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Herbivore-induced plant volatiles mediate defense regulation in maize leaves but not in maize roots

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Abstract

Plant leaves that are exposed to herbivore-induced plant volatiles (HIPVs) respond by increasing their defenses, a phenomenon referred to as priming. Whether this phenomenon also occurs in the roots is unknown. Using maize plants, *Zea mays*, whose leaves respond strongly to leaf HIPVs, we measured the impact of belowground HIPVs, emanating from roots infested by the banded cucumber beetle, *Diabrotica balteata*, on constitutive and herbivore-induced levels of defense-related gene expression, phytohormones, volatile and non-volatile primary and secondary metabolites, growth and herbivore resistance in roots of neighbouring plants. HIPV exposure did not increase constitutive or induced levels of any of the measured root traits. Furthermore, HIPV exposure did not reduce the performance or survival of *D. balteata* on maize or its ancestor teosinte. Cross-exposure experiments between HIPVs from roots and leaves revealed that maize roots, in contrast to maize leaves, neither emit nor respond strongly to defense-regulating HIPVs. Together, these results demonstrate that volatile-mediated defense regulation is restricted to the leaves of maize. This finding is in line with the lower diffusibility of volatiles in the soil and the availability of other, potentially more efficient, information conduits below ground.

KEYWORDS
belowground plant-herbivore interactions, plant-plant interactions, priming, root defenses

1 | INTRODUCTION

Upon herbivory, plants emit volatile organic compounds that can repel herbivores and attract their natural enemies (Baldwin, 2010; Turlings & Erb, 2018). These herbivore-induced plant volatiles (HIPVs) can also be perceived by unattacked plant tissues and neighbouring plants, resulting in the direct activation and/or priming of defense and resistance (Baldwin, Halitschke, Paschold, von Dahl, & Preston, 2006; Bouwmeester, Schuurink, Bleeker, & Schiestl, 2019; Erb, 2018;

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Farmer, 2001; Frost, Mescher, Carlson, & de Moraes, 2008; Heil, 2014; Heil & Ton, 2008; Turlings & Erb, 2018). Numerous HIPVs have been found to regulate defenses, including green leaf volatiles such as (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexenyl acetate (HAC) aromatic compounds such as indole, and terpenoids such as (*E*)-β-ocimene (Ameye et al., 2018; Engelberth, Alborn, Schmelz, & Tumlinson, 2004; Erb et al., 2015; Farmer, 2001; Riedlmeier et al., 2017). HIPVs can regulate redox signalling genes (González-Bosch, 2018), early defense signalling genes and proteins such as mitotic-activated protein (MAP) kinases, the biosynthesis of stress hormones such as jasmonates and the expression of direct and indirect defenses (Freundlich & Frost, 2018; González-Bosch, 2018; Hu et al., 2018; Hu, Ye, & Erb, 2018; Kim & Felton, 2013; Martinez-Medina et al., 2016; Mauch-Mani, Baccelli, Luna, & Flors, 2017; Tugizimana, Mhlongo, Piater, & Dubery, 2018; Ye, Glauser, Lou, Erb, & Hu, 2019).

Although defense regulation by HIPVs has been documented extensively in plant leaves, much less is known about this phenomenon in the roots (Delory, Delaplace, Fauconnier, & Du Jardin, 2016). To the best of our knowledge, no study so far investigated the impact of root HIPVs on defense and resistance of neighbouring plants. Roots emit specific volatile blends when attacked by herbivores (Ali, Alborn, & Stelinski, 2010; Delory et al., 2016; Rasmann et al., 2005). These volatiles can diffuse through the soil and alter the behaviour of herbivores and natural enemies (Gfeller et al., 2019; Hiltpold & Turlings, 2008; Xavier, Campos-Herrere, Jaffuel, Roder, & Turlings, 2017). Recent work also found that constitutively released root volatiles can affect growth and defense expression in neighbouring plants (Gfeller et al., 2019; Huang, Zwimpfer, Hervé, Bont, & Erb, 2018). Thus, it is conceivable that roots may also respond to root HIPVs in anticipation of an attack by belowground herbivores.

To test this hypothesis, we investigated HIPV-mediated root interactions in maize, one of the three most important crops worldwide (Shiferaw, Prasanna, Hellin, & Bänziger, 2011). Maize plants are regularly attacked by root herbivores such as rootworms (*Diabrotica* sp.), which can cause substantial damage and yield losses (Tinsley et al., 2016). Upon herbivore attack, maize roots emit distinct blends of HIPVs that contain terpenes such as (*E*)-β-caryophyllene, humulene and copaene (Rasmann et al., 2005; Robert et al., 2012; Robert et al., 2012), but no detectable amounts of indole or GLVs. (*E*)-β-caryophyllene can diffuse up to 20 $cm.h^{-1}$ in the soil matrix (Xavier et al., 2017). To test if maize roots can use root HIPVs to prepare their defense system for incoming herbivore attack, we first assessed the impact of root HIPVs on maize primary metabolism and defense markers in the absence of herbivory. Second, we assessed the impact of root HIPVs on root-herbivory-induced changes in primary metabolism and defense markers. Third, we tested the effect of HIPVs on plant growth and resistance in maize and its ancestor teosinte. Fourth, we conduced cross-exposure experiments to assess the impact of leaf HIPVs on root resistance and vice versa. Together, these experiments yielded no evidence for HIPV-mediated induction of root defenses and suggest that roots do not respond to HIPVs by increasing their resistance to herbivores.

2 | MATERIALS AND METHODS

2.1 | Plants and insects

Maize seeds (*Zea mays* L., var. "Delprim") were provided by Delley Semences et Plantes (DSP, Delley, CHE). Teosinte seeds (*Zea mays parviglumis*) were provided by Ted Turlings, University of Neuchâtel. All plants were germinated in plastic pots (diameter, 4 cm; height, 11.2 cm; Patz GmbH Medizintechnik, Dorsten-Wulfen; DE) as described in Erb et al. (2011). The plants were grown in a greenhouse (26 ± 2°C; 14:10 hr, light [8 a.m.–10 p.m.]: dark; 55% relative humidity). For all experiments, plants with three fully developed leaves were removed from plastic pots and transplanted into L-shaped glass pots (diameter: 5 cm; depth: 11 cm; Verre & Quartz Technique SA, Neuchâtel, CHE) filled with moist quartz sand (10% w/v, Genossenschaft Migros Aare, Urtenen-Schönbühl, CHE). L-pots were wrapped in aluminium foil to keep the root system in the dark and prevent degradation of light-sensitive compounds. After their transfer to L-pots, all seedlings were fertilised twice a week with Hauert Typ K (N:P:K: 16:6: 26%, Hauert HBG SA, Grossaffoltern, CHE). Larvae of the banded cucumber beetle *Diabrotica balteata* (Coleoptera: Chrysomelidae) and of the Egyptian cotton leafworm *Spodoptera littoralis* (Lepidoptera) were used in bioassays below or above the ground, respectively. Eggs of *D. balteata* were kindly provided by Oliver Kindler (Syngenta, Stein, CHE). Hatching larvae were reared on freshly germinated maize seedlings (var. Akku, DSP, CHE). Second-instar larvae were used in the experiments. The larval instars were determined according to the head capsule size as previously described (George & Hintz, 1966). Plant infestations were performed by placing six larvae in two 4–5 cm deep holes in the sand. Eggs of *S. littoralis* were provided by the group of Ted Turlings, University of Neuchâtel and reared on artificial diet until use. Plant infestations with *S. littoralis* caterpillars were conducted by adding three-fourth-instar larvae per plant.

2.2 | Characterisation of root HIPV emission by emitter plants

To assess whether belowground herbivory alters root volatile emissions, 12-day-old plants were transferred in moist white sand (Migros, CHE) in spherical pots (7 cm diameter, Verre & Quartz Technique SA, Neuchâtel, CHE), as described by Hiltpold, Erb, Robert, and Turlings (2011). The spherical pots were wrapped in aluminium foil. Two days later, the plants were either infested with 6 second-instar *D. balteata* or remained uninfested as controls (n = 4 per treatment). The root volatiles were collected 4 days later following the procedure described by Hiltpold et al. (2011). Briefly, the spherical pots were connected with multiple air delivery systems and the volatiles were trapped on SuperQ filters (25 mg of Super-Q adsorbent, 80–100 mesh; Alltech Assoc., Deerfield, IL). Cleaned humidified air was pushed through the system at a rate of 1 L min^{-1} and pulled through the superQ traps at a rate of 0.7 L min^{-1}. Root volatiles were

collected overnight from 7 p.m. to 7 a.m. (12 hr). After his period, the superQ filters were rinsed with 150 μL of dichloromethane. N-octane and nonyl-acetate (Sigma, Buchs, Switzerland) were further added as internal standards (200 ng in 10 μL dichloromethane). The root volatiles were analysed by gas chromatography coupled to mass spectrometry (Agilent 7820A GC coupled to an Agilent 5977E MS, Agilent Technologies, Santa Clara, CA). The aliquot was injected in the injector port (230°C) and pulsed in a spitless mode onto an apolar column (HP-5MS 5% Phenyl Methyl Silox, 30 m × 250 μm internal diameter × 0.25 μm film thickness, J&W Scientific, Agilent Technologies SA, Basel, Switzerland). Helium at a constant flow of 1 mL min^{-1} (constant pressure 8.2317 psi) was used as carrier gas. After injection, the column temperature was maintained at 60°C for 1 min, and then increased up to 250°C at 5°C min^{-1}. Integration parameters were set as follows: initial area reject: 0%, peak width: 0.017 min, initial threshold: 16.5 cps/mAU/mV. Putative volatile identification was obtained by comparing mass spectra with those of the NIST05 Mass Spectra Library.

2.3 | Characterisation of HIPV in roots

To determine HIPVs present in ground roots over time, 12-day-old maize plants were transplanted into L-shaped glass pots. Two days later, half of the plants were infested with 6 second-instar *D. balteata* larvae. Control and infested maize roots were harvested after 1, 2, 3, 4 or 8 days (n = 5–7 per treatment and per time point). The roots were gently washed with tap water and then ground in liquid nitrogen using a mortar and a pestle. An aliquot of 100 mg ground root material was used to characterise root volatiles by solid phase micro extraction gas chromatography coupled to mass spectrometry (SPME-GC–MS, Agilent 7820A GC coupled to an Agilent 5977E MS, Agilent Technologies, Santa Clara, CA). Briefly, the frozen root material was added to a glass vial (20 mL Precision Thread Headspace-Vial) and a 100 μm polydimethylsiloxane SPME fiber (Supelco, Bellefonte, PA) was inserted through the septum of the vial lid (UltraClean 18 mm Screw caps, Gerstel GmbH & Co., Mülheim an der Ruhr, DE) and exposed to the vial headspace for 40 min at 20°C. The fiber was then inserted into the GC injection port (220°C) and desorbed. Chromatography was performed using an apolar column (HP-5MS 5% Phenyl Methyl Silox, 30 m × 250 μm internal diameter × 0.25 μm film thickness, J&W Scientific, Agilent Technologies SA, Basel, Switzerland). Helium was used as carrier gas at a constant pressure of 50.6 kPa. The column temperature was maintained at 60°C for 1 min and then increased to 250°C at 5°C min^{-1} followed by a final stage of 4 min at 250°C. Integration parameters were set as described above. Putative volatile identification was obtained by comparing mass spectra with those of the NIST05 Mass Spectra Library and retention times with those of previous analyses. (*E*)-β-Caryophyllene was identified and quantified using a standard curve of the pure compound diluted in ethyl acetate (Merck KGaA, Darmstadt, DE).

2.4 | Root herbivore migration timing

To determine the most realistic experimental timing for the response phase of neighbouring plants, we evaluated the time window during which *D. balteata* root herbivores are most likely to migrate from an infested to a neighbouring plant. Maize plants were potted into 100 mL pots with 5 mm diameter openings at the bottom. Each pot was placed in a plastic cup (12 × 25 × 10 cm WxLxH, OBI Group Holding SE & Co.KGaA, Schaffhausen, CHE) filled with a 3 cm high layer of tap water. All plants (n = 6) were infested with 6 second-instar *D. balteata* larvae. The larvae moving away from the plant through the openings or from the top of the pot were therefore trapped in water and collected daily. After 1 day, 23.3% of the larvae were recovered outside the pots, and after 4 days, more than 60% had migrated away from the plant (Figure S1). For all subsequent experiments, response plants were thus pre-exposed to root HIPVs for 4 days.

2.5 | Root exposure to belowground HIPVs

To test whether plant exposure to belowground HIPVs induces a response in neighbouring plants, we carried out four sets of experiments (see below) using belowground two-arm olfactometers following previously described methods (Robert, Erb, Duployer, et al., 2012; Robert, Erb, Hibbard, et al., 2012). Briefly, for each experiment maize plants were transplanted into L-shaped glass pots as described above and 2 days later, pots containing plants of similar sizes were connected in pairs using two Teflon connectors and one glass connector (length, 8 cm; diameter, 2.2 cm, VQT, Neuchâtel, CHE). The Teflon connectors contained a fine metal screen (2,300 mesh; Small Parts Inc., Miami Lakes, FL) to restrain the larvae from moving to the second plant. The glass connectors remained empty to only allow volatile compounds to diffuse through the system. Each pair included one emitter plant and one receiver plant. Emitter plants were either infested with 6 second-instar *D. balteata* larvae or remained uninfested as controls. Infesting emitter plants with six *D. balteata* larvae reflects natural herbivore densities. Receiver plants were exposed to emitter plants for 4 days prior to any treatment. After this four-day exposure period, receiver plants were either infested with root herbivores, leaf herbivores or left uninfested depending on the experiment. All paired plants were left connected until harvest.

2.6 | Root responses to root HIPVs

To evaluate how exposure to HIPVs affects the metabolism of maize plants in absence and presence of herbivores, two independent experiments were conducted. In the first experiment, primary metabolism and defenses of receiver plants were characterized after 4 days exposure to volatiles from control or infested plants (HIPVs, n = 9 per treatment). In the second experiment, receiver plants were all infested with 6 second-instar *D. balteata* larvae, and primary metabolism and

defenses were measured 1, 3, 6, 9 and 12 hr later in independent replicates. Because of the limited number of two arm-olfactometers, this experiment was carried out once to measure the plant response at 1, 3 and 6 hr after herbivory (n = 3–4) and once to measure the plant response at 6, 9 and 12 hr after herbivory (n = 3–4). As the plant response in the two experiments was similar at 6 hr, both experiments were pooled (n = 3–7).

In all experiments, maize roots were collected, gently washed with tap water, dried with tissue paper, flash frozen in liquid nitrogen and ground to a fine powder for further analyses. Plant primary metabolism was assessed by measuring sucrose, glucose, fructose and starch using enzymatic assays (Machado et al., 2013; Smith & Zeeman, 2006; Velterop & Vos, 2001), soluble proteins using colorimetric assays (Bradford, 1976; Jongsma, Bakker, Visser, & Stiekema, 1994), free amino acids using derivatisation (AccQ Tag, Waters, Milford, MA) and HPLC-MS (Li et al., 2018) and the expression of the carbohydrate transporters *Zm-stp1*, *Zm-zifl2* by q-RT-PCR (Robert, Erb, Duployer, et al., 2012; Robert, Erb, Hibbard, et al., 2012) (Table S1). A more detailed description of these genes can be found in Table S1. Plant secondary metabolism was characterised by performing untargeted metabolomic analyses by UHPLC-qTOF-MS (Hu, Mateo, et al., 2018), targeted analysis and quantification of concentrations by UHPLC-qTOF-MS (Hu, Ye, & Erb, 2018) and volatile emissions by GC–MS as described above. Full names of benzoxazinoids can be found in Table S2. Plant defense expression was characterised by measuring stress hormones by UHPLC–MS/MS (Glauser, Vallat, & Balmer, 2014) and defense marker genes, including genes involved in volatile production (*Zm-tps23*, *Zm-igl*); hormonal signalling (*Zm-saur2*, *Zm-nced*, *Zm-orp7*, *Zm-lox5 Zm-acs6*) and direct defenses (*Zm-cysII*, *Zm-cyst*, *Zm-serpin*, *Zm-mpi*, *Zm-bx1*, *Zm-pal*, *Zm-pr1*) by q-RT-PCR (Robert, Erb, Hibbard, et al., 2012). For a more detailed description of these genes, refer to Robert, Erb, Hibbard, et al. (2012) and Table S1.

2.7 | Plant and herbivore performance following root exposure to root HIPVs

To determine whether exposure to root HIPVs impacts the performance of root herbivores, belowground two-arm olfactometers were used as described above. After 4 days exposure to control or infested emitter plants, all receiver plants were infested with six preweighed root herbivore larvae (n = 18 per treatment). Four days later, all larvae feeding on receiver plants were recovered and weighed. Maize roots from the plants were collected for damage evaluation (Oleson, Park, Nowatzki, & Tollefson, 2005) and weighed.

2.8 | Cross-exposure experiment

To assess whether priming is tissue-specific, a full factorial design cross exposure experiment was conducted by exposing roots or leaves to volatiles emitted by either control or infested roots or to control or infested leaves of emitter plants (n = 4–5 per treatment). All plants were potted in L-pots as described above. Emitter plants were either infested with 6 second-instar *D. balteata* (root herbivory), three fourth-instar *S. littoralis* larvae (leaf herbivory) or left uninfested. All plants were covered with polyester oven bags (Bratbeutel Tangan N°34, Genossenschaft Migros Aare, Urtenen-Schönbühl, CHE). Emitter and receiver plants were paired as above, but the glass connectors were either used to connect roots to roots, roots to leaves, leaves to roots or leaves to leaves. To connect a leaf compartment, a 3 cm opening was made in the polyester bag to insert the connector. The bag was then sealed around the glass connector with a rubber band and tape. The leaf headspace of emitter plants was connected to a multiple air-delivery system via Teflon tubing. Purified air was pushed through the system at a flow rate of 0.3 L min^{-1} between emitter leaves and receiver leaves or roots. This air flow and time of exposure were chosen to mirror previously published experimental set ups investigating aboveground priming in maize (Erb et al., 2015; Hu, Mateo, et al., 2018; Hu, Ye, & Erb, 2018). No airflow was applied between the root headspace of emitter plants and leaves or roots of exposed plants. After 17 hr exposure to emitter plants (from 5 p.m. to 10 a.m. the next day), all systems were disconnected, and bags removed. Three pre-weighed *S. littoralis* or six pre-weighed second-instar *D. balteata* larvae were added to receiver plants and new polyester bags were added to all plants. After 2 days, all larvae were collected and weighed.

2.9 | Statistical analyses

Statistical analyses were conducted using R (version 3.5.3, https://www.r-project.org) and Sigma Plot (version 13, Systat Software, San Jose, CA). All data sets were tested for normality and heteroscedasticity of residuals using Shapiro–Wilk and Brown-Forsythe tests. Data sets fitting these assumptions were analysed using Student t-tests and analyses of variance (ANOVA). Other data sets were analysed using Mann–Whitney Rank Sum tests (U tests) and ANOVAs on ranks. Unbalanced replicate numbers were due to either uneven number of apparatus or to the pool of two experiments each including one reference treatment. Pooling data sets from different experiments was performed when no effect of the experiment on the reference treatment was observed. Metabolomic and volatile data were analysed using principal component analyses (PCA) followed by powered partial least squares–discriminant analysis (PPLS-DA). The log-abundances (a value of 0.001 was added to each value to avoid zeros) of the same mass features shared across different samples were autoscaled to allow for unbiased comparison of relative profile differences between samples. PCA was performed using the function rda in the statistical package vegan for R. PPLS-DA was performed using functions cppls in package pls and evaluated by estimating the classification error rate using cross-model validation in MVA.cmv and testing the significance of discrimination using permutation tests in MVA.test. Both MVA.cmv and MVA.test were from the package RVAideMemoire. The heat maps represent the log fold change between the different treatments compared to plants infested with root herbivores for 1 hr following exposure to control

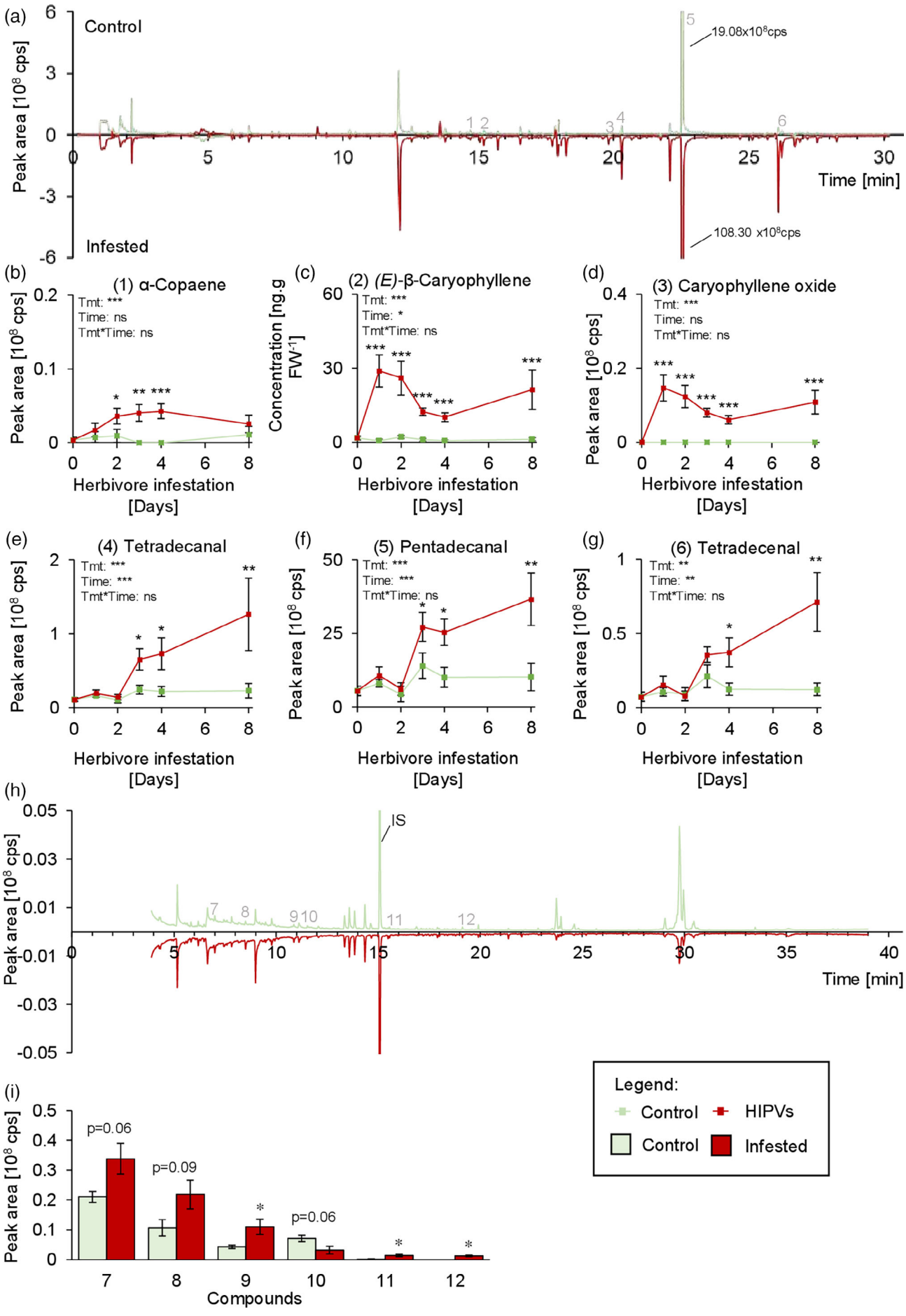


FIGURE 1 Legend on next page.

plants. All heat maps were created using the heatmap.2 function using the statistical packages gplot and RColorBrewer.

3 | RESULTS

3.1 | Root herbivory induces root volatiles

Root herbivory induced distinct volatile metabolites in frozen-ground-thawed roots, including high concentrations of *(E)*-β-caryophyllene, caryophyllene oxide and α-copaene over the entire exposure period (Figure 1a–g and Table S3). To verify whether this shift in the root volatile profiles reflected a shift in volatile emissions, we characterised volatile emissions from control and infested roots in vivo. Although this procedure remains quite challenging belowground, it yields reliable data about actual volatile emissions in the rhizosphere (Grunseich et al., 2020; Gulati, Ballhausen, Kulkarni, Grosch, & Garbeva, 2020; Hiltpold et al., 2011). We detected 25 volatile compounds, none of which overlapped with the compounds found using SPME on frozen-ground-thawed roots. Out of these 25 compounds, 3 were emitted in higher abundance upon herbivory, 2 showed a trend to be released in higher amounts, and 1 was less emitted upon herbivory than in control plants (Figure 1h,i). None of these compounds could be identified using typical known mass fragments or the NIST05 library. The mass spectra of these compounds can be found in Figure S2.

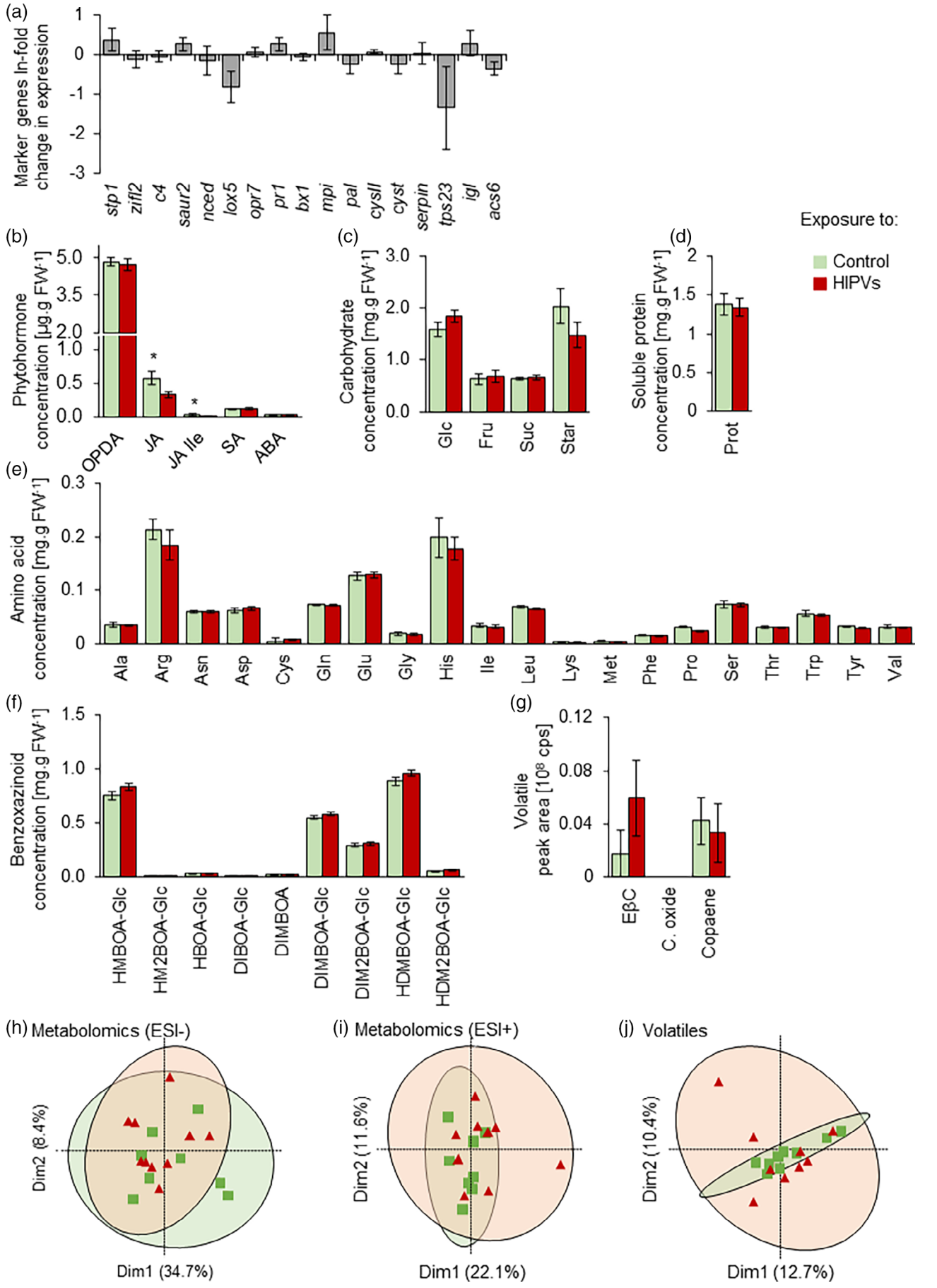
3.2 | Root HIPVs do not directly induce defenses in neighbouring root systems

To evaluate whether belowground exposure to root HIPVs induces physiological changes in neighbouring plants, we characterised the primary metabolism and defenses of maize roots exposed to volatiles emanating from control or root-herbivore infested plants over 4 days. The expression of marker genes involved in plant primary or secondary metabolism was not significantly altered by exposure to root HIPVs (Figure 2a). Phytohormone concentrations were similar between control and HIPV-exposed roots, except for jasmonic acid (JA) and its isoleucine conjugate (JA-Ile), for which levels were slightly lower in HIPV-exposed roots than control roots (Figure 2b). Individual and total soluble sugars, starch, protein, and amino acid concentrations were not affected by exposure to root HIPVs (Figures 2c–e). Also, no significant effects on benzoxazinoids, the most abundant maize root secondary metabolites (Robert, Erb, Duployer, et al., 2012; Robert, Erb, Hibbard, et al., 2012), were observed (Figure 2f). Untargeted metabolomics (511 and 1763 mass features were detected in negative and positive modes, respectively) did not reveal differential clustering of chemicals (Figures 2h,i). Finally, the profile of volatiles in frozen-ground-thawed roots remained unchanged between control and HIPV-exposed plants (Figures 2g,j). For a statistical summary, see Table S4.

3.3 | Root HIPVs do not change root defense induction in neighboring root systems

To investigate whether belowground HIPV-exposure alters responses to herbivory in the roots of neighboring plants, we compared root responses to infestation by *D. balteata* of maize roots exposed to control or to root-herbivore infested volatiles over 4 days. Marker genes involved in plant response to root herbivory (Robert, Erb, Duployer, et al., 2012; Robert, Erb, Hibbard, et al., 2012) responded similarly in control and HIPV-exposed maize plants, with the exception of the ethylene biosynthesis gene *acs6* which was expressed significantly more relative to control plants early after infestation (Figures 3a and S3). Carbohydrate concentrations were similar between control and in HIPV-exposed plants, although HIPV-exposed plants overall had lower fructose concentrations than control plants (Figures 3b and S3). Soluble proteins and amino acids responded to herbivory independently of HIPV exposure (Figures 3b and S3). The production of abscisic acid (ABA), oxo-phytodienoic acid (OPDA) and JA and JA-Ile increased upon root herbivory but was not influenced by HIPV exposure (Figure 3c and S3). Untargeted metabolomics (443 and 1906 features detected in negative and positive modes, respectively) and benzoxazinoid profiling did not reveal differential clustering or differences in concentrations (Figures 3c–e). Volatiles measured in roots by SPME were similarly altered by herbivory, independently of previous exposure to HIPVs (Figures 3c,f). For a statistical summary, see Table S5.

FIGURE 1 Root herbivory triggers the production and emission of a distinct volatile bouquet by maize roots. (a) Representative chromatograms of volatiles produced by control roots (green) and roots infested with *Diabrotica balteata* (dark red) for 4 days. The peak numbering (1–6) corresponds to the compounds significantly different between treatments as listed in Figure 1b–g. (b) α-copaene (1), (c) *(E)*-β-caryophyllene (2), (d) caryophyllene oxide (3), (e) tetradecanal (4), (f) pentadecanal (5), and (g) tetradecenal (6) production by control (green) and infested maize roots (dark red) over 8 days (Mean ± SE, Two-way ANOVA, $n = 5–7$). *(E)*-β-Caryophyllene was identified and quantified using a standard curve of the pure compound. Other compounds were tentatively identified by using the NIST05 library (Match >85%) and retention times correspondence with previous analyses. Tmt, treatment; cps, counts per second; ns, non-significant. (h) Average chromatograms of root volatile emissions of control (green) and infested (dark red) plants 4 days after infestation. The peak numbering 7–11) indicates peaks whose emission was changed ($p < .10$) upon root herbivory. Peaks 10 and 11 were at the limit of quantification. (i) Volatile compounds whose emission was changed ($p < .10$) upon root herbivory (Student *t*-tests and Mann–Whitney Rank Sum tests, $n = 4$). The peak numbering corresponds to compounds whose emission was significantly different between treatments as numbered in Figure 1h. cps, counts per second. Stars indicate significant differences (*$p \leq .05$, **$p \leq .01$; ***$p \leq .001$)



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3.4 | Belowground HIPVs do not increase plant resistance to root herbivory in maize and teosinte

To investigate whether exposure to root HIPVs increases plant resistance in maize or its wild ancestor teosinte, we measured herbivore performance and root damage on control and HIPV-exposed root systems. Exposure to HIPVs emitted by neighbouring plants did not alter the herbivore performance, survival, root damage and root fresh mass in both maize and teosinte (Figures 4& S4 and Table S6).

3.5 | Roots are impaired in the emission and perception of resistance-inducing HIPVs

The fact that roots did not respond to belowground HIPVs could be explained by two mechanisms. A first hypothesis is that root HIPVs are not priming-inducing volatiles. A second hypothesis is that root-HIPVs are priming-inducing agents but roots cannot perceive them. To disentangle between these two possibilities, we conducted an unrealistic cross-exposure experiment. Because leaves can emit and perceive priming-inducing volatiles, we expected that (i) if root-HIPVs were priming agents, maize leaves would respond to their presence, and/or that (ii) if roots were able to perceive priming-inducing HIPVs, they would respond to the leaf HIPV blend. Leaf exposure to leaf HIPVs, but not to root HIPVs, leads to a decreased performance of *S. littoralis* caterpillars (Figure 5a). Root exposure to either leaf or root HIPVs prior infestation did not affect the root herbivore performance (Figure 5b). Thus, root HIPVs do not trigger resistance in roots or leaves, and roots, in contrast to leaves, do not respond to leaf HIPVs through an increase in resistance. This result suggests that maize roots are impaired in both emission and perception of resistance-inducing HIPVs. Statistical data are provided in Table S7.

4 | DISCUSSION

The current work shows that HIPV-mediated defense priming occurs in maize leaves, but not roots. The lack of root HIPV response contrasts with the well-characterised responses in maize leaves to leaf HIPVs (Engelberth et al., 2004; Erb et al., 2015; Heil & Silva Bueno, 2007; Lu, Ye & Erb, 2018; Skoczek et al., 2017) and is discussed in detail below.

Leaves of many different species are known to respond to HIPVs by increasing their defense investment, and, sometimes also reduce their growth. A recent study furthermore found that volatiles that are constitutively emitted by *Centaurea stoebe* lead to changes in root carbohydrate and protein levels in *Taraxacum officinale* (Gfeller et al., 2019; Huang, Gfeller, & Erb, 2019). Importantly, *C. stoebe* is an unusually strong constitutive emitter of root terpenes, thus whether plants respond to herbivory-induced changes in volatile as a form of "eavesdropping" remains unknown. Our study demonstrates that HIPV-exposed maize roots do not display any of the defense responses displayed by maize leaves and leaves of other plant species (Baldwin et al., 2006; Bouwmeester et al., 2019; Erb, 2018; Farmer, 2001; Frost et al., 2008; Heil, 2014; Heil & Ton, 2008; Rodriguez-Saona, Mescher, & de Moraes, 2013; Rodriguez-Saona, Rodriguez-Saona, & Frost, 2009; Turlings & Erb, 2018). Despite prolonged exposure of maize roots to distinct blends of root HIPVs, we did not observe direct induction or priming of stress hormones, primary and secondary metabolites in these roots. On the contrary, we observed that root HIPVs slightly suppressed constitutive JA-Ile levels. This suppression however was gone 1 h after herbivore attack. The majority of evaluated defense marker genes were likewise not differentially expressed, with the exception of the ethylene biosynthesis gene *acs6*, whose suppression upon herbivore attack was delayed in HIPV pre-exposed roots. However, these differences were not associated with measurable changes in metabolite accumulation, resistance or plant growth, despite the well-established roles of

FIGURE 2 Belowground herbivore-induced plant volatiles (HIPVs) do not affect plant metabolism in absence of herbivory. (a) Ln fold changes in gene expression (Mean ± SE, Student's *t*-tests and Mann–Whitney U tests, $n = 9$) in maize roots exposed for 4 days to plants infested with six *Diabrotica balteata* larvae (HIPVs) relative to maize roots exposed to control plants. The description of the selected marker genes can be found in Table S1. (B) Phytohormone concentrations (Mean ± SE, Mann–Whitney U tests, $n = 9$) in maize roots exposed for 4 days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red). OPDA, cis-12-oxo-phytodienoic acid; JA, jasmonic acid; JA-Ile, jasmonic acid isoleucine conjugate; SA, salicylic acid; ABA, abscisic acid. (c–f) Concentrations (Mean ± SE, Student's *t*-tests and Mann–Whitney U tests, $n = 9$) of (c) carbohydrates: Glc, glucose; Fru, fructose; Suc, sucrose; Star, starch; (d) proteins, (e) amino acids (Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine), and (f) benzoxazinoids in roots of maize plants exposed for 4 days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red). Benzoxazinoid full names can be found in Table S2. (g) terpene volatiles emissions by roots of maize plants exposed for 4 days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red). Each symbol represents a single replicate. (h,i Principal Component Analysis of all features detected (PCA, $n = 9$) in roots of maize plants exposed for 4 days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red) using untargeted metabolomic analysis in (h) negative (511 features) and (i) positive modes (1763 features). Each symbol represents a single replicate. (j) Principal Component Analysis of volatile emissions (PCA, $n = 9$). EβC, (*E*)-β-caryophyllene; C. oxide, caryophyllene oxide. Stars indicate significant differences, $^*p \leq .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

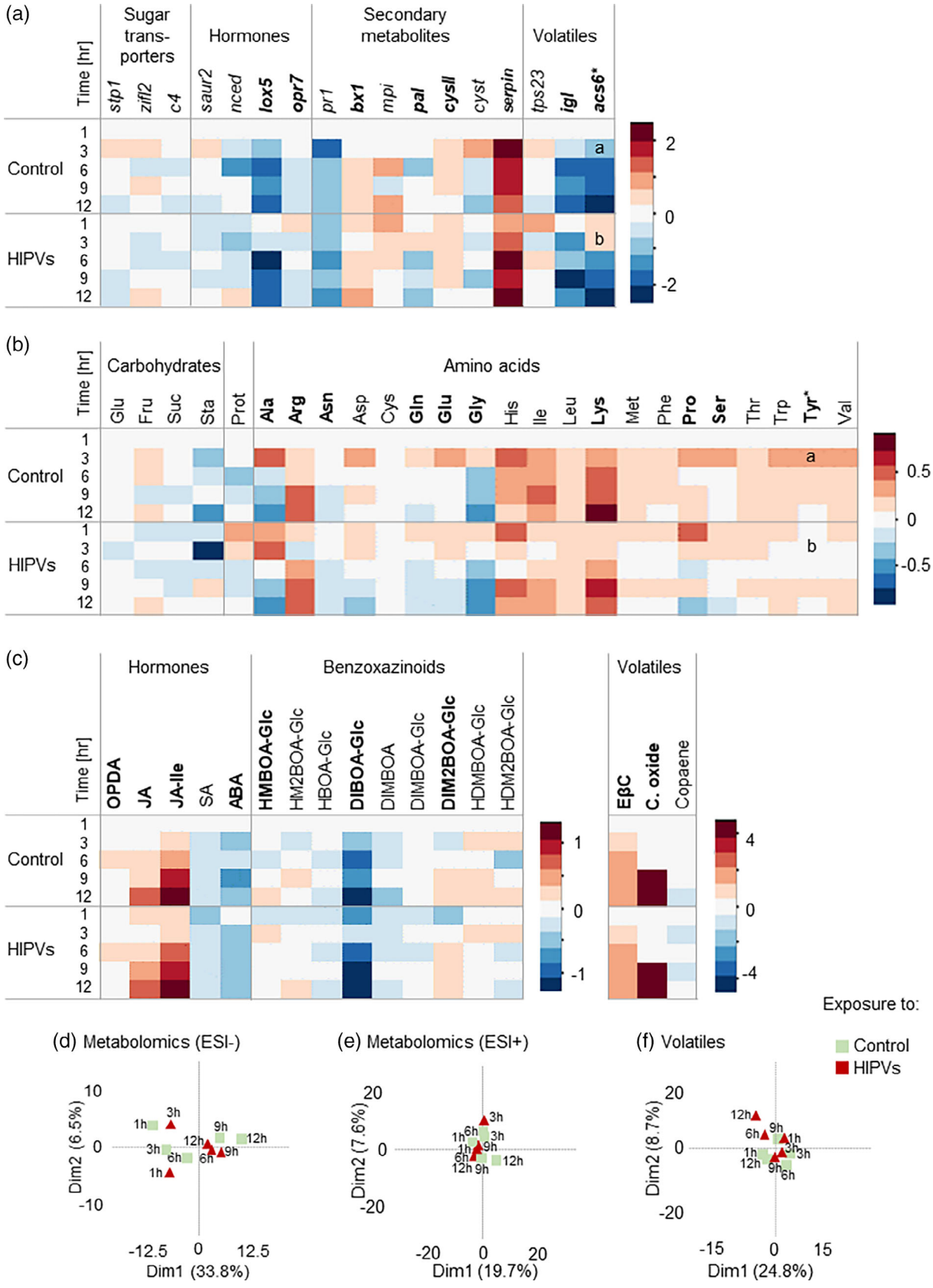

FIGURE 3 Legend on next page.

jasmonates and ethylene in root growth (Dubois, van den Broeck, & Inzé, 2018; H. Huang, Liu, Liu, & Song, 2017; Schaller, 2012; Staswick, Su, & Howell, 1992) and defense (Bonaventure, VanDoorn, & Baldwin, 2011; Erb, Glauser, & Robert, 2012; McConn, Creelman, Bell, Mullet, & Browse, 1997). This absence of phenotypic consequences could be because the changes in Ja-Ile and ethylene biosynthesis were too small and/or transient. Root resistance and plant growth were not affected in teosinte either suggesting that the absence of HIPV responsiveness in maize roots is not due to plant domestication. From these results, we conclude that maize roots, in contrast to leaves, do not strongly respond to root HIPVs.

What are the physiological mechanisms that could be responsible for the tissue-specific absence of responsiveness of maize roots to root HIPVs? Our experiments suggest two mutually non-exclusive mechanisms: Absence of defense-inducing HIPVs and lack of HIPV responsiveness. Regarding the first mechanism, our experiments show that maize roots do not release any HIPVs that have been shown to mediate priming in maize leaves: GLVs and indole (Ameye et al., 2018; Engelberth et al., 2004; Erb et al., 2015; Farmer, 2001; Riedlmeier et al., 2017). Instead, their HIPV profile is dominated by sesquiterpenes (Robert, Erb, Duployer, et al., 2012; Robert, Erb, Hibbard, et al., 2012). Interestingly, and in contrast with a previous study (Hiltpold et al., 2011), we did not detect any (*E*)-β-caryophyllene emissions in vivo. This difference may be explained by methodological differences in herbivory durations, plant age and/or soil microbiota and requires further investigation. Sesquiterpenes have been associated with priming in tomato, beans (Arimura et al., 2000; Arimura, Ozawa, Horiuchi, Nishioka, & Takabayashi, 2001; Zhang et al., 2019), but not in maize (Ruther & Fürstenau, 2005). This suggests that maize roots do not produce HIPV blends capable of triggering defense responses in conspecific neighbours. GLVs are produced via the hydroperoxide lyase (HPL) branch of the oxylipin pathway (Kenji, 2006). The first step of GLV biosynthesis is to deacylate galactolipids to release the omega-3 and omega-6 fatty acids, α-linolenic acid and linoleic acid (Kombrink, 2012; Matsui, Kurishita, Hisamitsu, & Kajiwara, 2000). The hydroperoxidation of α-linolenic and of linoleic acid results in the production of *Z*-3-hexenal and *n*-hexanal, respectively (Moataz, Katsuyuki, Takayuki, Takao, & Kenji, 2017). Yet, maize roots contain only trace amounts of linolenic acid in favour of high concentrations of linoleic acid (Bernklau & Bjostad, 2008). This limitation in linolenic acid contents in the roots may explain the absence of *Z*-3-hexenal, as well as its alcohol and acetyl GLV downstream products (*Z*-3 and *E*-2 hexenol, *Z*-3 and *E*-2 hexenyl acetate). The lack of indole release is likely due to a different mechanism, as indole-3-glycerol-phosphate, the precursor of indole, benzoxazinoids and tryptophane (Frey, Schullehner, Dick, Fiesselmann, & Gierl, 2009), is abundant in maize roots. However, the indole-3-glycerol phosphate lyase (*Igl*), which is responsible for volatile indole production (Frey et al., 2000), is slightly suppressed upon *D. balteata* attack in the roots, which may explain the absence of volatile indole in the headspace of attacked roots. Regarding the second mechanism, our experiments show that maize roots do not seem capable of increasing their resistance in response to bioactive HIPV blends which are capable of inducing resistance in the leaves. This suggests that maize roots can either not perceive or not translate HIPVs into resistance responses. A better understanding of HIPV perception and early signalling will help to test these hypotheses in the future.

FIGURE 3 Exposure to an infested neighboring plant does not change the plant response to *D. balteata's* attack. (a) Heatmap comparison of control- and HIPV-exposed root gene expression upon herbivory. The heatmap visually represents fold changes in marker gene expression of maize roots exposed for 4 days to plants infested with six *Diabrotica balteata* larvae plants prior attack by *D. balteata* for 1–12 hr and maize roots exposed to control plants prior attack by *D. balteata* for 1–12 hr. All data are represented relatively to plants exposed to control plants and then infested for 1 hr (Mean, Two-way ANOVA, $n = 3–7$). Marker genes whose expression was time-dependent are indicated in bold. Marker genes whose expression was affected by previous exposure are labelled with a star. Significant post-hoc comparisons between treatments and within time are indicated with different letters on the corresponding locations on the heatmap. (b) Heatmap comparison of control- and HIPV-exposed root primary metabolism upon herbivory. The heatmap visually represents fold changes in primary metabolite concentrations in maize roots exposed for 4 days to plants infested with six *Diabrotica balteata* larvae plants prior attack by *D. balteata* for 1–12 hr and maize roots exposed to control plants prior attack by *D. balteata* for 1–12 hr. All data are represented relatively to plants exposed to control plants and then infested for 1 hr (Mean, Two-way ANOVA, $n = 3–7$). Glc, glucose; Fru, fructose; Suc, sucrose; Star, starch; Prot, proteins; Ala, Alanine; Arg, Arginine; Asn, Asparagine; Asp, Aspartic acid; Cys, Cysteine; Gln, Glutamine; Glu, Glutamic acid; Gly, Glycine; His, Histidine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Met, Methionine; Phe, Phenylalanine; Pro, Proline; Ser, Serine; Thr, Threonine; Trp, Tryptophan; Tyr, Tyrosine; Val, Valine. Compounds whose levels were time-dependent are indicated in bold. Compounds whose levels were affected by previous exposure are labelled with a star. Significant post-hoc comparisons between treatments and within time are indicated with different letters on the corresponding locations on the heatmap. (c) Heatmap comparison of control- and HIPV-exposed root secondary metabolism upon herbivory. The heatmap visually represents fold changes in hormone levels, secondary metabolite concentrations and volatile present in frozen-ground-thawed roots of maize plants exposed for 4 days to plants infested with six *Diabrotica balteata* larvae plants prior attack by *D. balteata* for 1–12 hr and maize roots exposed to control plants prior attack by *D. balteata* for 1–12 hr. All data are represented relatively to plants exposed to control plants and then infested for 1 hr (Mean, Two-way ANOVA, $n = 3–7$). OPDA, cis-12-oxo-phytodienoic acid; JA, jasmonic acid; JA-Ile, jasmonic acid isoleucine conjugate; SA, Salicylic acid; ABA, abscisic acid. Benzoxazinoid full names can be found in Table S2. EβC, *(E)*-β-caryophyllene; C. oxide, caryophyllene oxide. Compounds whose levels were time-dependent are indicated in bold. (d–f) Principal Component Analysis of all features detected (PCA, $n = 3–7$) in maize roots exposed for 4 days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red) prior attack by *D. balteata* for 1–12 hr, using untargeted metabolomic analysis in (d) negative (443 features) and (e) positive modes (1906 features). (f) Principal Component Analysis of volatile emissions (PCA, $n = 3–7$). In PCAs, each point represents the average per treatment per time point. No interaction between time and exposure was found to be significant in any of the tested markers [Colour figure can be viewed at wileyonlinelibrary.com]

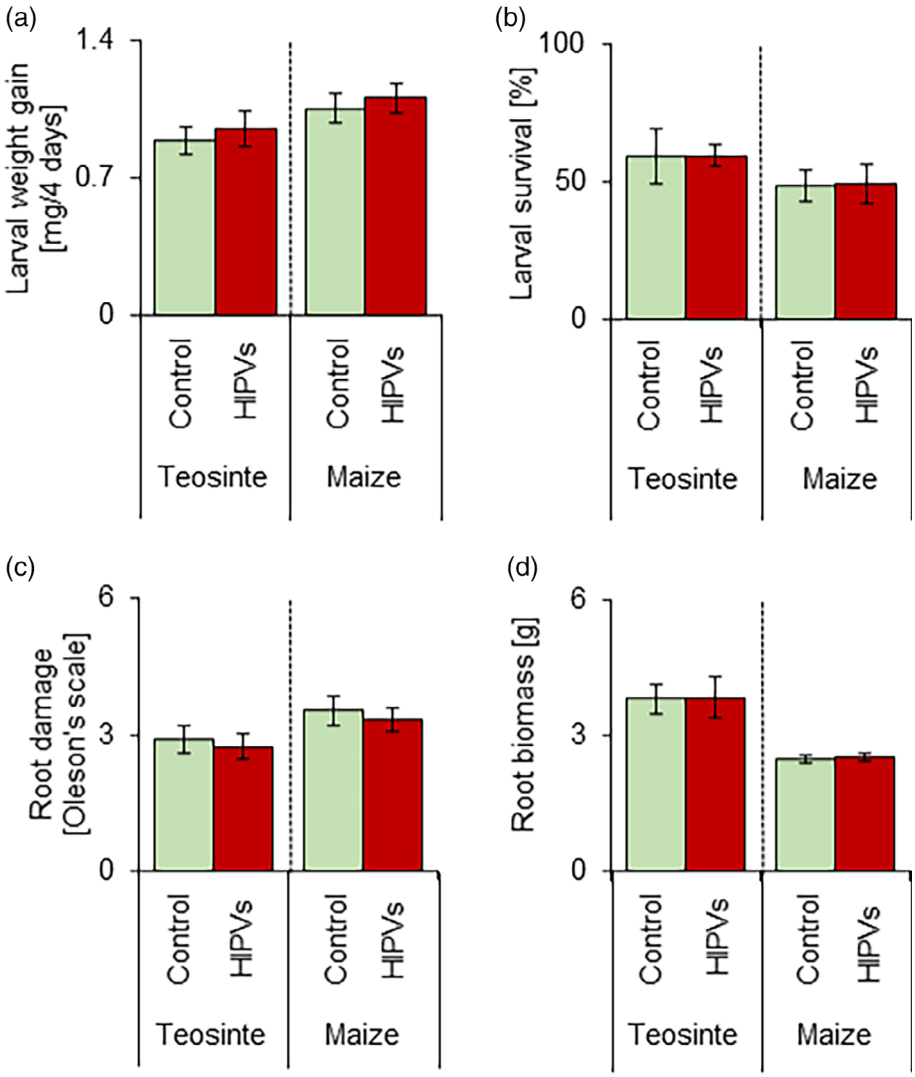

FIGURE 4 Exposure to an infested neighboring plant does not alter plant defense to herbivory. (a) Larval weight gain (Mean ± SE, Student's *t*-tests) of the root herbivore *Diabrotica balteata* feeding for 4 days on maize (n = 17–18) or teosinte (n = 8–9) previously exposed for 4 days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red). (b) Proportions (Mean ± SE, Student's *t*-tests) of *D. balteata* recovered after 4 days infested on maize (n = 18) and teosinte (n = 9) previously exposed for 4 days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red). (c) *D. balteata* damage scaling (Mean ± SE, Student's *t*-tests) after 4 days infestation of maize (n = 18) and teosinte (n = 9) plants previously exposed for 4 days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red). (d) Root fresh mass after 4 days infestation by the root herbivore *D. balteata* (Mean ± SE, Student's *t*-tests) of maize (n = 18) and teosinte (n = 9) previously exposed for 4 days to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red). Spotted lines indicate that maize and teosinte were tested in independent experiments. No significant difference was observed [Colour figure can be viewed at wileyonlinelibrary.com]

Volatile-mediated defense regulation belowground may have failed to evolve if the transfer of HIPVs between plants in the rhizosphere is unreliable. First, volatile dispersal, conversion or degradation in the soil strongly depends on matrix properties (Hayward, Muncey, James, Halsall, & Hewitt, 2001; Hiltpold & Turlings, 2008; Owen, Clark, Pompe, & Semple, 2007; Peñuelas et al., 2014; Perry, Alford, Horiuchi, Paschke, & Vivanco, 2007; Ramirez, Lauber, & Fierer, 2010; Seo, Keum, & Li, 2009; Xavier et al., 2017). Volatile compounds, such as the monoterpenes linalool, α-pinene, and limonene, can be degraded and used as source of carbon for soil dwelling micro-organisms (Misra, Pavlostathis, Perdue, & Araujo, 1996; Owen et al., 2007). The monoterpene alcohol, α-terpineol, can be degraded by micro-organisms immediately upon release and at a rate reaching 13 mg/L/hr (Misra et al., 1996). Second, root HIPVs may be less reliable signals, as soil microorganisms produce a wide variety of volatile compounds. Terpenes such as copaene, (*E*)-β-caryophyllene and caryophyllene oxide, for instance, are also produced by soil dwelling micro-organisms (Delory et al., 2016; Insam & Seewald, 2010; Schenkel, Lemfack, Piechulla, & Splivallo, 2015; Wenke, Kai, & Piechulla, 2010). Thus, we propose that the unreliable transfer and the low specificity of root HIPVs may have impeded the evolution of HIPV-mediated defense regulation and/or priming in maize roots. Instead, alternative strategies to eavesdrop on neighbours may have emerged, including soluble exudates (Chamberlain et al., 2001; Dicke & Dijkman, 2001) or mycorrhizal networks (Perry, 1995; Selosse, Richard, He, & Simard, 2006; Van der Heijden & Horton, 2009).

In summary, our work shows that plant–plant interactions mediated by herbivore-induced plant volatiles may be tissue specific and restricted to the leaves in wild and cultivated maize, and that this tissue-specificity is likely driven by a lack of bioactive cues and a lack of perception capacity of roots. We suggest that the low reliability and specificity of volatiles as danger cues in the rhizosphere together with the availability of other information transfer networks may have impeded the evolution of eavesdropping mechanisms in plant roots.

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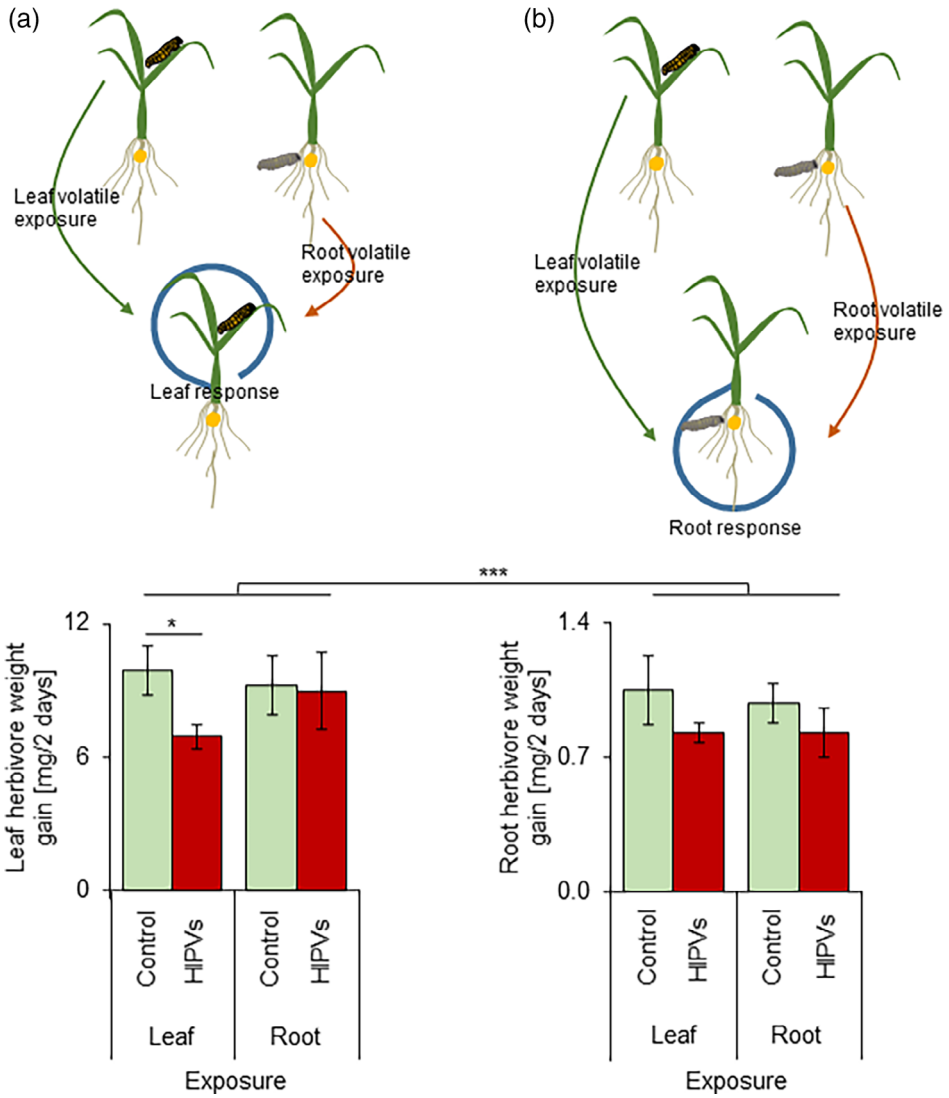

FIGURE 5 Only leaf exposure to leaf HIPVs leads to a decreased performance of *Spodoptera littoralis* caterpillars. (a) Larval weight gain (Mean ± SE, n = 4–5) of the leaf herbivore *S. littoralis* feeding for 2 days on leaves previously exposed for one night to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red). (b) Larval weight gain (Mean ± SE, Two-way ANOVA, n = 4–5) of the root herbivore *D. balteata* feeding for 2 days on roots previously exposed for one night to control plants (control, green) or to plants infested with six *D. balteata* larvae (HIPVs, dark red). Stars indicate significant differences (*$p \leq .05$; ***$p \leq .001$) [Colour figure can be viewed at wileyonlinelibrary.com]

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CONFLICT OF INTEREST

The authors declare having no conflict of interest.

AUTHOR CONTRIBUTIONS

CAMR designed the project. CAMR supervised the project. CvD, TZ, CM, XZ, RARM, PM, MY, BCJS, GG and JDB performed the experiments. CvD, CAMR, TZ, RARM and GG analysed the data. CvD and CAMR wrote the first draft. All authors reviewed and approved the manuscript.

DATA AVAILABILITY STATEMENT

All data are provided as supplementary information.

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SUPPORTING INFORMATION

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