13	1.25 ns	12	8	0.45 ns
$G_{4d.f.}$ = 1.3^{ns}			$G_{4d.f.}$ = 2.3^{ns}			$G_{4d.f.}$ = 1.2^{ns}		
	Control	0	11	9	0.05 ns	9	11	0.05 ns
(*Z*)-3-HAC	1	60	12	8	0.45 ns	10	10	0.05 ns
	2	300	10	10	0.05 ns	13	7	1.25 ns
	3	1500	16	4	6.05* (a)	16	4	6.05* (a)
	4	7500	3	17	8.45** (r)	4	16	6.05* (r)
$G_{4d.f.}$ = 35***			$G_{4d.f.}$ = 19***			$G_{4d.f.}$ = 17**		
	Control	0	12	8	0.45 ns	7	13	1.25 ns
(*Z*)-OCI	1	60	7	13	1.25 ns	13	7	1.25 ns
	2	300	11	9	0.05 ns	6	14	2.45 ns
	3	1500	14	6	2.45 ns	10	10	0.05 ns
	4	7500	3	17	8.45** (r)	5	15	4.05* (r)
$G_{4d.f.}$ = 15**			$G_{4d.f.}$ = 17**			$G_{4d.f.}$ = $8.9^{0.06}$		
	Control	0	10	10	0.05 ns	8	12	0.45 ns
LIN	1	60	12	8	0.45 ns	9	11	0.05 ns
	2	300	11	9	0.05 ns	10	10	0.05 ns
	3	1500	3	17	8.45** (r)	4	16	6.05* (r)
	4	7500	2	18	11.3*** (r)	3	17	8.45** (r)
$G_{4d.f.}$ = 28***			$G_{4d.f.}$ = 21***			$G_{4d.f.}$ = $9.1^{0.06}$		
	Control	0	11	9	0.05 ns	12	8	0.45 ns
BAC	1	60	14	6	2.45 ns	12	8	0.45 ns
	2	300	10	10	0.05 ns	13	7	1.25 ns
	3	1500	7	13	1.25 ns	11	9	0.05 ns
	4	7500	11	9	0.05 ns	9	11	0.05 ns
$G_{4d.f.}$ = 3.9^{ns}			$G_{4d.f.}$ = 5.2^{ns}			$G_{4d.f.}$ = 1.9^{ns}		

r, repellent; a, attractant; +, Y – tube arm with tested amount of the compound, volatile diluted in hexane emitted from filter paper; –, Y – tube arm only with hexane emitted from filter paper; (*Z*)-3-HAL, (*Z*)-3-hexenal; (*Z*)-3-HOL, (*Z*)-3-hexenol; (*Z*)-3-HAC, (*Z*)-3-hexenyl acetate; (*Z*)-OCI, (*Z*)-*β*-ocimene; LIN, linalool; BAC, benzyl acetate.

(1)level of significance, ns – not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

et al. 2009) or nearby uninjured plants (Engelberth et al. 2004; Baldwin et al. 2006; Ton et al. 2007). However, some VOCs, especially GLVs, can induce VOCs from plants as shown in earlier studies (Farag and Paré 2002; Farag et al. 2005; Ruther and Kleier 2005). This lends some support to more recent studies that have reported uninjured plant VOC induction after exposure to plants that were injured (Piesik et al. 2010) or infected (Piesik et al. 2011b; current study). As methyl salicylate (Shulaev et al. 1997) and GLVs (Shiojiri et al. 2006) reduce pathogen growth and promote plant resistance to pathogens, an infected plant may have concentric rings of surrounding plants that have progressively smaller degrees of VOC induction and possibly defence priming around it. The plants nearest to a pathogen-infected plant have the

highest degree of induction as suggested by our results and so may have the highest degree of resistance as they have the highest probability of attack from a nearby spreading pathogen. This may be one mechanism by which eavesdropping plants can benefit from VOC induction. Priming, or even induction, of plant defences to help cope with biotic attack may have population- and community-level consequences.

Studies have shown that VOC 'induction' in some cases may be passive, where neighbouring uninjured/uninfected plants adsorb and later re-release relatively low volatility compounds (Choh et al. 2004; Himanen et al. 2010). This was shown to happen with a few VOCs in experiments by Choh et al. (2004), where protein inhibitors blocked VOC induction of injured plants but did not block VOC 'induction' in neighbouring uninjured plants to suggest VOC adsorption and re-release. Choh et al. (2004) also found uninjured plant induction of other VOCs was an active process. Another study to examine passive VOC adsorption and re-release showed that concentrations of induced VOC were not constitutively produced by birch (*Betula* spp.) but were detected when Rhododendron plants were nearby neighbours (Himanen et al. 2010). However, the concentrations of passively re-released VOCs from birch plants were ~ 0.1% of the emitted concentrations from rhododendron plants (Himanen et al. 2010). For many of the VOCs reported in the current study and Piesik et al. (2010, 2011b), levels in uninjured/uninfected neighbour plants at 1 m were 25–50% of the injured/infected plant and 10–25% when at 3 m. These emission rates are much higher than levels expected to reach a neighbouring plant for passive adsorption and re-release, when considering a 1 or 3 m radius half sphere coming from an infected, induced VOC-emitting plant. Thus, we contend that our uninfected, neighbouring plants had active VOC induction (Piesik et al. 2010, 2011b; current study). Plants at 3 m likely receive lower concentrations of emitted VOCs from an injured/infected plant, so the degree of neighbouring uninjured/uninfected plant VOC induction seems dose dependent. However, we emphasize that these experiments were conducted in the greenhouse (Piesik et al. 2010, 2011b, current study), so they would need to be studied under open-field conditions.

Responses of an herbivore to single VOC induced by barley and wheat

The third part of our experiment showed that a cereal leaf beetle, *O. cyanella*, was attracted to two GLVs ((*Z*)-3-HAC and (*Z*)-3-HAL) at doses comparable with herbivore-injured plant emission levels (Piesik et al. 2010), but > 2× (often much lower) lower than emission levels from *Fusarium* infected plants (current study). In contrast, at the highest test dose, *O. cyanella* was repelled by these two GLVs and two terpenes ((*Z*)-OCI and LIN). Thus, these results might only have agricultural or ecological relevance if *O. cyanella* adults could be attracted to, or repelled by, a close group of infected plants. Some GLVs ((*Z*)-3-HAC and (*Z*)-3-HOL) are attractive to adult females of the parasitoid wasp, *Cotesia marginiventris*, after herbivory on maize (D'Alessandro and Turlings 2005; Hoballah and Turlings 2005) and (*Z*)-3-HAC induced (Kost and Heil 2006) or primed (Heil et al. 2008) extrafloral nectar secretion.

In contrast to *O. cyanella* dose responses, *O. melanopus* had dose-dependant attractive responses at the lowest doses to the same two GLVs ((*Z*)-3-HAC and (*Z*)-3-HAL) and at higher doses for LIN and (*E*)-β-CAR (Piesik et al. 2011b). The amounts attractive to *O. melanopus* for (*Z*)-3-HAC, (*Z*)-3-HAL), and LIN falls within the induced concentration levels from *Fusarium* spp.-infected maize (Piesik et al. 2011b) and barley and wheat (current study). Also, both *O. melanopus* and *O. cyanella* dose-dependent attraction was in the range of VOC induction for the compounds from barley, oat and wheat plants injured by *O. melanopus* (Piesik et al. 2010). It may also be of interest that *O. melanopus* has dose-dependent attraction to four tested VOCs and is a severe pest species of cereal crops. In contrast, *O. cyanella* doses for attraction and repellence tend to be higher, and this species is not a severe agricultural cereal crop pest. Of course, a comparison of host preferences of these two congeneric beetle species will require future comparison with actual injured, infected and control plant odours and testing with specific VOC blends.

Plant responses to biotic and abiotic perturbation often reduce the severity of future damage through complex changes. These changes include VOC induction that can directly repel herbivore feeding or oviposition (De Moraes et al. 2001; Kessler and Baldwin 2001; Beale et al. 2006; Laothawornkitkul et al. 2008; Unsicker et al. 2009). Also, essential oils can be active against fungal pathogens via contact and exposure to headspace VOC to significantly reduce fungal hyphal growth (Alvarez-Castellanos et al. 2001). VOC induction has fascinated researchers because compounds can indirectly act by attraction of natural enemies of arthropod pests aerially (Turlings et al. 1990; McCall et al. 1993; De Moraes et al. 1998; Mumm et al. 2008; Dicke et al. 2009; Unsicker et al. 2009) or in the soil (Rasmann et al. 2005) that can increase

herbivore parasitism (Thaler 1999). Yet, our study supports another study (Piesik et al. 2011b) to suggest that biotic attack by one organism (pathogen infection) might influence plant attractiveness to a second type of biotic attacker (herbivore) to a plant (Halitschke et al. 2008). Thus, ecological and fitness consequences of VOC induction are complex, because a VOC bouquet (or some subset of it) can attract or deter multiple trophic levels, including biotic attacking agents of a plant and natural enemies of those agents (Halitschke et al. 2008; Dicke et al. 2009; Kessler and Heil 2011).

VOC induction effects on cereal grasses will need further investigation to explore the ecological role of these active substances (Halitschke et al. 2008; Heil and Karban 2010; Kessler and Heil 2011). It is well known that manipulation of volatile emission in plants has enormous potential in relationship to pest management in agricultural contexts (Turlings and Ton 2006). Manipulating these signals may help increase the effectiveness of attracting parasitoid and predatory natural enemies with induced VOC to more effectively serve as biological control agents in agroecosystems (Turlings and Ton 2006) or possibly have plant defences primed before enemies can attack. Also, injured plant VOC induction may prime or induce VOC induction and other defensive responses in nearby uninjured plants, which could also be a useful component of host plant resistance within an IPM context (Turlings and Ton 2006). Yet, our studies suggest VOC induction may have a double edge, as plant enemies may also be attracted (Halitschke et al. 2008; Kessler and Heil 2011). Plant VOC induction in response to pathogen infection might help with pathogen resistance, but possibly at the expense of plants fully achieving maximum yields if there is a significant resource allocation cost to such extended VOC induction.

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