plant biology

Plant Biology ISSN 1435-8603

RESEARCH ARTICLE

Volatile-mediated signalling in barley induces metabolic reprogramming and resistance against the biotrophic fungus *Blumeria hordei*

S. Laupheimer[1,*], L. Kurzweil[2,*], R. Proels[1], S. B. Unsicker[3], T. D. Stark[2], C. Dawid[2] & R. Hückelhoven[1]

1 Chair of Phytopathology, TUM School of Life Sciences, Technical University of Munich, Freising, Germany
2 Chair of Food Chemistry and Molecular Sensory Science, TUM School of Life Sciences, Technical University of Munich, Freising, Germany
3 Department of Biochemistry, Max Planck Institute for Chemical Ecology (MPI-CE), Jena, Germany

Keywords
(*Z*)-3-hexenyl acetate; green leaf volatiles; *Hordeum vulgare* L.; induced resistance; lipids; metabolomics; powdery mildew.

Correspondence
R. Hückelhoven, Chair of Phytopathology, TUM School of Life Sciences, Technical University of Munich, Emil-Ramann-Str. 2, 85354 Freising, Germany.
E-mail: hueckelhoven@tum.de

C. Dawid, Chair of Food Chemistry and Molecular Sensory Science, TUM School of Life Sciences, Technical University of Munich, Liese-Meitner-Str. 34, 85354 Freising, Germany.
E-mail: corinna.dawid@tum.de

*These authors contributed equally to this work.

Editor
U. Wittstock

Received: 25 June 2022;
Accepted: 7 November 2022

doi:10.1111/plb.13487

ABSTRACT

- Plants have evolved diverse secondary metabolites to counteract biotic stress. Volatile organic compounds (VOCs) are released upon herbivore attack or pathogen infection. Recent studies suggest that VOCs can act as signalling molecules in plant defence and induce resistance in distant organs and neighbouring plants. However, knowledge is lacking on the function of VOCs in biotrophic fungal infection on cereal plants.
- We analysed VOCs emitted by 13 ± 1-day-old barley plants (*Hordeum vulgare* L.) after mechanical wounding using passive absorbers and TD-GC/MS. We investigated the effect of pure VOC and complex VOC mixtures released from wounded plants on the barley–powdery mildew interaction by pre-exposure in a dynamic headspace connected to a powdery mildew susceptibility assay. Untargeted metabolomics and lipidomics were applied to investigate metabolic changes in sender and receiver barley plants.
- Green leaf volatiles (GLVs) dominated the volatile profile of wounded barley plants, with (*Z*)-3-hexenyl acetate (*Z*3HAC) as the most abundant compound. Barley volatiles emitted after mechanical wounding enhanced resistance in receiver plants towards fungal infection. We found volatile-mediated modifications of the plant–pathogen interaction in a concentration-dependent manner. Pre-exposure with physiologically relevant concentrations of *Z*3HAC resulted in induced resistance, suggesting that this GLV is a key player in barley anti-pathogen defence.
- The complex VOC mixture released from wounded barley and Z3HAC induced *e.g.* accumulation of chlorophyll, linolenic acid and linolenate-conjugated lipids, as well as defence-related secondary metabolites, such as hordatines in receiving plants. Barley VOCs hence induce a complex physiological response and disease resistance in receiver plants.

INTRODUCTION

Volatile organic compounds (VOCs) are known signalling molecules in plant communication (Ninkovic *et al.* 2021). They attract pollinators or enhance plant defence against insect herbivores and pathogens. The emission of VOCs can be both constitutive and stress-induced. In the latter case, amount and composition of VOCs depend on the kind of damage (Paré & Tumlinson 1996; Mithöfer *et al.* 2009; Ameye *et al.* 2018). According to Dudareva *et al.* (2006), the release of plant VOC has several functions when caused by biotic stress: inter- and intraspecific plant communication, allelopathy or cross-kingdom chemical signals for herbivores or natural enemies of the attacking herbivore. Volatile emission by herbivore-damaged plants may not only trigger defence responses directly, but instead prime the plant itself and neighbouring plants upon further biotic stress. The plant defence response is then usually much faster and stronger in primed plants (Baldwin & Schultz 1983; Ton *et al.* 2007; Ameye *et al.* 2015; Ye *et al.* 2021).

Green leaf volatiles (GLVs) are ubiquitously produced by all green plants (Hatanaka 1993; Maffei 2010) and constitutively emitted in small amounts from intact plant tissue (Piesik *et al.* 2011). Increased synthesis and release of GLVs occur after tissue damage caused by herbivory (Scala *et al.* 2013), and GLVs might act as long-distance signals, provoking induced resistance (Scala *et al.* 2013; López-Gresa *et al.* 2018; Quintana-Rodriguez *et al.* 2018). In GLV biosynthesis, C_6- and C_9-aldehydes, alcohols and esters originate from lipid degradation in chloroplasts. In the lipoxygenase pathway, membrane-bound hydroperoxide lyases catalyse the release of two structurally different aldehydes from unsaturated fatty acid hydroperoxides into the cytosol, namely hexanal and (*Z*)-3-hexenal. An isomerase factor is responsible for the spontaneous

Plant Biology **25** (2023) 72–84 © 2022 The Authors. *Plant Biology* published by John Wiley & Sons Ltd on behalf of German Society for Plant Sciences, Royal Botanical Society of the Netherlands.

conversion of (*Z*)-3-hexenal to its isomer, (*E*)-2-hexenal (Scala *et al.* 2013; Yactayo-Chang *et al.* 2022). Next, the aldehyde is reduced to its corresponding alcohol by alcohol dehydrogenases. The alcohols are further processed by alcohol acyltransferases that catalyse formation to the corresponding acetate esters (Matsui 2006; D'Auria *et al.* 2007). GLVs exert insecticidal and fungicidal effects (Nakamura & Hatanaka 2002; Kishimoto *et al.* 2008; Yousafi *et al.* 2022). Engelberth *et al.* (2004) discovered the protective potential of plant priming with the pure GLVs, (*Z*)-3-hexenal, (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate. GLV-treated corn (*Zea mays cv.* Delprim) had higher jasmonic acid concentrations in response to herbivore damage than control plants. A similar response and induced resistance was shown in wheat (*Triticum aestivum*) pre-treated with (*Z*)-3-hexenyl acetate and challenged with the hemibiotroph fungus *Fusarium graminearum* (Ameye *et al.* 2015). Hammerbacher *et al.* (2019) reviewed the defence role of volatiles against microbial pathogens. Most studies investigated the role of GLVs in plant–insect interaction and the potential of GLVs in plant pathogen interaction with hemibiotrophic or necrotrophic fungi (Vicedo *et al.* 2009; Kravchuk *et al.* 2011; Scala *et al.* 2013; Finiti *et al.* 2014; Gorman *et al.* 2020). The biotrophic powdery mildew fungus is one of the most common fungal pathogens of cereal crops, with the potential to cause drastic losses in yield worldwide. However, there is little research investigating resistance modulation by naturally occurring external signals, such as GLVs, of cereal plants against biotrophic fungi.

We used the biotrophic ascomycete *Blumeria hordei* (*Bh*) and its natural host barley (*Hordeum vulgare* L.) as a model system. The epiphytic growth stages of *Bh* are well known and allow easy monitoring of the fungal development on plant tissues (KIta *et al.* 1981; Schulze-Lefert & Vogel 2000). Fungal spores grow through a defined programme upon leaf inoculation. After penetration of the host cell wall, *Bh* develops a specialized hyphal structure to enter the intact epidermal cell and create a biotrophic cellular interface within 24 h. This so-called haustorium serves mainly to absorb nutrients from living host cells and to release effectors (Panstruga 2003). The haustorium is the only structure that feeds the pathogen. Hence, intact host leaf tissue is required for fungal survival. After successful fungal establishment, mycelium formation and growth occur on leaf tissue 3–7 days after infection (Eichmann & Hückelhoven 2008). Diverse forms of induced resistance are known for the barley pathosystem, and respective gene expression or physiological markers have been described (Beßer *et al.* 2000; Kogel & Langen 2005; Dey *et al.* 2014; Käsbauer *et al.* 2018).

The aim of this study was to investigate the volatile bouquet of mechanical wounded barley plants and the effect of emitted VOCs on the resistance behaviour of receiver plants to subsequent inoculation with powdery mildew spores. We addressed the questions: (i) whether volatiles trigger the plant defence response in receiving plants, and (ii) which metabolic changes are activated in both emitting and receiving plants.

MATERIAL AND METHODS

Plant material

Experiments were performed with 13 ± 1-day-old barley plants (*Hordeum vulgare* L. *cv.* Golden Promise and Ingrid). Seeds of *cv.* Golden Promise were germinated for 3 days on wet filter paper in Petri dishes in the dark at room temperature then transferred to non-sterilized soil; seeds of *cv.* Ingrid were directly grown in the soil (CL ED73; Werke Patzer, Sinntal, Germany). Depending on the experiment, 10 to 15 individuals were grown together in a 7 × 7 cm plastic pot and watered every day. Plants were cultivated in the climate chamber ATC26 (Conviron, Winnipeg, Canada) with a 16 h:8 h (day: night) photoperiod at a temperature of 18 ± 2 °C, a light intensity of 150 ± 2 µmol m^{-2} s^{-1} and a relative humidity of 65%.

Cultivation of *Blumeria hordei*

The powdery mildew fungus *Blumeria hordei* (*Bh*) Em. Marchal race A6 served as pathogen. It was maintained on barley *cv.* Golden Promise in a climate chamber MLR-351H (Sanyo, Moriguchi, Japan) at a temperature of 18 ± 2 °C, a light intensity of 150 µmol m^{-2} s^{-1} and 65% relative humidity.

VOC collection and analysis

Volatiles in the static headspace of barley plants were collected in polydimethylsiloxane (PDMS) tubes. The PDMS tubes were prepared as described in Kallenbach *et al.* (2014). Briefly, PDMS hoses (1 mm internal diameter × 1.8 mm external diameter; PDMS, Carl Roth, Karlsruhe, Germany) were cut into 5-mm long pieces and stored in acetonitrile/methanol 4/1 (v/v). After 3 h incubation at 25 °C, the PDMS pieces were transferred out of the solvent into a 200 ml glass column containing a glass frit. The column was connected to a nitrogen gas flow with 5 l min^{-1} to flush solvent and contaminants and then heated under nitrogen flow for 1.5 h at 210 °C. After cooling, the PDMS tubes were transferred into 4 ml screw-neck glass vials containing argon, screwed tightly, and sealed with polytetrafluorethylene (PTFE). VOC collection was performed in the climate chamber ATC26 (Conviron). Collection and analysis of volatiles emitted by barley *cv.* Golden Promise were done for time periods over 1, 3, 5.5, 8, 10.5, 13 and 24 h after wounding in a static headspace. Additionally, VOCs were collected from non-wounded control plants and from pots without any plant as a system control. A biological replicate consisted of 10 individuals grown together in one pot. We measured seven biological replicates in independently repeated experiments for each treatment. The total leaf tissue of all individuals was damaged with a sterile bluntish razor blade, applying cuts and crushes every few millimetres across the entire leaf blade. Immediately after wounding, an oven bag (50 × 31 cm; Toppits, Minden, Germany) was wrapped around each plant (including the pot). For each time point, three PDMS tubes, as technical replicates, were strung on an aluminium wire (Ø 0.05 mm) and hung inside the oven bag without touching the bags. Then, the bags were tightly closed with a cable binder on the upper side of the bag. An elastic band was installed right at the upper rim of the pots. After 1, 3, 5.5, 8, 10.5, 13 and 24 h of VOC collection, one wire containing three PDMS tubes was transferred to 1.5 ml screw neck glass vials and stored at −20 °C until further analysis. PDMS tubes measured the gaseous equilibrium in the static headspace and reflect the relative compound abundance when one wire was sampled.

The VOCs were analysed with a gas chromatography coupled to mass spectrometry (GC–MS) system QP2010 Ultra (Shimadzu, Kyoto, Japan). PDMS tubes were placed in 89 mm glass thermo-desorption tubes (Sigma Aldrich, St. Louis, USA). Analytes were released from the adsorbent using a thermo desorption unit (TD20; Shimadzu) and a helium flow of 60 ml min^{-1} at 200 °C for 8 min. The substances were cryo-focused onto a trap (Tenax®-GR adsorbent resin; Tenax, Baltimore, USA) at −20 °C. This trap was heated to 230 °C within 10 s and analytes were injected onto a Rtx-5MS column (30 m × 0.25 mm, film thickness 0.25 µm; Restek, Bellefonte, USA). Helium was used as carrier gas, with a constant linear velocity of 44.3 cm s^{-1}. The GC programme was set to 45 °C for 3 min, followed by 180 °C, with a thermal increase of 6 °C min^{-1}. Finally, the temperature was increased to 320 °C with 100 °C min^{-1} and held for 15 min to clear the column. Electron impact mass spectrometry data was detected in the mass range from 33 to 350 Da, with a collision energy of 70 eV and a scan speed of 1666 Da s^{-1}. MS data was analysed using GCMS solution (version 4.20; Shimadzu) based on the Wiley Registry of Mass Spectral Data (8th Edition; Shimadzu) and NIST11 database (National Institute of Standards and Technology, Gaithersburg, MD, USA). A method file with a compound list containing target ions at a specific retention time with automatic peak area integration was created. The used PDMS methods provide semi-quantitative results. An internal standard for quantification was not used. Results were evaluated in peak area per g fresh weight (peak area g_{FW}^{-1}).

VOC-mediated signalling in a dynamic headspace

A VOC exposure experiment was performed for 24 h or 48 h with a dynamic headspace sampling system. Two oven bags were connected by a pump system which allows an active VOC treatment under controlled conditions (Tholl *et al.* 2006). Activated charcoal-filtered air was continuously pushed into the first bag at 700 ml min^{-1} and an airflow of 400 ml min^{-1} transported the air from the first bag to the second bag, which contains the receiver plants (15 individuals per pot and biological replicate). After VOC application, the first bag was tightly closed with a cable binder at both ends. The second bag was not closed tightly to avoid CO_2 depletion and accumulation of air or humidity. A higher influx flow rate was necessary to avoid air contamination. The first bag contained either emitter barley plants *cv*. Ingrid (15 individuals per pot) or different concentrations of (*Z*)-3-hexenyl acetate (*Z*3HAC; 0.1, 1, 10, 25 or 50 µM). This corresponds to a volume of 0.5, 5, 50, 125 or 250 µl of pure Z3HAC (≥ 98% purity; Merck Millipore, Darmstadt, Germany). The concentrations were related to the total volume of the dynamic system (30 l) and represented the predicted maximum concentration for a short amount of time at the beginning of the exposure time of 24 or 48 h. The emitter plants were mechanically damaged, or Z3HAC was applied on a filter paper, marking the starting point of the exposure time. The total leaf tissue was crushed with a sterile bluntish razor blade. A native non-wounded plant served as a control emitter. The experiment was run in the climate chamber ATC26 (Conviron) with a 16 h:8 h (day:night) photoperiod at a temperature of 18 ± 2 °C, a light intensity of 150 ± 2 µmol m^{-2} s^{-1}, and relative humidity of 65%, without any contamination with *Bh* spores during the exposure experiment.

Blumeria hordei susceptibility assay

The 13 ± 1-day-old barley plants exposed to *Z*3HAC, or the bouquet of wounded emitter plants were used in a susceptibility assay against *Bh*. At the end of the volatile exposure time, pots were removed from the closed headspace and the plants acclimated for 30 min. The second leaves of three selected barley individuals remained attached to the intact plants in the pot. We repeated this experiment three times. Other plant individuals in the pot were removed. To ensure a homogeneous spore inoculation, the upper leaf surfaces were spread out and placed flat by fixing them with small metal rods (Ø 2 mm). Barley individuals were inoculated in an inoculation tower (50 × 50 × 200 cm) with 6–10 spores mm^{-2} by gently blowing fresh spores from strongly infested plant tissue into the inoculation tower. After 3 days of incubation in the climate chamber ATC26 (Conviron), 2-cm long leaf segments were evaluated by macroscopically counting fungal microcolonies developing into *Bh* pustules. In total, experimental datasets represent nine technical replicates from three independent biological experiments. One technical replicate represents one barley individual.

Metabolomics and lipidomics with liquid chromatography coupled to mass spectrometry with time of flight mass analyser (LC-TOF-MS)

The sample preparation and analysis method for barley metabolites was modified according to Giavalisco *et al.* (2011). The experimental datasets represent three independent biological experiments. One biological replicate represents five pooled barley individuals. 100 mg frozen plant tissue were homogenized in 2 ml bead beater tubes (CKMix-2 ml; Bertin Technologies, Montingny-le-Bretonneux, France) filled with ceramic balls (zirconium oxide; mix beads of 1.4 mm and 2.8 mm) with 1 ml cold methanol/*tert*-butyl methyl ether/water 1/3/1 (v/v/v) in a Precellys® homogenizer (Bertin Technologies; 6500 rpm, 3 × 30 s, 15 s pause). The supernatant was removed, stored in the fridge and the extraction was repeated for three cycles. 3250 µl methanol/water 1/3 (v/v) were added to the combined extracts, vortexed and centrifuged for 5 min at 4 °C to achieve phase separation. The three phases – upper organic phase containing the lipids, lower aqueous phase containing the metabolites and a pellet of starch and proteins at the bottom – were isolated, dried under nitrogen and redissolved in 150 µl water (aqueous extract) or 500 µl acetonitrile/2-propanol 7/3 (v/v) (lipid extract).

The aqueous extract was used for analysis of secondary metabolites. Chromatographic separation was achieved on an ACQUITY UPLC® I-Class system (Waters, Eschborn, Germany) equipped with an ACQUITY UPLC-BEH C18 column (150 mm × 2.1 mm, 1.7 µm; Waters) as stationary phase and a mobile phase consisting of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B) at a flow rate was 0.4 ml min^{-1} and an injection volume of 3 µl. Mobile phase composition changed as follows: hold 1%B for 1 min, 6 min linear gradient from 1%B to 35%B, 1 min linear gradient from 35%B to 70%B, 1 min linear gradient from 70%B to 99%B, hold 99%B for 3 min, 0.5 min linear gradient from 99% B to 1%B, hold 1%B for 1 min (total run time: 13.5 min). The autosampler temperature was set on 10 °C, column temperature was 45 °C.

The lipid extract was used for lipidomics analysis on a C8 column (100 mm × 2.1 mm, 1.7 µm; Phenomenex, Aschaffenburg, Germany) and a mobile phase of water with 1% 1 M ammonium acetate and 0.1% acetic acid (eluent A) and acetonitrile/2-propanol 7/3 (v/v) with 1% 1 M ammonium acetate and 0.1% acetic acid (eluent B). The autosampler temperature was set at 10 °C, column temperature was 45 °C. The ultra high performance liquid chromatography (UPLC) gradient was as follow: hold 55%B for 1 min, 1 min linear gradient from 55%B to 65%B, 6 min linear gradient from 65%B to 89% B, 1 min linear gradient from 89%B to 99%B, hold 99%B for 3 min, 0.3 min linear gradient from 99%B to 55%B, hold 55% B for 0.7 min (total run time: 13 min) with a flow rate of 0.4 ml min^{-1}.

Mass spectrometry was accomplished using the Synapt G2-S HDMS mass spectrometer (Waters, Manchester, UK) in the high-resolution mode with electrospray ionization (ESI). Scan time for the MS^E method (centroid) was set to 0.1 s. The instrument was operated in positive (metabolomics) or negative (lipidomics) ionization mode, applying the following ion source parameters: capillary voltage +2.5 kV (ESI^+), −3.0 (ESI^-), sampling cone 20 V, source offset 40 V, source temperature 120 °C, desolvation temperature 450 °C, cone gas flow 2 l h^{-1}, nebuliser 6.5 bar and desolvation gas 850 l h^{-1}, collision energy ramp 20–40 eV. All data were lock mass corrected on the pentapeptide leucine enkephaline (Tyr-Gly-Gly-Phe-Leu, *m/z* 554.2615, $[M-H]^-$) in a solution (1 ng μl^{-1}) of acetonitrile/0.1% formic acid 1/1 (v/v). Scan time for the lock mass was set to 0.3 s, an interval of 15 s and three scans to average with a mass window of ±0.3 Da. Calibration of the Synapt G2-S in the range from *m/z* 50 to 1200 was performed using a solution of sodium formate (5 mmol l^{-1}) in 2-propanol/water 9/1 (v/v).

Isolation of hordatine glucosides from barley grains

Commercial barley grains (Davert, Ascheberg, Germany) were ground and extracted with 2-propanol/water 80/20 (v/v) for 10 min in an ultrasonic bath. The extract was decanted and the residue extracted again two times with 2-propanol/water 80/20 (v/v). The combined supernatants were filtered and concentrated on a rotary evaporator at 30 °C under reduced pressure. A possibly existing lipid phase was removed by membrane filtration. The extract was fractionated *via* medium pressure liquid chromatography (MPLC) using a Sepacore system (Büchi, Flawil, Switzerland) consisting of two C-605 pumps, a C-620 control unit, a C-660 fraction collector and a C-635 UV detector. Stationary phase was a 150 × 40 mm PP cartridge (40 mm × 150 mm) filled with 25–40 µm LiChroprep RP18 material (Merck, Darmstadt, Germany). The mobile phase consisted of 0.1% formic acid in water (eluent A) and methanol (eluent B). Separation was achieved using a flow rate of 40 ml min^{-1} and the following gradient: hold 5%B for 3 min, linear gradient from 5%B to 28%B in 10 min, hold 28%B for 5 min, linear gradient from 28%B to 40%B in 5 min, linear gradient from 40%B to 100%B in 3 min, hold 100%B for 4 min (total run time: 30 min). The effluent was monitored at 280 nm, data were recorded by using Sepacore Control Chromatography Software (version 1.0; Büchi, Flawil, Switzerland).

The MPLC fractions were freeze-dried, and further subfractionated *via* semi-preparative high performance liquid chromatography (HPLC) on a Jasco HPLC system (Jasco, Groß-Umstadt, Germany) consisting of two PU-2087 Plus pumps, a DG-2080-53 degaser, and a MD-2010 Plus diode array detector monitoring the effluent at 280 nm using Chrompass 1.8.6.1 (Jasco) as software. For sample injection, a 7725i type Rheodyne injection valve (Rheodyne, Bensheim, Germany) was used in preparative mode. As stationary phase, a Luna PFP(2) 100 Å column (250 × 10 mm, 5 µm; Phenomenex, Aschaffenburg, Germany) was used. Mobile phase consisted of 30 mM phosphate buffer (pH 2.4, eluent A) and methanol (eluent B). Separation was achieved using a flow rate of 4.4 ml min^{-1} and the following gradient: hold 23%B for 4 min, linear gradient from 23–40%B in 30 min, linear gradient from 40–23%B in 1 min (total run time: 35 min).

The HPLC fractions were dried, dissolved in alkalized water (pH 11) and solid phase extraction (SPE) was carried out to remove the phosphate buffer using Chromabond® C_{18}ec columns (45 µm, 70 ml 10,000 mg^{-1}; Macherey-Nagel, Düren, Germany) which were conditioned with 30 ml methanol, and 30 ml alkalized water (pH 11). After application of the HPLC fraction, buffer was eluted with 30 ml alkalized water (pH 11), and hordatine glucosides were eluted with 30 ml acidified methanol (pH 2.8).

The isolated structures were elucidated using LC-TOF-MS, ^{1}H-NMR, ^{13}C-NMR and 2D-NMR experiments (COSY, HSCQ, HMBC) on a 500 MHz ultrashield plus Avance III spectrometer with a Triple Resonance Cryo Probe TCI probehead (Bruker, Rheinstetten, Germany). Chemical shifts are quoted in parts per million (ppm) relative to the solvent D_2O signal. The pulse sequences for recording 2D-NMR experiments were taken from the Bruker software library. Data were processed using Topspin (version 3.1; Bruker) and MestReNova (version 14.2.3–29241; Mestrelab Research, Santiago de Compostela, Spain).

Reagents and chemicals

The (*Z*)-3-hexenyl acetate (≥ 98%) was purchased from Merck Millipore (Darmstadt, Germany). The reference substances α-linolenic acid (≥ 99%), linoleic acid (≥ 99%), glyceryl trilinolenate (≥ 97%), chlorophyll *a* (≥ 95%) and chlorophyll *b* (≥ 95%) were obtained from Sigma Aldrich (Steinheim, Germany). A mixture of mono- and di-galactosyldiacylglycerides (product nr 840524 and 840523) differing in fatty acids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Acetonitrile, methanol, 2-propanol (Fisher Scientific, Schwerte, Germany), formic acid and acetic acid (≥ 99%; HIPer Solv Chromanorm®, VWR International, Darmstadt, Germany) were of liquid chromatography/mass spectrometry (LC–MS) grade. Water used for LC–MS was purified with an AQUA-Lab-B30-Integrity system (AQUA-Lab, Ransbach-Baubach, Germany).

Statistical analysis

All data were checked for statistical assumptions, *i.e.* homogeneity of variances and normal distribution. To disentangle the most important VOCs that differentiate the volatile profile of differently treated barley plants, the random forest algorithm was investigated (Breiman 2001). Analysis was performed with

RStudio (version 0.98.978; RStudio, Boston, USA). For data of developing *Bh* pustules, an unpaired *t*-test was conducted using GraphPad Prism® 6 (version 6.01). Mass spectrometry data were analysed using MassLynx software (version 4.1 SCN 901; Waters, Manchester). Principal components analysis (PCA) for untargeted metabolomics and lipidomics was conducted using Progenesis QI software (version 3.0; Waters, Manchester) applying the following peak picking conditions: all runs, limits automatic, sensitivity 3, and retention time limits 0.5–11.5 min. Features used for PCA were filtered by means of ANOVA *P*-value ≤0.05 and a max fold change of ≥2. The processed data were exported to EZinfo (version 3.0; Waters, Manchester) and the matrix was analysed by PCA with Pareto scaling. Group differences were calculated using orthogonal partial least squares discriminant analysis (OPLS-DA) and visualized in S-plots.

RESULTS

Z3HAC dominates VOC bouquet of mechanically wounded barley

To evaluate the dynamic behaviour of VOC emission in 13 ± 1-day-old barley plants, we compared the headspace levels of mechanically wounded plants and untreated plants. VOCs were collected with passive absorbers over time periods from 1 to 24 h in a static headspace. PDMS tubes measured the gaseous equilibrium in the static headspace and reflect the relative compound abundance for a certain time point. This method allows a qualitative identification and a semi-quantitative assessment of headspace levels given as peak area normalized by gram fresh weight (peak area g_{FW}^{-1}). A screening of the total ion current (TIC) of all chromatograms was performed using a custom-built method. Only VOCs with a substance matching quality >90% in the data libraries were extracted. In addition, earlier results of other barley studies were used to confirm our VOC identification (Metcalf & Kogan 1987; Piesik *et al.* 2010). Originating from the barley plant itself, 11 compounds could be detected in the headspace of untreated and wounded plants: (*Z*)-3-hexenal, (*Z*)-3-hexenol, (*E*)-2-hexenol, hexanoic acid, (*Z*)-3-hexenyl acetate, heptanoic acid, nonanoic acid, α-terpinolene, β-caryophyllene, butane and 2,3-butanediol (Table S1; Fig. S1). Among VOCs, the group of GLVs was highly elevated after mechanical wounding and dominated the volatile blend, with (*Z*)-3-hexenyl acetate (*Z*3HAC) as the most abundant compound. Directly after mechanical wounding, the relative amount of *Z*3HAC (23,500 peak area g_{FW}^{-1}) was around seven-fold higher than the relative amount in the headspace of untreated control plants (3300 peak area g_{FW}^{-1}). The maximum was reached at 3 hpw (40,000 peak area $g_{FW\text{-}1}$). Subsequently, we recorded a successive decrease of the relative Z3HAC content until 24 hpw (Fig. 1a).

Random Forest analysis of the volatile blend at 1 and 3 hpw classified *Z*3HAC and *Z*3HOL as the most important compounds discriminating between wounded and control plants (Fig. S2). *Z*3HOL, the precursor of *Z*3HAC in the biosynthetic pathway, showed a similar time course, but lower relative headspace levels, with 9400 peak area g_{FW}^{-1} in wounded plants and 260 peak area g_{FW}^{-1} in non-wounded control plants (Fig. 1b).

Z3HAC modifies susceptibility of barley against the biotrophic fungus Bh in a concentration-dependent manner

To investigate the role of *Z*3HAC in the barley pathogen response, we performed a *Bh*-susceptibility assay with barley plants pre-exposed to different concentrations of pure *Z*3HAC. Thereby, we focused on the second leaf of young barley plants and an exposure time of 24 h. Barley plants *cv.* Ingrid were placed in a dynamic setup (Fig. 2) and exposed for 24 h to different headspace concentrations of *Z*3HAC. An empty emitter container served as control. The headspace concentration of 0.1 µM was calculated, referring to previous quantitative experiments, and represents the physiological emission level of young barley plants that are mechanically wounded (Fig. S3). By using a dynamic system, the concentration of *Z*3HAC is expected to decrease rapidly after application. Hence, a dilution factor must be considered with a constant flow rate of 700 ml min^{-1} influx and 400 ml min^{-1} efflux. Therefore, the plants were additionally exposed to 1 µM or 10 µM *Z*3HAC.

We observed an enhanced resistance of barley plants pre-exposed to *Z*3HAC compared to control plants (Fig. 3). All three applied concentrations caused a significantly lower number of developing *Bh* pustules compared to the control, with a mean of 75 developing *Bh* pustules cm^{-2}. The concentration of 0.1 µM Z3HAC affected fungal colonization most strongly, allowing only 42 developing *Bh* pustules cm^{-1}, which represents a reduction of 40%. The number of developing pustules per cm^2 was reduced by about 35% after 1 or 10 µM Z3HAC exposure.

We speculate that the headspace level of Z3HAC is a crucial factor for responses of barley against the biotrophic fungus *Bh*. Therefore, we additionally applied higher concentrations (25 µM or 50 µM) of Z3HAC. Plants pre-exposed to 25 µM Z3HAC showed no difference in disease resistance to non-exposed control plants, whereas a significantly enhanced susceptibility against *Bh* was observed in plants pre-exposed with 50 µM Z3HAC (Fig. 4). The number of developing pustules per cm^2 was around 30% higher than on control plants.

Induced resistance to Bh mediated by plant–plant communication

We also determined resistance in barley induced by complex volatile profiles emitted from mechanically wounded plants using the same experimental setup (Fig. 2). The VOC bouquet emitted by wounded plants elicited a defence response in receiving plants similar to that observed for the Z3HAC treatment with physiologically relevant concentrations. Plants perceiving the volatile wounding bouquet showed a significant decrease of 14% in the number of developing *Bh* pustules per cm^2 compared to control receiver plants (Fig. 5a). To further investigate the effect of exposure time on the defence behaviour, the exposure time was prolonged to 48 h. Again, pre-exposure with volatiles from wounded emitter plants limited the number of developing *Bh* pustules in receiver plants by 45% (Fig. 5b).

Volatiles induce metabolic alterations

To gain a deeper understanding of the observed defence responses at a molecular level, we performed an untargeted

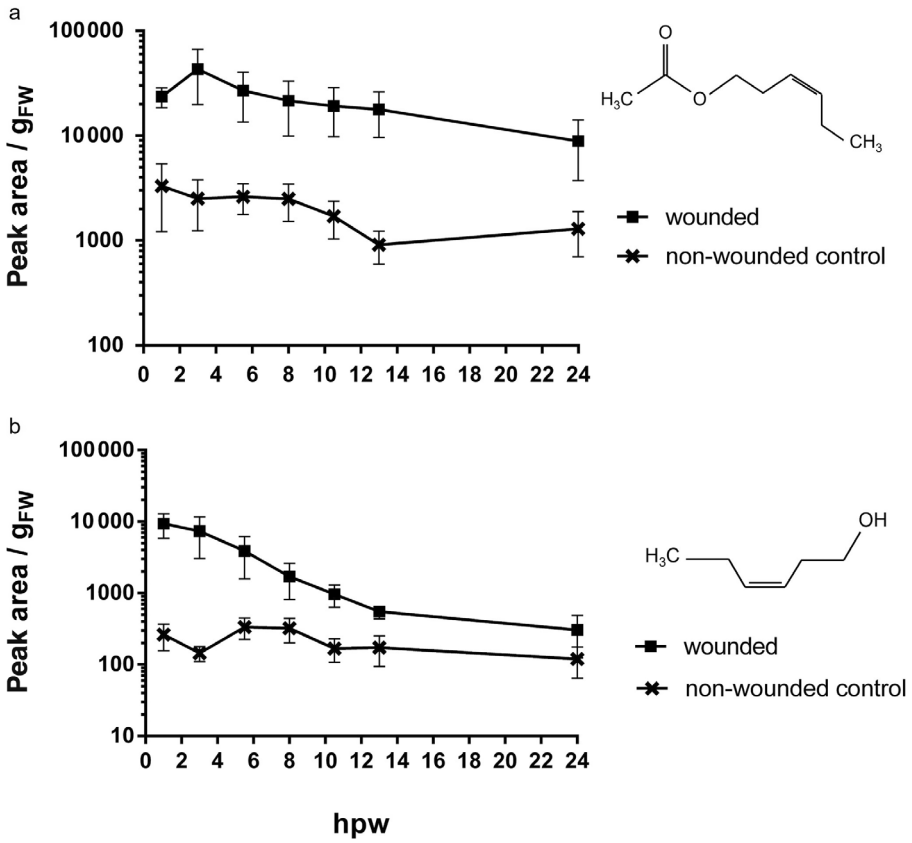

Fig. 1. Chemical structure and relative amounts in the headspace of (*Z*)-3-hexenyl acetate (a) and (*Z*)-3-hexenol (b) after mechanical wounding in comparison to a non-wounded control over time from 1 to 24 h post wounding (hpw). VOCs were collected in a closed headspace with passive absorbers and identified *via* TD-GC/MS (n = 7). For wounding, leaves were strongly damaged with a blunted razor blade, applying consecutive cuts and crushes every few millimetres alongside the leaf blade. Y-axis shows peak area per g fresh weight (peak area g_{FW}^{-1}). Error bars are mean ± SE.

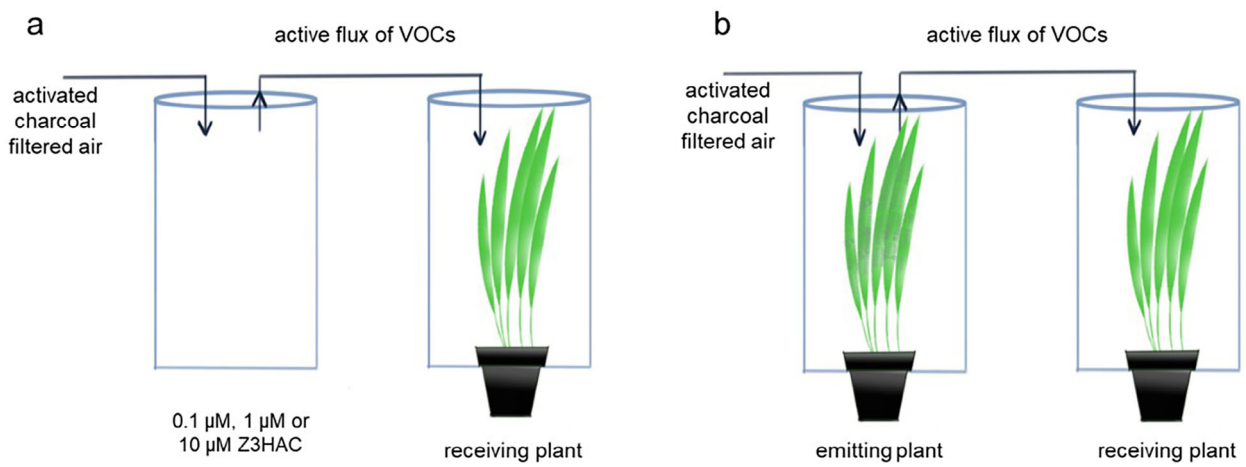

Fig. 2. Schematic setup for investigating VOC-mediated signalling in a dynamic headspace. Activated charcoal-filtered air is actively pushed at 700 ml min^{-1} into the first bag containing Z3HAC dosages (a) or the emitting plant (b), which is either a mechanically wounded plant or a non-wounded control plant. The first bag is closed tightly at both sides including the pot and the soil. A second pump pushes the air at 400 ml min^{-1} from the first bag into the second bag, which contains the receiving plant. The second bag allows passive outflow of air to avoid accumulation of air and humidity. The total volume of this system is 30 l.

metabolomics and lipidomics analysis. Therefore, the metabolome of barley plants pre-exposed to 0.1 µM, 1 µM *Z*3HAC or the complex VOC blend emitted by mechanically wounded plants was compared to untreated control plants using UPLC-TOF-MS measurements. Compounds used for PCA were filtered by means of ANOVA *P*-value ≤0.05 and max fold

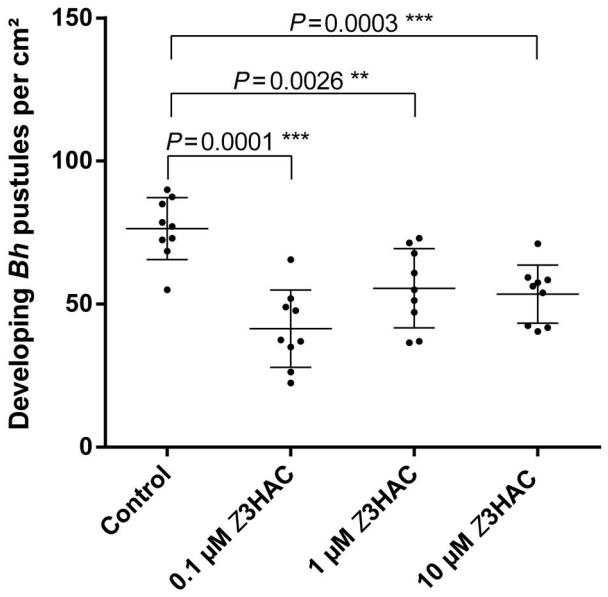

Fig. 3. Developing *Bh* pustules after exposure to 0.1 µM, 1 µM or 10 µM Z3HAC for 24 h. Pre-exposed second leaves of young barley plants (*cv. Ingrid*) were inoculated with *Bh* spores. After 3 days of incubation, developing *Bh* pustules were counted for untreated controls and plants pre-treated with 0.1 µM, 1 µM or 10 µM *Z*3HAC (n = 9). Error bars are mean ± SD ($P < 0.001$ *** or 0.005 **, unpaired *t*-test). Data are 9 technical replicates from three independent biological experiments. One technical replicate represents one barley individual.

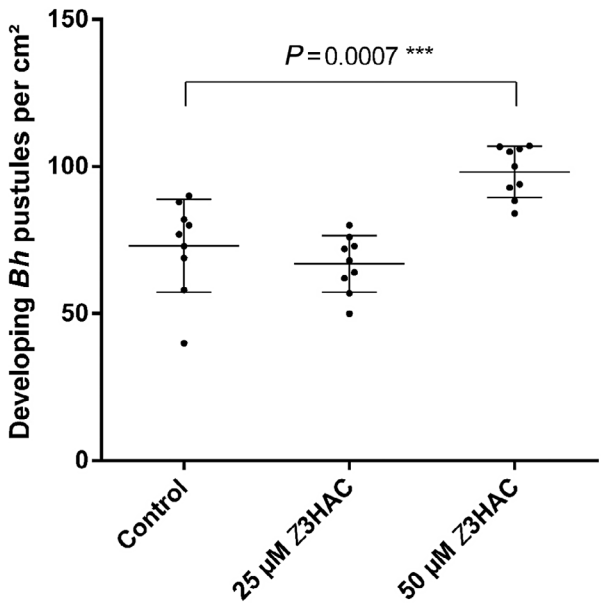

Fig. 4. Developing *Bh* pustules after exposure to 25 µM or 50 µM Z3HAC for 24 h. Pre-exposed second leaves of young barley plants (*cv. Ingrid*) were inoculated with *Bh* spores. After 3 days of incubation, developing *Bh* pustules were counted (n = 9). Error bars are mean ± SD ($P < 0.001$ ***, unpaired *t*-test). Data represent 9 technical replicates from three independent biological experiments. One technical replicate represents one barley individual.

change ≥2. Each treated group was separated from the control group (Fig. S4). Z3HAC-treated barley plants were compared with untreated control plants by means of orthogonal partial least squares discriminant analysis (OPLS-DA) to determine differences in metabolite levels between treatment and control group. Each dot in the resulting S-plot represents a mass-to-charge-retention-time pair (*m/z*-Rt) which corresponds to a molecular compound. Substances with positive loadings that appear on the upper right side of the S-plot are upregulated in *Z*3HAC-treated plants, whereas those with negative loadings on the lower left side are downregulated. We selected marker compounds and characterized them by accurate mass, retention time, MS^2 fragmentation pattern and verification with reference substances. Phosphoglycerols were identified from their specific MS^2 fragmentation, which was in good agreement with the accurate masses of precursor and fragment ions predicted by the product ion calculation tool LIPID MAPS® (mass error ≤5 ppm; Table S4).

The compound *Z*3HAC, as well as the volatile profile of wounded barley, induced an upregulation of both isomers of potentially hordatine A and B and hordatine B glucoside (Fig. 6; Table S2). The presence of hordatine glucosides was verified using reference compounds isolated from barley grains and fully characterized *via* nuclear magnetic resonance spectroscopy (NMR) as well as mass spectrometry (Table S5). The aglycones, hordatine A and B were detected as $[M + H]^+$ and $[M + 2H]^{2+}$ adduct, with the latter being the more intensive ion. This indicated a similar MS^2 fragmentation pattern as the corresponding glucosides (Table S6). The observed data were in agreement with previously published data (Spreng & Hofmann 2018). In addition, *p*-coumaroylagmatine, a precursor in the hordatine biosynthesis, was upregulated in plants exposed to 1 µM *Z*3HAC, but not in plants perceiving the VOC mixture emitted by a mechanically wounded plant. Mass spectrometry data of *p*-coumaroylagmatine (Table S7) were in agreement with the literature (von Röpenack *et al.* 1998).

Z3HAC induces upregulation of chlorophyll- and linolenic acid-containing lipids

In addition to the metabolomics analysis, the lipidome of barley plants exposed to 1 µM *Z*3HAC was examined in comparison to an untreated control. Plants exposed to Z3HAC had elevated levels of chlorophyll *a* and *b* (Fig. 7a). Another, yet unidentified, chlorophyll species (*m/z* 951.5505) shows similar fragment ions as chlorophyll *a* (Table S3) and has a mass difference corresponding to an additional formyl group.

Apart from chlorophyll, the unsaturated fatty acids linolenic acid (18:3) and linoleic acid (18:2) and several lipids with linolenic acid side chains were upregulated in the *Z*3HAC-treated plants. 18:3-fatty acid residues could be observed in lipid components, such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). The proposed metabolites 1,2-dilinolenoyl-3-O-(β-D-digalactopyranosyl)-*sn*-glycerol (DGDG (18:3/18:3)) and 1,2-dilinolenoyl-3-O-(β-D-galactopyranosyl)-*sn*-glycerol (MGDG(18:3/18:3)) were identified using surrogate standards, as described previously (Stark *et al.* 2020). These polar lipids are constituents of the thylakoid membrane and synthesized exclusively in the chloroplasts. Two linolenic acid side chains (18:3/18:3) are the most common combination found in these substances (Reszczyńska & Hanaka 2020).

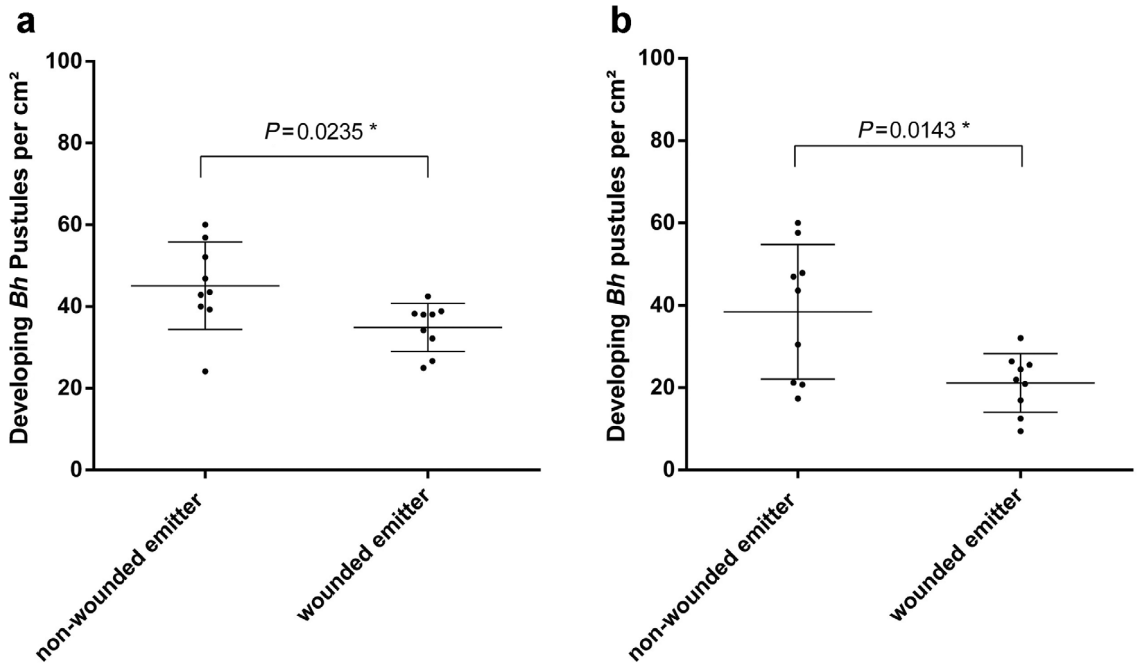

Fig. 5. Developing *Bh* pustules after exposure to the complex bouquet of wounded barley plants for 24 h (a) or 48 h (b). Pre-exposed second leaves were inoculated with *Bh* spores. After 3 days of incubation, developing *Bh* pustules were counted for plants exposed to the bouquet of emitter plants either non-wounded controls or mechanically wounded emitter plants (n = 9). Error bars are mean ± SD ($P < 0.05$ *; unpaired *t*-test- for 24 h, unpaired *t*-test with Welch's correction for 48 h). Data represent 9 technical replicates from three independent biological experiments. One technical replicate represents one barley individual.

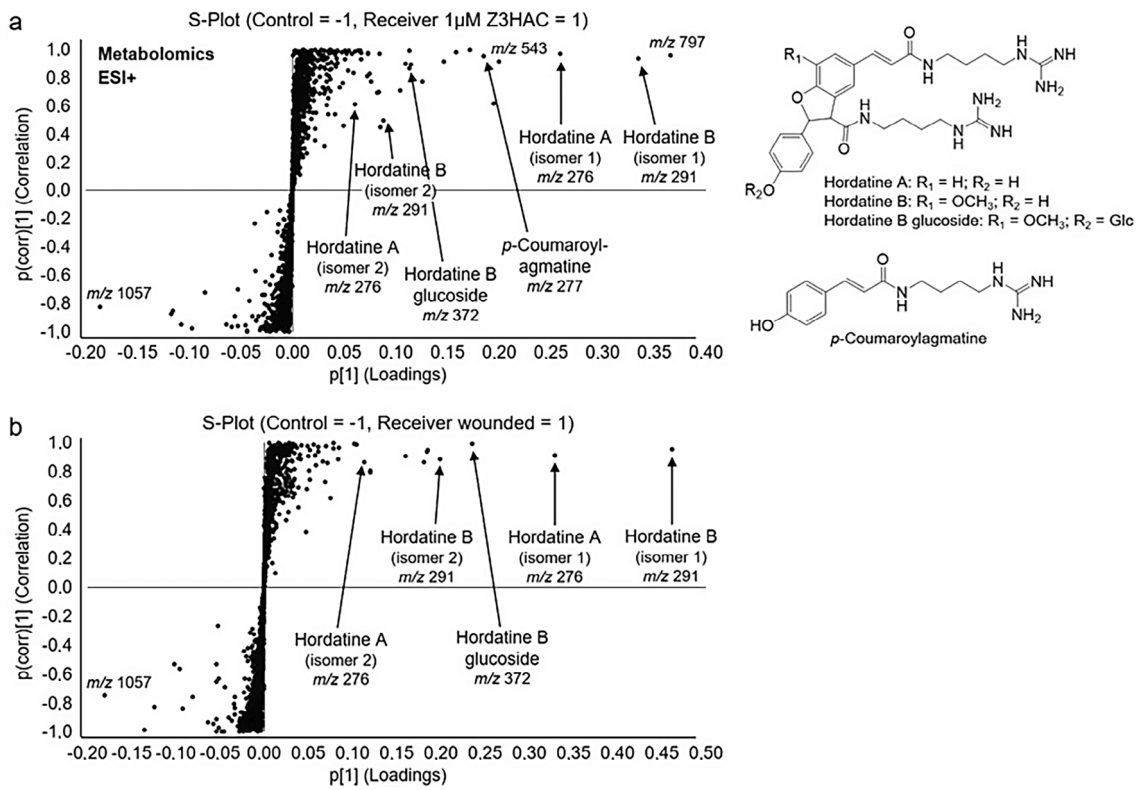

Fig. 6. S-plot of the metabolomics analysis in positive ionization mode of barley plants exposed to 1 µM Z3HAC (a) or the VOC bouquet released by a mechanically wounded emitter plant (b). Substances on the upper right side are upregulated in treated plants, those on the lower left side are downregulated (ANOVA *P*-value ≤0.05, max fold change ≥2). Data represent three independent biological experiments. One biological replicate represents five pooled barley individuals.

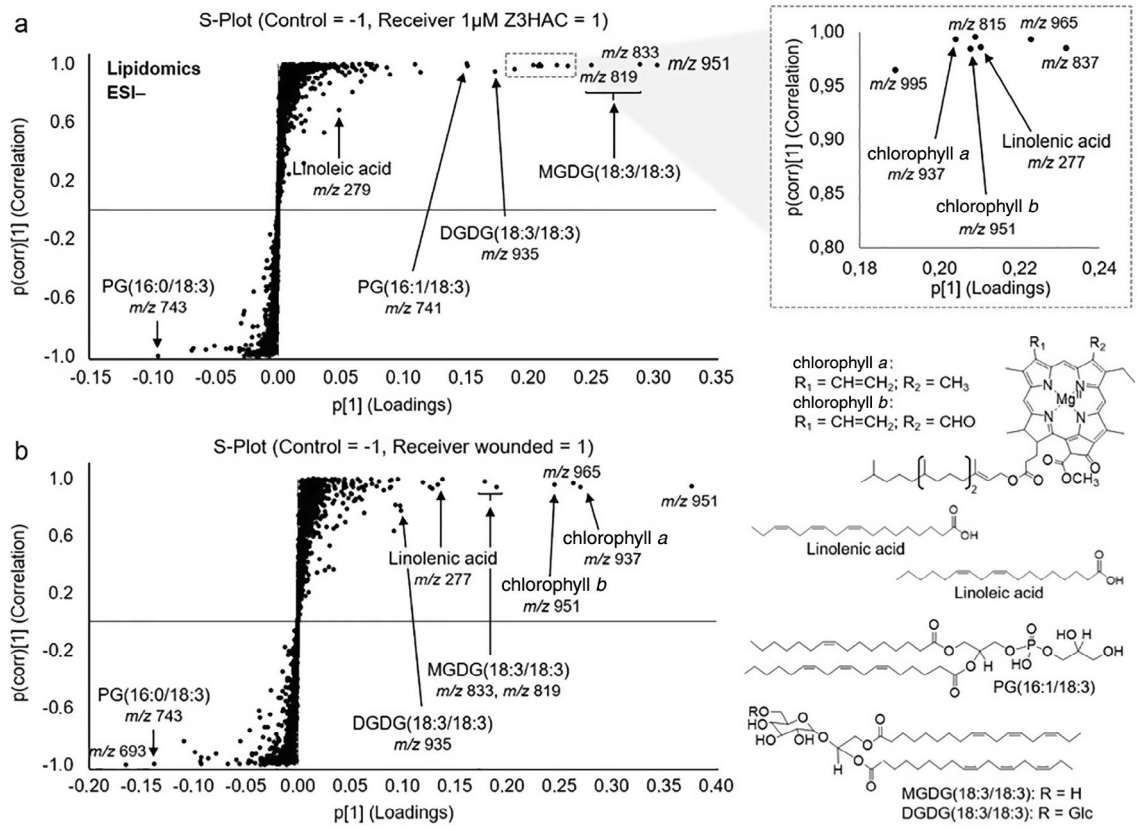

Fig. 7. S-plot of the lipidomics analysis in negative ionization mode of barley plants exposed to 1 µM Z3HAC (a) or the VOC bouquet released by a mechanically wounded emitter plant (b). Substances on the upper right side are upregulated in treated plants, those on the lower left side are downregulated (ANOVA *P*-value ≤0.05, max fold change ≥2). Data represent threeindependent biological experiments. One biological replicate represents five pooled barley individuals.

Phosphoglycerol (PG) lipids are also among the main lipid components of chloroplasts. *Z*3HAC treatment resulted in an upregulation of PG(16:1/18:3) and downregulation of PG (16:0/18:3). Both phosphoglycerols possess an 18:3-side chain and a C_{16}-fatty acid residue. The position at which the linolenic acid is bound cannot be predicted using mass spectrometry. However, naturally occurring phospholipids contain saturated fatty acids at *sn1* and unsaturated at *sn2* (Alfieri *et al.* 2017). A similar picture emerges from recipient plants exposed to a mechanically wounded emitter plant (Fig. 7b).

Emitter and receiver plants reveal contrasting and similar regulation of marker substances

The VOC play an important role in plant-to-plant communication. The volatile signalling molecules are released by a wounded plant tissue and recognized by the surrounding parts of the same or neighbour plants, where defence responses are quickly activated (Heil & Karban 2010; Ameye *et al.* 2018). Nevertheless, the underlying molecular mechanisms in emitter and receiver plants are not well studied. We applied untargeted metabolomics and lipidomics to compare the metabolome of mechanically wounded barley plants with non-wounded control plants. Chlorophyll *a* and *b*, the polar chloroplast lipid DGDG (18:3/18:3) and the phosphogylcerol PG (16:1/18:3) were downregulated after wounding (Table 1). Noticeably, these were among the compounds upregulated in the receiver plants. PG (16:0/18:3) was downregulated in both emitter and receiver plants; the free fatty acids linolenic acid and linoleic acid were upregulated in both emitter and receiver plants.

Table 1. Comparison of significant up- (↑) or down- (↓) regulation of selected compounds in wounded emitter and non-wounded receiver plants.

compound	*m/z*	retention time [min]	emitter up-/down-regulation	receiver up-/down-regulation
Chlorophyll *a*	937.5351 891.5276	8.92	↓	↑
Chlorophyll *b*	951.5139 905.5091	8.16	↓	↑
DGDG (18:3/18:3)	935.5753	6.06	↓	↑
PG (16:1/18:3)	741.4714	5.97	↓	↑
PG (16:0/18:3)	743.4869	6.16	↓	↓
Linolenic acid	277.2162	3.37	↑	↑
Linoleic acid	279.2326	3.94	↑	↑

DISCUSSION

We identified the volatile profile of 13 ± 1-day-old barley plants *cv*. Golden Promise and found that mechanically damaged plants mainly emit GLVs. This VOC group is known as a common chemical defence and signalling measure of plants

and is in the focus of research for biocontrol of pests and diseases (Ninkovic *et al.* 2021). Most studies have focused on later physiological stages and cereal grains or root tissue to characterize VOC emissions from barley (Piesik *et al.* 2011; Gfeller *et al.* 2013; Delory *et al.* 2016). GLVs dominated the total volatile blend, with *Z*3HAC and *Z*3HOL as typical examples of this VOC class. Piesik *et al.* (2010) observed a similar composition of VOCs after mechanical wounding in 6-week-old barley *cv.* Rasik plants. However, the amount and ratio of emitted compounds differed. This might be caused by the different cultivars and developmental stages (Torres *et al.* 2017). We elucidated highly dynamic headspace amounts of GLVs within a time frame of 24 h. The maximum of GLVs were reached in the first 3 h after mechanical damage, and the proportionate abundance of *Z*3HAC and *Z*3HOL gradually decreased until 24 h. It should be noted that, beside the decreasing VOC emission of the plant itself, other reasons such as re-absorption, could cause the observed decrease in the static headspace. PDMS tubes only reflect the relative compound abundance for a certain time period.

The GLVs are suggested as general responses after biotic and abiotic stresses and therefore might play an important role as long-distance signals in stressful environments. Signalling between plants or distinct plant organs has been reported to be mediated by GLVs (Baldwin & Schultz 1983; Ameye *et al.*, 2018; Brosset & Blande 2022). However, the biological function of GLVs from signal perception to signal transduction and physiological responses is poorly understood. Nevertheless, GLVs likely can act as signals to activate defence responses in stressed and in surrounding plants. We show that exposure to the GLV *Z*3HAC or the bouquet of volatiles released by a mechanically wounded emitter plant enhances the resistance of young barley plants to infection with *Bh*. After 24 h or 48 h of VOC exposure, the plants were challenged with *Bh* spores. First developing microcolonies, indicative of successful fungal development, were visible after 3 days. In the case of 48 h exposure time, the volatile profile of a mechanically wounded plant led to a significantly reduced number (45%) of developing pustules compared to a non-pre-exposed control plant. Induced resistance in barley against *Bh* has already been demonstrated after abiotic stresses such as heat (Vallélian-Bindschedler *et al.* 1998), osmotic stress, drought (Wiese *et al.* 2004) and chemicals like chitosan and benzothiadiazole (Beßer *et al.* 2000; Faoro *et al.* 2008). Induction of resistance triggered by VOCs, especially GLVs, has been evidenced for example in maize (Engelberth *et al.* 2004), lima bean (Kost & Heil 2006), poplar (Frost *et al.* 2008), tomato (Finiti *et al.* 2014), potato (Najdabbasi *et al.* 2021) and wheat (Ameye *et al.* 2015).

In addition, the level of included resistance depended on the applied concentration of *Z*3HAC. Physiologically relevant concentrations (0.1 µM, 1 µM or 10 µM) were efficient in resistance induction against fungal infection, whereas a higher concentration (50 µM) induced susceptibility. These observations suggest a concentration-dependent response in volatile-receiving young barley plants. Thus, the VOC composition, exposure time, dose and headspace volume may have an important impact on the resistance response. In nature or greenhouses this may translate into induced resistance or susceptibility, depending on the strength of the emission and the distance of receiver tissue, organ or plants from the emitting GLV source.

The GLVs are involved in a range of interactions and possess antibacterial or antifungal activity (Nakamura & Hatanaka 2002; Kishimoto *et al.* 2008). It has been demonstrated that GLV-priming of plants modulates phytohormonal levels and stress-related gene expression (Engelberth *et al.* 2004; Frost *et al.* 2008; Ameye *et al.* 2015; Najdabbasi *et al.* 2021; Brambilla *et al.* 2022). These responses, and our observations, suggest an important role of GLVs in inducing resistance in barley. In our trials, the number rather than the size of developing *Bh* pustules was reduced in VOC-treated barley, which makes a direct antifungal effect of VOCs unlikely. This is underlined by the fact that higher concentrations of Z3HAC supported the susceptibility and thus did not harm the fungus. From earlier studies, it appears likely that a reduced number of microcolonies can be explained by reduced success in initial cell wall penetration and haustoria formation, which is the pivotal step in early powdery mildew pathogenesis and is crucial for further pathogen development and reproductive success (Eichmann & Hückelhoven 2008). Most recently, Brambilla et al. (Brambilla *et al.* 2022) described that nonanal and β-ionone, emitted by *Pseudomonas syringae* pv. *japonica*-infected barley, show a high potential to induce resistance to *Bh* infection in plants exposed to these VOCs. This shows the potential of VOCs to induce resistance against biotrophic *Bh* in barley. Up to now, it is unclear how plants perceive GLVs (Wang & Erb, 2022), and which specific subsequent physiological responses are activated. Volatile treatments impede pathogen success in barley, but the mechanisms of action and metabolic changes in the receiving plant remain elusive. Therefore, we applied a combined metabolomics and lipidomics approach to obtain insight into up- or downregulation of defence substances and metabolic pathways involved in plant–plant communication.

Both *Z*3HAC and the VOC blend released by a wounded emitter plant induced upregulation of the polyunsaturated fatty acids linolenic acid and linoleic acid, as well as linolenate-conjugated lipids in receiver and emitter plants. Both linolenic and linoleic acids are precursors in the biosynthesis of membrane lipids, but also of oxylipins and GLVs through the lipoxygenase pathway (Matsui *et al.* 2012). Linolenic acid is a better substrate for chloroplast lipoxygenase than linoleic acid (Hatanaka *et al.* 1987). Fatty acids have been described as having antifungal activity (Liu *et al.* 2008). Especially, the accumulation of linolenic acid is associated with stress tolerance (Zhang & Tian 2010) and might be mediated through its oxidation products (Devi *et al.* 2000; Yara *et al.* 2008). If 18:3 fatty acids were released from membrane lipids, this could explain a compensatory upregulation of linolenic acid-containing lipids in receiver plants. By contrast, wounded tissue appears to generally downregulate these chloroplast-associated lipids and chlorophylls, a response that may be associated with stress-induced degradation of photosystems and thylakoid membranes. Both *Z*3HAC and the wounding-induced volatiles caused an upregulation of chlorophyll *a* and *b* in receiver plants. Chlorophylls have an essential role in photosynthesis, which is regulated in plant defence against biotic stress (Pérez-Bueno *et al.* 2019). Tian *et al.* (2019) showed that peanut seedlings pre-exposed to Z3HAC possessed enhanced resistance to salinity stress and a higher net photosynthesis rate and photochemical efficiency of photosystem II. It would also be interesting to study the function of VOCs from wounded tissues in local *versus* systemic tissues and effects on photosynthetic

capacities within one plant. We speculate that VOCs could act as signals for resource re-allocation during response to local tissue damage.

Exposure of plants to Z3HAC or the VOC blend of a wounded emitter plant triggered accumulation of defence-related secondary metabolites, such as hordatines, and their precursor *p*-coumaroylagmatine, in the receiver plant. Hordatine A is a homodimer of *p*-coumaroylagmatine, while hordatine B is a heterodimer of *p*-coumaroylagmatine and feruloylagmatine (Stoessl 1967; Burhenne *et al.* 2003; Gorzolka *et al.*, 2014). Hordatines are described as antifungal compounds against several pathogenic fungi, such as *Botrytis allii*, *Colletotrichum coccodes*, *Fusarium solani*, *Glomerella cingulata*, *Helminthosporium sativum* and *Monilinia fructicola* (Stoessl & Unwin 1970), whereas the activity of its precursor, *p*-coumaroylagmatine, is weak (von Röpenack *et al.* 1998). The exact biological function of the barley-specific hordatines and if they are directly involved in anti-pathogen defence or mediated through their degradation products or integration into the cell wall remains unknown. Nevertheless, the accumulation of antifungal compounds in receiver plants might contribute to the observed increased resistance to fungal infection. Additionally, upregulation or depletion of barley metabolites reveals a global physiological response to the respective volatiles of the emitter, strongly supporting their biological function in plant–plant communication (Table 1). Interestingly, maize plants exposed to *Z*3HAC or indole showed accumulation of defence-related hormones, defence gene transcripts and chemical defence compounds in the class benzoxazinoids. This response and induced resistance to feeding of *Spodoptera littoralis* caterpillars was stronger when a combination of both volatiles was applied (Hu *et al.* 2019). Hence, it seems possible that additive or synergistic activities of diverse volatiles could be exploited to optimize plant protection strategies.

Our data clearly show that VOCs provoke physiological responses in barley and can modify susceptibility to powdery mildew. Nevertheless, the spatial expansion of VOC perception in the neighbourhood of damaged barley tissue in the field remains to be demonstrated. VOCs may also act locally and in short distance signalling and can be considered as resistance-inducing factors in barley. There is an increasing need for alternatives to chemical fungicides in disease management of crop plants. Plant VOCs could be exploited as potential plant protection measures to supplement future agronomic or horticultural glasshouse practices. This may require further research into the underlying molecular mechanisms, controlled application technology and breeding for high VOC sensitivities or for emission of efficient inducer bouquets.

ACKNOWLEDGEMENTS

This research was funded by the Deutsche Bundesstiftung Umwelt (DBU). The work in the Dawid lab was funded by the German Research Foundation in the frame of SFB924/TP-B12. Open Access funding enabled and organized by Projekt DEAL.

AUTHOR CONTRIBUTIONS

Conceptual and experimental design: SL, CD, RH. Funding acquisition: SL, CD, RH. Experimentation, data acquisition, processing and visualization. Writing – original draft: SL, LK. Review and editing: SL, LK, RP, SU, TS, CD, RH. Supervision: RP, CD, RH. Methodological support: SU, TS.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. VOCs originated from untreated and wounded 13-day old barley plants cv. Golden Promise. Green leaf volatiles (GLVs), Terpenoids and Others (origin is unknown) were collected with passive absorbers (PDMS tubes) in a closed headspace and identified by TD-GC/MS in untreated and wounded barley plants. A unique analysis method for barley plants was established with the specific mass-to-charge ratio of the target ion (*m/z*) and the retention time (min) for the listed compounds. The molecular weight in g/mol is given (Pubchem Open Chemistry Datebase, 2016).

Table S2. Secondary metabolites upregulated in barley exposed to Z3HAC.

Table S3. Lipids up- or downregulated in barley exposed to Z3HAC.

Table S4. ESI^--TOF-MS^2 data of phosphoglycerols compared with the accurate masses predicted by the product ion calculation tool LIPID MAPS®.

Table S5. ^1H-/^{13}C-NMR data (500/125 MHz, D_2O, 300 K) of hordatine A glucoside (a), and hordatine B glucoside (b) isolated from barley grains.

Table S6. ESI^+-TOF-MS^2 data of hordatine A and B.

Table S7. ESI^+-TOF-MS^2 data of *p*-coumaroylagmatine.

Figure S1. Gas chromatography (GC) profile of (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate emitted from mechanically wounded or untreated control barley plants 3 h post wounding.

Figure S2. Relative importance of each VOC in the volatile blend of a 13-day old barley plants 1 or 3 h after wounding (hpw) calculated by the random forest algorithm in R!. The 11 compounds emitted by wounded barley plants were sorted by a random forest algorithm according to their relative importance in the complex volatile blend. Results are given in mean decrease accuracy (MDA). VOCs were collected in a closed headspace with passive absorbers (PDMS tubes) and identified *via* TD-GC/MS (n = 7).

Figure S3. Flux of Z3HAC after mechanical wounding in *cv.* Golden Promise compared to non-wounded controls plants. Error bars represent mean ± SD (n = 12). The volatiles emitted by wounded barley were quantified using a dynamic headspace sampling system installed in a climate chamber (York, Johnson Controls, Milwaukee, WI, USA) that allows VOC collection under controlled conditions (Tholl *et al.* 2006). Activated charcoal filtered air was continuously pushed into the bags with a flow rate of 0.6 l/min and an airflow of 0.3 l/min was constantly pulled out of the bag for 120 min. The VOCs were trapped with a glass filter containing 50 mg Super-Q absorbent (Analytical Research Systems, Gainesville, Florida, USA). After collection, the absorbent was washed twice with 200 µl of dichloromethane containing an internal standard (10 ng/µl nonylacetate) in a 1.5 ml screw neck glass vial and stored at 4 °C. Detection was performed using two identical gas chromatographs (GC HP 6890, Agilent, Santa Klara, California, USA). One was connected to an Agilent 5973 mass spectrometry unit for detection of fragmented molecular ions. The other

GC was coupled to a flame ionization detector (FID, H9200 hydrogen generator, Agilent, Santa Klara, California, USA) for quantification.

Figure S4. Principle Components Analysis (PCA) of barley plants treated with 1 μM Z3HAC (green), 0.1 μM Z3HAC (red), and untreated controls (blue) based on an UPLC-ESI-TOF-MS profiling. Data represent 3 independent biological experiments. One biological replicate represents five pooled barley individuals.

REFERENCES

Alfieri A., Imperlini E., Nigro E., Vitucci D., Orrù S., Daniele A., Buono P., Mancini A. (2017) Effects of plant oil interesterified triacylglycerols on lipemia and human health. *International Journal of Molecular Sciences*, **19**, 104.

Ameye M., Allmann S., Verwaeren J., Smagghe G., Haesaert G., Schuurink R.C., Audenaert K. (2018) Green leaf volatile production by plants: a meta-analysis. *New Phytologist*, **220**, 666–683.

Ameye M., Audenaert K., Zutter N., Steppe K., van Meulebroek L., Vanhaecke L., Vleesschauwer D., Haesaert G., Smagghe G. (2015) Priming of wheat with the green leaf volatile Z-3-hexenyl acetate enhances defense against *fusarium graminearum* but boosts deoxynivalenol production. *Plant Physiology*, **167**, 1671–1684.

Baldwin I.T., Schultz J.C. (1983) Rapid changes in tree leaf chemistry induced by damage: Evidence for communication between plants. *Science*, **221**, 277–279.

Beßer K., Jarosch B., Langen G., Kogel K.H. (2000) Expression analysis of genes induced in barley after chemical activation reveals distinct disease resistance pathways. *Molecular Plant Pathology*, **1**, 277–286.

Brambilla A., Sommer A., Ghirardo A., Wenig M., Knappe C., Weber B., Amesmaier M., Lenk M., Schnitzler J.-P., Vlot A.C. (2022) Immunity-associated volatile emissions of β-ionone and nonanal propagate defence responses in neighbouring barley plants. *Journal of Experimental Botany*, **73**, 615–630.

Breiman L. (2001) Random forests. *Machine Learning*, **45**, 5–32.

Brosset A., Blande J.D. (2022) Volatile-mediated plant–plant interactions: Volatile organic compounds as modulators of receiver plant defence, growth, and reproduction. *Journal of Experimental Botany*, **73**, 511–528.

Burhenne K., Kristensen B.K., Rasmussen S.K. (2003) A new class of N-hydroxycinnamoyltransferases. Purification, cloning, and expression of a barley agmatine coumaroyltransferase. *Journal of Biological Chemistry*, **278**, 13919–13927.

D'Auria J.C., Pichersky E., Schaub A., Hansel A., Gershenzon J. (2007) Characterization of a BAHD acyltransferase responsible for producing the green leaf volatile (Z)-3-hexen-1-yl acetate in *Arabidopsis thaliana*. *The Plant Journal*, **49**, 194–207.

Delory B.M., Delaplace P., Du Jardin P., Fauconnier M.-L. (2016) Barley (*Hordeum distichon* L.) roots synthesise volatile aldehydes with a strong age-dependent pattern and release (E)-non-2-enal and (E,Z)-nona-2,6-dienal after mechanical injury. *Plant Physiology and Biochemistry*, **104**, 134–145.

Devi P.U.M., Reddy P.S., Rani N.U., Reddy K.J., Reddy M.N., Reddanna P. (2000) Lipoxygenase metabolites of α-linolenic acid in the development of resistance in pigeonpea, *Cajanus cajan* (L.) millsp, seedlings against *fusarium udum* infection. *European Journal of Plant Pathology*, **106**, 857–865.

Dey S., Wenig M., Langen G., Sharma S., Kugler K.G., Knappe C., Hause B., Bichlmeier M., Babaeizad V., Imani J., Janzik I., Stempfl T., Hückelhoven R., Kogel K.-H., Mayer K.F.X., Vlot A.C. (2014) Bacteria-triggered systemic immunity in barley is associated with WRKY and ETHYLENE RESPONSIVE FACTORs but not with salicylic acid. *Plant Physiology*, **166**, 2133–2151.

Dudareva N., Negre F., Nagegowda D.A., Orlova I. (2006) Plant volatiles. Recent advances and future perspectives. *Critical Reviews in Plant Sciences*, **25**, 417–440.

Eichmann R., Hückelhoven R. (2008) Accommodation of powdery mildew fungi in intact plant cells. *Journal of Plant Physiology*, **165**, 5–18.

Engelberth J., Alborn H., Schmelz E., Tumlinson J. (2004) Airborne signals prime plants against insect herbivore attack. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 1781–1785.

Faoro F., Maffi D., Cantu D., Iriti M. (2008) Chemical-induced resistance against powdery mildew in barley: The effects of chitosan and benzothiadiazole. *BioControl*, **53**, 387–401.

Finiti I., de la O Leyva M., María V.B., Gómez-Pastor R., López-Cruz J., García-Agustín P., Real M.D., González-Bosch C. (2014) Hexanoic acid protects tomato plants against *Botrytis cinerea* by priming defence responses and reducing oxidative stress. *Molecular Plant Pathology*, **15**, 550–562.

Frost C.J., Mescher M.C., Dervinis C., Davis J.M., Carlson J.E., De Moraes C.M. (2008) Priming defense genes and metabolites in hybrid poplar by the green leaf volatile cis-3-hexenyl acetate. *New Phytologist*, **180**, 722–734.

Gfeller A., Laloux M., Barsics F., Kati D.E., Haubruge E., Du Jardin P., Verheggen F.J., Lognay G., Wathelet J.-P., Fauconnier M.-L. (2013) Characterization of volatile organic compounds emitted by barley (*Hordeum vulgare* L.) roots and their attractiveness to wireworms. *Journal of Chemical Ecology*, **39**, 1129–1139.

Giavalisco P., Li Y., Matthes A., Eckhardt A., Hubberten H.-M., Hesse H., Segu S., Hummel J., Köhl K., Willmitzer L. (2011) Elemental formula annotation of polar and lipophilic metabolites using (13) C, (15) N and (34) S isotope labelling, in combination with high-resolution mass spectrometry. *The Plant Journal*, **68**, 364–376.

Gorman Z., Christensen S.A., Yan Y., He Y., Borrego E., Kolomiets M.V. (2020) Green leaf volatiles and jasmonic acid enhance susceptibility to anthracnose diseases caused by *Colletotrichum graminicola* in maize. *Molecular Plant Pathology*, **21**, 702–715.

Gorzolka K., Bednarz H., Niehaus K. (2014) Detection and localization of novel hordatine-like compounds and glycosylated derivates of hordatines by imaging mass spectrometry of barley seeds. *Planta*, **239**, 1321–1335.

Hammerbacher A., Coutinho T.A., Gershenzon J. (2019) Roles of plant volatiles in defence against microbial pathogens and microbial exploitation of volatiles. *Plants*, **42**, 2827–2843.

Hatanaka A. (1993) The biogeneration of green odour by green leaves. *Phytochemistry*, **34**, 1201–1218.

Hatanaka A., Kajiwara T., Sekiya J. (1987) Biosynthethic pathway for C6-aldehydes formation from linolenic acid in green leaves. *Chemistry and Physics of Lipids*, **44**, 341–361.

Heil M., Karban R. (2010) Explaining evolution of plant communication by airborne signals. *Trends in Ecology & Evolution*, **25**, 137–144.

Hu L., Ye M., Erb M. (2019) Integration of two herbivore-induced plant volatiles results in synergistic effects on plant defence and resistance. *Plant, Cell & Environment*, **42**, 959–971.

Kallenbach M., Oh Y., Eilers E.J., Veit D., Baldwin I.T., Schuman M.C. (2014) A robust, simple, high-throughput technique for time-resolved plant volatile analysis in field experiments. *The Plant Journal*, **78**, 1060–1072.

Käsbauer C.L., Pathuri I.P., Hensel G., Kumlehn J., Hückelhoven R., Proels R.K. (2018) Barley ADH-1 modulates susceptibility to Bh and is involved in chitin-induced systemic resistance. *Plant Physiology and Biochemistry*, **123**, 281–287.

Kishimoto K., Matsui K., Ozawa R., Takabayashi J. (2008) Direct fungicidal activities of C6-aldehydes are important constituents for defense responses in Arabidopsis against *Botrytis cinerea*. *Phytochemistry*, **69**, 2127–2132.

KIta N., Toyoda H., Shishiyama J. (1981) Chronological analysis of cytological responses in powdery-mildewed barley leaves. *Canadian Journal of Botany*, **59**, 1761–1768.

Kogel K.-H., Langen G. (2005) Induced disease resistance and gene expression in cereals. *Cellular Microbiology*, **7**, 1555–1564.

Kost C., Heil M. (2006) Herbivore-induced plant volatiles induce an indirect defence in neighbouring plants. *Journal of Ecology*, **94**, 619–628.

Kravchuk Z., Vicedo B., Flors V., Camañes G., González-Bosch C., García-Agustín P. (2011) Priming for JA-dependent defenses using hexanoic acid is an effective mechanism to protect Arabidopsis against *B. cinerea*. *Journal of Plant Physiology*, **168**, 359–366.

Liu S., Ruan W., Li J., Xu H., Wang J., Gao Y., Wang J. (2008) Biological control of phytopathogenic fungi by fatty acids. *Mycopathologia*, **166**, 93–102.

López-Gresa M.P., Payá C., Ozáez M., Rodrigo I., Conejero V., Klee H., Bellés J.M., Lisón P. (2018) A new role for green leaf volatile esters in tomato stomatal defense against *pseudomonas syringe* pv. *Tomato*. *Frontiers in Plant Science*, **9**, 1855.

Maffei M.E. (2010) Sites of synthesis, biochemistry and functional role of plant volatiles. *South African Journal of Botany*, **76**, 612–631.

Matsui K. (2006) Green leaf volatiles: Hydroperoxide lyase pathway of oxylipin metabolism. *Current Opinion in Plant Biology*, **9**, 274–280.

Matsui K., Sugimoto K., Mano J., Ozawa R., Takabayashi J. (2012) Differential metabolisms of green leaf volatiles in injured and intact parts of a wounded leaf meet distinct ecophysiological requirements. *PLOS ONE*, **7**, e36433.

Metcalf R.L., Kogan M. (1987) Plant volatiles as insect attractants. *Critical Reviews in Plant Sciences*, **5**, 251–301.

Mithöfer A., Boland W., Maffei M.E. (2009) Molecular aspects of plant disease resistance. *Chemical Ecology of Plant-Insect Interaction*, **34**, 261–291.

Najdabbasi N., Mirmajlessi S.M., Dewitte K., Ameye M., Mänd M., Audenaert K., Landschoot S., Haesaert G. (2021) Green leaf volatile confers management of late blight disease: A green vaccination in potato. *Journal of Fungi*, **7**, 312.

Nakamura S., Hatanaka A. (2002) Green-leaf-derived C6-aroma compounds with potent antibacterial action that act on both gram-negative and gram-positive bacteria. *Journal of Agriculture and Food Chemistry*, **50**, 7639–7644.

Ninkovic V., Markovic D., Rensing M. (2021) Plant volatiles as cues and signals in plant communication. *Plant, Cell & Environment*, **44**, 1030–1043.

Panstruga R. (2003) Establishing compatibility between plants and obligate biotrophic pathogens. *Current Opinion in Plant Biology*, **6**, 320–326.

Paré P., Tumlinson J. (1996) Plant volatile signals in response to hervbivore feeding. *Behavioral Ecology Symposium*, **76**, 93–103.

Pérez-Bueno M.L., Pineda M., Barón M. (2019) Phenotyping plant responses to biotic stress by chlorophyll fluorescence imaging. *Frontiers in Plant Science*, **10**, 1135.

Piesik D., Łyszczarz A., Tabaka P., Lamparski R., Bocianowski J., Delaney K.J. (2010) Volatile induction of three cereals: Influence of mechanical injury and insect herbivory on injured plants and neighbouring uninjured plants. *Annals of Applied Biology*, **157**, 425–434.

Piesik D., Pańka D., Delaney K.J., Skoczek A., Lamparski R., Weaver D.K. (2011) Cereal crop volatile organic compound induction after mechanical injury, beetle herbivory (*Oulema* spp.), or fungal infection (*fusarium* spp.). *Journal of Plant Physiology*, **168**, 878–886.

Quintana-Rodriguez E., Duran-Flores D., Heil M., Camacho-Coronel X. (2018) Damage-associated molecular patterns (DAMPs) as future plant vaccines that protect crops from pests. *Scientia Horticulturae*, **237**, 207–220.

Reszczyńska E., Hanaka A. (2020) Lipids composition in plant membranes. *Current Opinion in Plant Biology*, **78**, 401–414.

Scala A., Allmann S., Mirabella R., Haring M.A., Schuurink R.C. (2013) Green leaf volatiles: A plant's multifunctional weapon against herbivores and pathogens. *International Journal of Molecular Sciences*, **14**, 17781–17811.

Schulze-Lefert P., Vogel J. (2000) Closing the ranks to attack by powdery mildew. *Trends in Plant Science*, **5**, 343–348.

Spreng S., Hofmann T. (2018) Activity-guided identification of in vitro antioxidants in beer. *Journal of Agriculture and Food Chemistry*, **66**, 720–731.

Stark T.D., Weiss P., Friedrich L., Hofmann T. (2020) The wheat species profiling by non-targeted UPLC–ESI–TOF-MS analysis. *European Food Research and Technology*, **246**, 1617–1626.

Stoessl A. (1967) The antifungal factors in barley. IV. Isolation, structure, and synthesis of the hordatines. *Canadian Journal of Chemistry*, **45**, 1745–1760.

Stoessl A., Unwin C.H. (1970) The antifungal factors in barley. V. Antifungal activity of the hordatines. *Canadian Journal of Botany*, **48**, 465–470.

Tholl D., Boland W., Hansel A., Loreto F., Röse U.S.R., Schnitzler J.-P. (2006) Practical approaches to plant volatile analysis. *The Plant Journal*, **45**, 540–560.

Tian S., Guo R., Zou X., Zhang X., Yu X., Zhan Y., Ci D., Wang M., Wang Y., Si T. (2019) Priming with the green leaf volatile (Z)-3-hexeny-1-yl acetate enhances salinity stress tolerance in peanut (*Arachis hypogaea* L.) seedlings. *Frontiers in Plant Science*, **10**, 785.

Ton J., D'Alessandro M., Jourdie V., Jakab G., Karlen D., Held M., Mauch-Mani B., Turlings T.C.J. (2007) Priming by airborne signals boosts direct and indirect resistance in maize. *The Plant Journal*, **49**, 16–26.

Torres D.P., Proels R.K., Schempp H., Hückelhoven R. (2017) Silencing of RBOHF2 causes leaf age-dependent accelerated senescence, salicylic acid accumulation, and powdery mildew resistance in barley. *Molecular Plant-Microbe Interactions*, **30**, 906–918.

Vallélian-Bindschedler L., Schweizer P., Mösinger E., Métraux J.-P. (1998) Heat-induced resistance in barley to powdery mildew (*Blumeria graminis* f.sp. *hordei*) is associated with a burst of active oxygen species. *Physiological and Molecular Plant Pathology*, **52**, 185–199.

Vicedo B., Flors V., de la O Leyva M., María F.I., Kravchuk Z., Real M.D., García-Agustín P., González-Bosch C. (2009) Hexanoic acid-induced resistance against *Botrytis cinerea* in tomato plants. *Molecular Plant-Microbe Interactions*, **22**, 1455–1465.

von Röpenack E., Parr A., Schulze-Lefert P. (1998) Structural analyses and dynamics of soluble and cell wall-bound phenolics in a broad spectrum resistance to the powdery mildew fungus in barley. *Journal of Biological Chemistry*, **273**, 9013–9022.

Wang L., Erb M. (2022) Volatile uptake, transport, perception, and signaling shape a plant's nose. *Essays in Biochemistry*, **66**, 695–702.

Wiese J., Kranz T., Schubert S. (2004) Induction of pathogen resistance in barley by abiotic stress. *Plant Biology*, **6**, 529–536.

Yactayo-Chang J.P., Hunter C.T., Alborn H.T., Christensen S.A., Block A.K. (2022) Production of the green leaf volatile (*Z*)-3-hexenal by a *Zea mays* hydroperoxide lyase. *Plants*, **11**, 2201.

Yara A., Yaeno T., Montillet J.-L., Hasegawa M., Seo S., Kusumi K., Iba K. (2008) Enhancement of disease resistance to *Magnaporthe grisea* in rice by accumulation of hydroxy linoleic acid. *Biochemical and Biophysical Research Communications*, **370**, 344–347.

Ye M., Liu M., Erb M., Glauser G., Zhang J., Li X., Sun X. (2021) Indole primes defence signalling and increases herbivore resistance in tea plants. *Plant, Cell & Environment*, **44**, 1165–1177.

Yousafi Q., Bibi S., Saleem S., Hussain A., Hasan M.M., Tufail M., Qandeel A., Khan M.S., Mazhar S., Yousaf M., Moustafa M., Al-Shehri M., Khalid M., Kabra A. (2022) Identification of novel and safe fungicidal molecules against *fusarium oxysporum* from plant essential oils: In vitro and computational approaches. *BioMed Research International*, **2022**, 5347224–5347218.

Zhang C., Tian S. (2010) Peach fruit acquired tolerance to low temperature stress by accumulation of linolenic acid and N-acylphosphatidylethanolamine in plasma membrane. *Food Chemistry*, **120**, 864–872.