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Herbivore-specific plant volatiles prime neighboring plants for nonspecific defense responses

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Abstract

Plants produce species-specific herbivore-induced plant volatiles (HIPVs) after damage. We tested the hypothesis that herbivore-specific HIPVs prime neighboring plants to induce defenses specific to the priming herbivore. Since *Manduca sexta* (specialist) and *Heliothis virescens* (generalist) herbivory induced unique HIPV profiles in *Nicotiana benthamiana*, we used these HIPVs to prime receiver plants for defense responses to simulated herbivory (mechanical wounding and herbivore regurgitant application). Jasmonic acid (JA) accumulations and emitted volatile profiles were monitored as representative defense responses since JA is the major plant hormone involved in wound and defense signaling and HIPVs have been implicated as signals in tritrophic interactions. Herbivore species-specific HIPVs primed neighboring plants, which produced 2 to 4 times more volatiles and JA after simulated herbivory when compared to similarly treated constitutive volatile-exposed plants. However, HIPV-exposed plants accumulated similar amounts of volatiles and JA independent of the combination of priming or challenging herbivore. Furthermore, volatile profiles emitted by primed plants depended only on the challenging herbivore species but not on the species-specific HIPV profile of damaged emitter plants. This suggests that feeding by either herbivore species primed neighboring plants for increased HIPV emissions specific to the subsequently attacking herbivore and is probably controlled by JA.

KEYWORDS

defense priming, *Heliothis virescens*, herbivore specificity, herbivore-induced plant volatiles (HIPVs), indirect defense, jasmonic acid (JA), *Manduca sexta*

1 | INTRODUCTION

In response to herbivore damage, plants emit a complex blend of volatiles known as herbivore-induced plant volatiles (HIPVs). HIPVs mainly comprise green leafy volatiles (6-carbon compounds viz. aldehyde, alcohol, and esters), terpenes (mono-, homo- and sesquiterpenes), and some aromatic compounds (e.g. benzenoids). Some HIPVs have the capacity to deter herbivores and egg-laying moths as a direct defense, while others recruit natural enemies of herbivores as an indirect defense (Clavijo McCormick et al., 2014). Besides direct and indirect plant defenses, certain HIPVs might also serve as chemical signals for within and between plant signaling/priming (Engelberth, Alborn, Schmelz, & Tumlinson, 2004; Paschold, Halitschke, & Baldwin, 2006).

Exposure to natural or synthetic volatiles enables plants to mount defense responses to actually occurring herbivory more effectively—this process is called priming (Cofer, Seidl-Adams, & Tumlinson, 2018; Engelberth et al., 2004; Erb et al., 2015; Simpson et al., 2011; Ton et al., 2006; Xin, Li, Li, Chen, & Sun, 2016). During the primed state, receiver plants prepare themselves molecularly for future attack with minimal associated costs compared to direct activation of defense (van Hulten, Pelser, van Loon, Pieterse, & Ton, 2006). Over the last three decades, priming has been tested in laboratory or natural

settings for more than 30 plant species of 15 families and the majority of studies showed evidence for inter-plant priming of herbivore resistance (Heil & Karban, 2010; Karban, Yang, & Edwards, 2014).

Plant volatiles convey highly complex information such as genetic background, plant sex, and the identity of attacking herbivores. The extent of induction of plant defense priming in receiver plants depends on the nature of the information transmitted by the volatiles. *Artemisia tridentata* plants recognize "kin" and respond more strongly to volatile signals from clones and close relatives than from distant relatives (Karban & Shiojiri, 2009; Karban, Shiojiri, Ishizaki, Wetzel, & Evans, 2013). Similarly, the dioecious *Baccharis salicifolia* plants can differentiate the sex of conspecific neighbors and respond to their volatiles accordingly. Female receiver plants induce defense responses when exposed to the HIPVs from aphid-damaged female plants but not from similarly treated male plants (Moreira, Nell, Meza-Lopez, Rasmann, & Mooney, 2018a). Moreover, in the case of phloem-feeding insects, *B. salicifolia* plants recognize the herbivore species feeding on the neighboring plants by their HIPVs and induce defenses specifically against the same herbivore species (Moreira, Nell, Katsanis, Rasmann, & Mooney, 2018b).

Plant responses to herbivore attack are highly specific to the herbivore species, their feeding guild and diet breadth. For example, *Manduca sexta* (specialist) feeding on *Nicotiana attenuata* plants induces jasmonic acid (JA) and ethylene (ET) production, while *Spodoptera litura* (generalist) amplifies salicylic acid (SA) production (Diezel, von Dahl, Gaquerel, & Baldwin, 2009). Similarly, *Helicoverpa assulta* (specialist) induces more nicotine and peroxidase (POD) and less polyphenol oxidase (PPO) than *H. armigera* (generalist), but similar amounts of proteinase inhibitors (PIs) and JA in *Nicotiana tabacum* plants (Zong & Wang, 2007). These compounds are antinutritive agents that are toxic to the herbivore (Felton, Donato, Del Vecchio, & Duffey, 1989; War et al., 2012). As for phloem-feeding herbivores, specialist and generalist aphids differentially induce direct defenses in Arabidopsis (Mewis et al., 2006). Whether primed plants also differentially regulate defenses against generalist and specialist herbivores after perceiving herbivore-specific HIPVs from damaged conspecific neighbors has not received much attention (Moreira et al., 2018b).

Jasmonic acid (JA) is recognized as a general defense signal and a common plant defense response to herbivore attack; therefore it is often monitored as a representative plant defense response (Engelberth et al., 2004; Erb et al., 2015; Xin et al., 2016). JA plays a central role in the signaling network that regulates plant responses to herbivore attack. For example, several studies have found that plants that are either unable to produce or perceive JA produce fewer metabolites upon herbivore damage and consequently are highly susceptible to chewing herbivores (Howe, Lightner, Browse, & Ryan, 1996; Paschold, Halitschke, & Baldwin, 2007; Shoji, Ogawa, & Hashimoto, 2008; Thaler, Farag, Paré, & Dicke, 2002; Thaler, Stout, Karban, & Duffey, 2001; Ye et al., 2012). Rescuing JA-deficient mutant plants through exogenous application of JA restores their defense capabilities resulting in decreased abundance and survivorship of herbivores (Thaler et al., 2001). In addition to regulating direct plant defenses, JA also regulates the emission of HIPVs, especially terpenes (Halitschke & Baldwin, 2003).

HIPVs play an important role in indirect plant defenses to herbivores by attracting predators and parasitoids of actively feeding herbivores, thus reducing herbivore load on plants (McCormick, Unsicker, & Gershenzon, 2012; van Poecke & Dicke, 2004). A large number of predators (24 species of 12 insect families) and parasitoids (34 species of 10 insect families) are attracted to natural or synthetic volatiles in laboratory or natural settings (Aljbory & Chen, 2018). Predators and parasitoids prefer plants that emit high amounts of volatiles (Schnee et al., 2006). Non-insect invertebrates like predatory nematodes are also attracted to volatiles emitted by root herbivore damaged plants (Rasmann, Erwin, Halitschke, & Agrawal, 2011; Rasmann & Turlings, 2008).

Most of the research on plant-plant communication mediated by HIPVs is usually performed with a single herbivore species (Erb et al., 2015; Paschold et al., 2006; Ton et al., 2006). Since plants respond to herbivory with herbivore-specific defense responses, such as the emission of specific HIPVs, it begs the question as to whether herbivore-specific HIPVs prime neighboring plants for herbivore-specific defense responses. However, only one study conducted to date focused on herbivore specificity in HIPV-mediated plant defense priming (Moreira et al., 2018b). Adding to this small body of research, here we investigated herbivore-based specificity for chewing insects in plant defense priming. We used two herbivores of the same feeding guild but different diet breadths (*Manduca sexta* (specialist) and *Heliothis virescens* (generalist)) to induce a host plant *Nicotiana benthamiana* (tobacco) to emit herbivore-specific volatiles. We tested the primed state of receiver plants by using a uniform damage treatment and application of regurgitant from the respective herbivores to determine whether induced resistance (JA accumulation and HIPV induction) in primed plants differs with different herbivore species.

2 | MATERIALS AND METHODS

2.1 | Plants and insects

Tobacco plants, *Nicotiana benthamiana*, were grown in a greenhouse at 26 ± 2 °C under a 16:8 hr light: dark photoperiod. *N benthamiana* seeds were kindly provided by Dr. Sarah Hind, University of Illinois, IL. Five-week-old plants with four fully developed leaves were used for all experiments.

Heliothis virescens (Lepidoptera: Noctuidae) were obtained from Benzon Research (PA) and *Manduca sexta* (Lepidoptera: Sphingidae) were kindly provided by Dr. Andrew Stephenson (Pennsylvania State University, PA). Larvae were reared on a commercial artificial diet (Southland product Inc., AR) and maintained in a growth chamber at 25 °C under a 16:8 hr light: dark photoperiod. Early fifth-instar *H. virescens* larvae and early fourth-instar *M. sexta* larvae were used for all experiments.

2.2 | Chemicals

Hexane (>98.5%, J.T.Baker) and dichloromethane (99.9%, OmniSolv, Germany) were used to elute volatiles from Super-Q (Alltech) filters,

and to wash Super-Q filters and glassware. Nonyl acetate (>97%, Aldrich) was used as an internal standard to quantify volatiles. (*Z*)-3-hexenal (50%, SAFC, USA), (*Z*)-3-hexenol (>98%, Aldrich), (*Z*)-3-hexenyl acetate (>98%, Aldrich), (*E*)-2-hexenal (98%, Bedoukian Research Inc., USA), linalool (97%, Aldrich), limonene (97%, Aldrich), (1, 8-) cineol (99%, Sigma-Aldrich), myrcene (90%, Aldrich), α-pinene (99%, Aldrich), β-pinene (99%, Aldrich), (*E*)-β-farnesene (99%, mixtures of isomers, Sigma-Aldrich) were used to identify the compounds by comparing the mass spectra and retention times in GC-MS and GC-FID, respectively.

2.3 | Herbivore regurgitant collection

Herbivore regurgitant was collected from fourth to fifth instar larvae that had been feeding on *N. benthamiana* plants for 24 hr. Larvae were squeezed behind the head and regurgitant was collected into a 4-mL glass vial set in dry ice. Regurgitant was centrifuged at 10,000 rpm for 3 min, and the resulting supernatant was collected and diluted in water (1:1, by volume) before use.

2.4 | Generation of priming volatiles

We first analyzed the volatile profile produced by plants damaged either by *M. sexta* or *H. virescens* for 48 hr. These two herbivore larvae differ in size and feeding style. In order to obtain comparable plant damage, we synchronized the amount of tissue removed per day. Since the amount of tissue removed by four fifth-instar *H. virescens* larvae was the same as that by one fourth-instar *M. sexta* larva, this respective number of larvae was used in the feeding experiment and priming phase of priming and cross-priming experiments. The larvae continuously fed on the plant during volatile collections in the feeding experiment.

2.5 | Priming experiments with individual herbivores

2.5.1 | Volatile collection and analysis

To examine whether HIPVs emitted by insect damaged *N. benthamiana* prime neighboring plants, intact receiver plants were exposed to volatiles from herbivore-damaged emitter plants. Two *N. benthamiana* plants were placed inside a bell jar (10 L); one was treated as an emitter plant and the other as a receiver plant. The emitter plant was enclosed inside a wire cage to prevent herbivore escape. For HIPV-exposure treatments, receiver plants were exposed to emitter plants damaged either by four fifth-instar *H. virescens* larvae or by one fourth-instar *M. sexta* larva. For the control treatments, receiver plants were exposed to constitutive *N. benthamiana* volatiles (NbVOCs). After 48 hr of exposure to volatiles, individual receiver plants were transferred to a clean bell jar (8 L) with 4-ports for volatile collections. In order to reduce variability due to differential damage by insect herbivory, all receiver plants were challenged by mechanical wounding followed by herbivore regurgitant (R) or water (W) application. A fine cheese grater (3 cm × 2 cm) with 1 mm hole diameters was used to damage receiver plants (Figure **S1a**). The fine cheese grater made 30 holes cm^{-2} (i.e., 180 holes per wounding site) and simulated typical herbivore damage (Heil et al., 2001; Heil et al., 2012). However, a single wounding event is not sufficient to mimic continuous herbivore feeding because it elicits a quick burst of JA accumulation that reaches a maximum at 30 to 45 min after wounding and then drops back down to basal levels (Heil et al., 2001; Ziegler, Keinänen, & Baldwin, 2001). Therefore, to mimic continuous feeding by herbivores, receiver plants were repeatedly challenged every 4 hr during the light period (starting at 9:30 a.m. on the first day and 5:30 a.m. on the second day) for two days (Paschold et al., 2006). During each wounding event, two leaves were damaged by wounding once on each side of the mid rib (Figure **S1b**). Immediately after wounding, 10 μL regurgitant (diluted with water, 1:1 v/v) or 10 μL distilled water was applied to each wound. Volatiles emitted by receiver plants were collected in 4-hr intervals on SuperQ filters for 36 hr. SuperQ filters consist of a 5 cm long borosilicate glass tube (4 mm ID) with one end sealed with stainless steel mesh (type 304), inserted inside a 6.5-cm long Teflon tube. The glass tube is then packed with 30 mg of 80/100 mesh SuperQ absorbent (Alltech), held in place with a disc of stainless steel mesh (type 304). A 3.2-cm long glass tube is inserted below the mesh. Its extending edge is cut at a 45-degree angle to serve as a drip tip collection point for the elution buffer. During the entire experiment, there was continuous airflow inside the bell jar (push air: 1 L min^{-1}, pull air: 0.8 L min^{-1}). We started all the experiments at 9:30 a.m. unless otherwise mentioned in the figure legend.

Volatiles collected on a Super-Q filter were eluted with 120 μL of a hexane: dichloromethane (1:1 v/v) mixture containing 4 ng μL^{-1} of nonyl-acetate as an internal standard. Eluted volatiles were analyzed by gas chromatography with an Agilent 6890 GC-FID (Agilent, CA) equipped with a capillary column (HP-5, 30 m × 0.25 mm ID × 0.25 μm film thickness; Supelco, PA). An aliquot of 1 μL per sample was injected into the GC inlet by an automated injection system. The initial GC oven temperature was held at 40°C for 2 min, followed by a linear temperature increase of 4°C min^{-1} until 150°C was reached, after that the linear temperature increase was set to 40°C min^{-1} until 290°C was reached. Then the oven was baked out at 290°C for 3 min. Helium at a constant pressure of 14.79 psi was used as the carrier gas. GC Chemstation software was used to calculate the peak area of detected volatiles. The quantity of volatiles was calculated relative to the peak area of the internal standard. Identification of most volatiles was based on spectrometric analysis of selected samples with an Agilent 6890N GC interfaced with an Agilent 5973N mass spectrometer (MS) detector and equipped with the same column. The method parameters used in GC-MS were similar to those used in GC-FID. Peaks for (*Z*)-3-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, linalool, limonene, (1, 8-) cineol, myrcene, α-pinene, β-pinene, sabinene and (*E*)-β-farnesene were identified by comparing mass spectra and retention times with authentic synthetic compounds. Other compounds were identified by comparing the mass spectrum to those in the NIST GC-MS library (GCMS Solution, Shimadzu, MD).

2.5.2 | Phytohormone analysis

The phytohormone, jasmonic acid (JA), was measured in receiver *N. benthamiana* plants after 48 hr of exposure to volatiles and subsequent challenge. To challenge the receiver plant, the third leaf from the bottom was selected and marked. Two wounds, one on each side of the midrib, were made using a fine cheese grater (3 cm × 2 cm) with 1 mm hole diameters as described above. Immediately after wounding, 10 µL of regurgitant (diluted with water, 1:1 v/v) or distilled water was applied to each wound. Only the damaged portion of the leaf was harvested 30 min after challenge and flash frozen in liquid nitrogen. The tissue was stored at −80°C until analysis. JA was analyzed according to the hormone analysis protocol as described by Engelberth, Seidl-Adams, Schultz, and Tumlinson (2007). JA was extracted from approximately 100 mg ground-up plant tissue. Briefly, ground-up tissue was transferred into 2-mL screw-cap FastPrep tubes (Qbiogene, CA) containing 1 g of Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, NJ), 400 µL extraction solvent (isopropanol: H_2O, 2:1 v/v, adjusted to pH 3 with hydrochloric acid) and 200ng dihydrojasmonate (dhJA) as an internal standard. The samples were vortexed thoroughly and 1 mL of dichloromethane was added to each sample, shaken for 20 sec in a FastPrep FP 120 tissue homogenizer (Qbiogene) and centrifuged at 10,000 rpm for 2 min. The bottom organic phase (dichloromethane and isopropanol layer) was transferred to a 4-mL screw cap glass vial, dried under nitrogen gas, and redissolved in 200 µL diethyl ether: methanol (9:1 v/v). Carboxylic acids were esterified by adding 3 µL trimethylsilyldiazomethane solution (2 M in hexanes, Aldrich). The vial was then capped, vortexed, and incubated at room

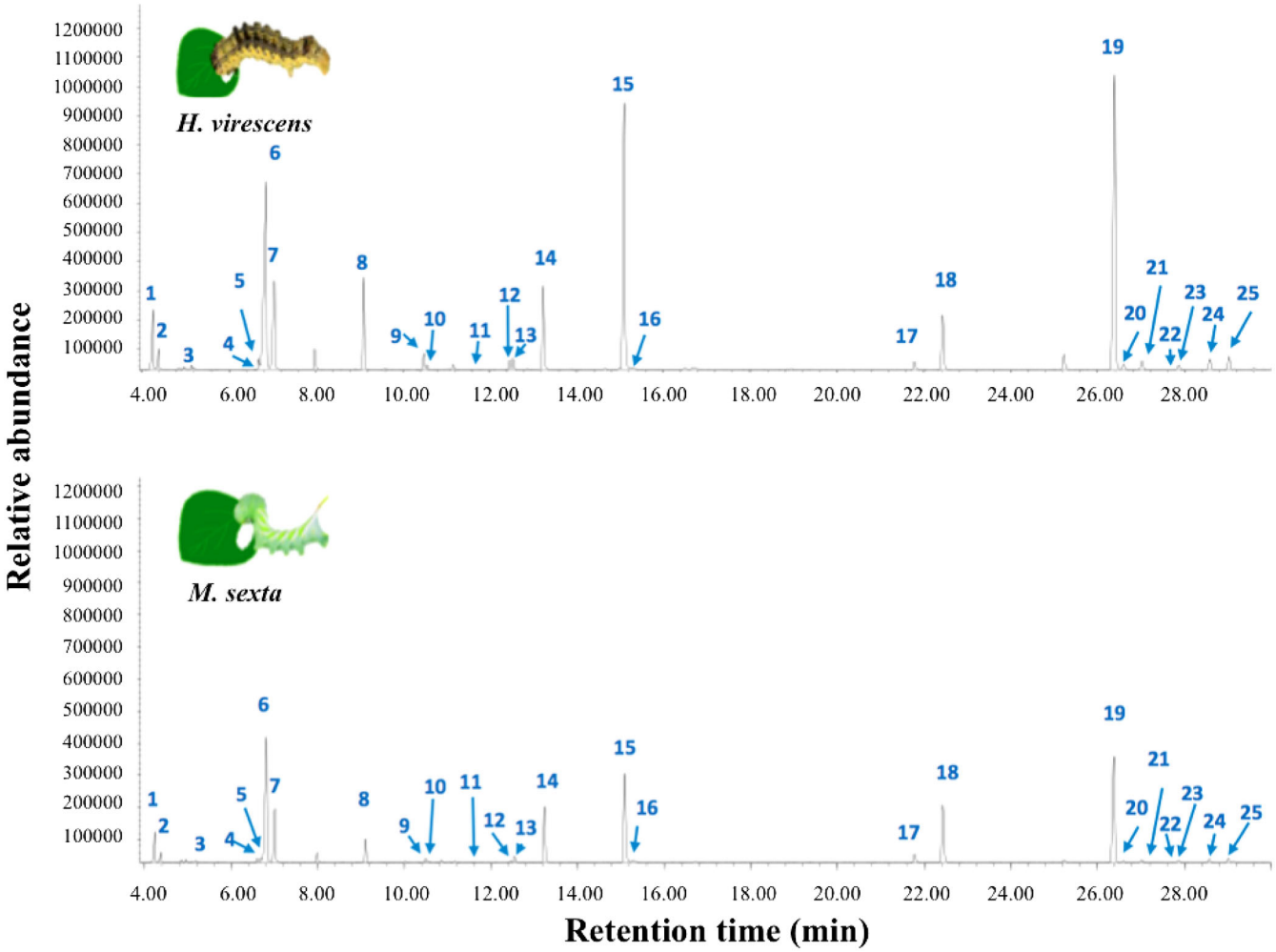

FIGURE 1 Chromatographic profiles of HIPVs collected from 24 to 28 hr after feeding by *H. virescens* or *M. sexta* on *N. benthamiana* plants. *N. benthamiana* plants were damaged by continuous feeding either by early fourth-instar *M. sexta* (one per plant) or by early fifth-instar *H. virescens* (four per plant) for 48 hr. HIPVs were collected in 4-hr intervals after feeding initiation. The chromatogram represents HIPV profile collected from 24 to 28 hr after feeding initiation by *H. virescens* (top) and *M. sexta* (bottom). Five major families of HIPVs were induced by herbivore feeding on *N. benthamiana* plants: **green leaf volatiles (GLVs)**: (*Z*)-3-hexenal [3], (*E*)-2-hexenal [4], (*Z*)-3-hexenol [5], (*Z*)-3-hexenyl acetate [11], **monoterpenes:** α-pinene [8], sabinene [9], β -pinene [10], limonene [12], 1,8-cineol [13], (*E*)-β-ocimene [14], linalool [15], nonanal [16], **sesquiterpenes**: (*E*)-α-bergamotene [19], unknown sesquiterpene#1 [20], (*E*)-β-farnesene [21], aristolochene (4,4-di epi) [22], unknown sesquiterpene#2 [23], β-bisabolene [24], β-sesquiphellandrene [25], **aldoximes**: propyl aldoxime, 2 methyl-,syn-[1], propyl aldoxime, 2 methyl-,anti-[2], butyl aldoxime, 2 methyl-,syn- [6], butyl aldoxime, 2 methyl-,anti-[7], and **aromatic compound**: indole [17]

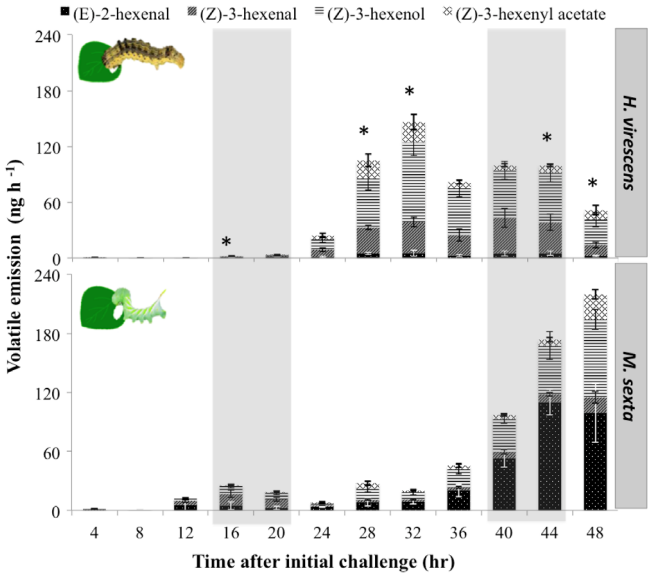

FIGURE 2 The emission pattern, concentration, and ratio of green leaf volatiles (GLVs) collected from *H. virescens* and *M. sexta* damaged *N. benthamiana* plants are different. *N. benthamiana* plants were fed on continuously for 48 hr either by early fifth-instar *H. virescens* (four per plant, top) or by early fourth-instar *M. sexta* (one per plant, bottom). Volatiles were collected in 4-hr intervals after feeding initiation. The graph shows the composition and ratio of GLVs. Shaded areas indicate night time volatile collections. Values represent means ± *SE* (n = 4). Data were analyzed with a mixed model for repeated measures. Asterisks indicate significant (p <.05) differences in total GLV emissions between treatments within time points, with Bonferroni's correction for multiple comparisons [Colour figure can be viewed at wileyonlinelibrary.com]

temperature for 30 min. Excess trimethylsilyldiazomethane was inactivated by adding 3 μL of 2 M acetic acid. The vial was again capped, vortexed, and incubated at room temperature for 30 min. To collect methylated and volatilized JA, a SuperQ filter attached to the vacuum source was inserted into the vial through a cut in the septum. The vial was heated in a heating block to 180°C for 2 min. The methylated and volatilized JA was then trapped on the SuperQ filter for 2 min at 400 mL min^{-1} flow rate. Trapped JA was eluded with 150 μL of dichloromethane and analyzed by gas chromatography with a GC-MS equipped with a capillary column (HP-1, 30 m × 0.25 mm ID × 0.25 μm film thickness; Supelco, PA) as described by Engelberth et al. (2003). In brief, an aliquot of 1 μL per sample was injected into the GC inlet by an automated injection system. The initial GC oven temperature was held at 40°C for 1 min, followed by a linear temperature increase of 15°C min^{-1} until 300°C was reached. The oven was baked out at 300°C for 5 min. Helium at a flow rate 0.7 mL min^{-1} was used as a carrier gas. The quantity of extracted JA was calculated relative to the peak area of the dhJA (internal standard) using GC Chemstation software.

2.6 | Cross priming experiments

For the cross-priming experiment, we assigned emitter plants to one of the following treatments: feeding damage by *H. virescens*, *M. sexta* or undamaged control. After 48 hr of exposure to volatiles, individual receiver plants were transferred to a clean bell jar and challenged by mechanical wounding and application of either *M. sexta* or *H. virescens* regurgitant as described above. Volatiles emitted by the receiver plants were collected for 36 hr in 4-hr intervals using SuperQ filters. For JA analysis, a challenged portion of the leaf tissue was harvested 30 min after challenge. There was continuous airflow inside the bell jar (push air: 1 L min^{-1}, pull air: 0.8 L min^{-1}) throughout the priming and challenging periods.

2.7 | Data extraction

We used Python programming language to extract compounds of interest from GC-FID data files into a CSV file (Method **S1**, the code is available at https://github.com/bipspau/extract_gc_fid_data.git).

2.8 | Statistics

The data of all time course experiments were analyzed using PROC MIXED procedure (Saxton, 1998) with an estimation of degrees of freedom with the Kenward Rodgers procedure in SAS (SAS Institute Inc. 2013; SAS/STAT 9.4 User's Guide, NC) as described by Seidl-Adams et al. (2015). The best covariance structure within repeated measure analysis was selected based on the Akaike information criterion (AIC) and Bayesian information criterion (BIC). Volatile experiment data were divided into three subsets (day-1, night-1, and day-2) because volatile emissions follow a diurnal rhythm. Two separate hypotheses for each experiment were tested: Hypothesis 1: mean volatile emissions at any particular time point are the same for all treatments, and Hypothesis 2: mean volatile emissions for a particular treatment are the same at all time points. The comparisons of interest were extracted (comparison between different treatments at the same time point and comparison between different time points for the same treatment) and checked for significance, adjusted with Bonferroni's method (Table **S1**).

Treatment effects on JA accumulation were determined using one-way ANOVA followed by Tukey's HSD *post hoc* test in R version 3.4.3 (R Core Team, 2017) with Agricolae package (de Mendiburu, 2019). For normal data with unequal variance, the Welch one-way test followed by Games-Howell *post hoc* test was performed using Userfriendlyscience R package (Peters, 2018).

3 | RESULTS

3.1 | Plants damaged by generalist *H. virescens* and specialist *M. sexta* produce unique volatile profiles

To compare the HIPVs emitted by *N. benthamiana* in response to *H. virescens* and *M. sexta* damage, we collected HIPVs emitted from herbivore-damaged plants over a period of 48 hr after feeding

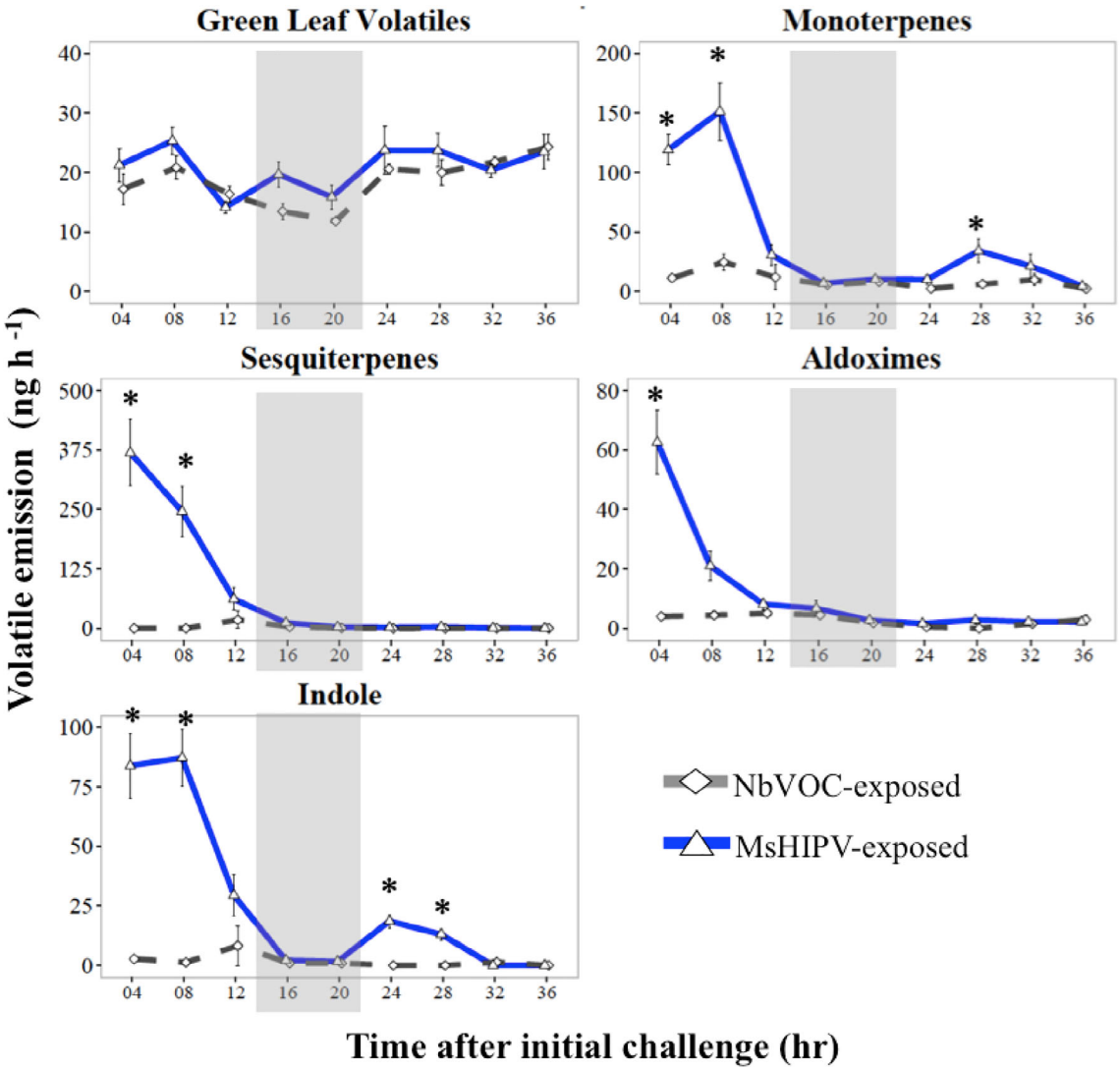

FIGURE 3 After the initial release of four major volatile families, MsHIPV-exposed undamaged plants only emit monoterpenes and indole on the second day. *N. benthamiana* plants were exposed to *M. sexta* damaged (MsHIPV-exposed, blue solid line) or undamaged (NbVOC-exposed, gray dotted line) plants for 48 hr. After 48 hr of exposure to volatiles, the receiver plants were transferred to individual clean bell jars and volatiles were collected for 36 hr in 4-hr intervals. The graph shows total amounts of five major families of volatiles for MsHIPV-exposed and NbVOC-exposed plants: green leaf volatiles, monoterpenes, sesquiterpenes, aldoxime, and indole. Values represent means ± *SE* (n = 4). Data were analyzed with a mixed model for repeated measures. Asterisks indicate significant (p <.05) differences between treatments within time points, with Bonferroni's correction for multiple comparisons. Shaded areas indicate night time volatile collections [Colour figure can be viewed at wileyonlinelibrary.com]

initiation. Plants damaged by these two herbivores emit unique HIPVs in different concentrations and ratios. *H. virescens* damaged plants produced two to four times more HIPVs in total relative to *M. sexta* damaged plants despite similar amounts of damage (Figure 1, Table **S2**). One of the major differences between the HIPV blends was the composition and concentration of GLVs. The major GLV produced by *M. sexta* damaged plants was (*E*)-2-hexenal whereas (*Z*)-3-hexenol was the major GLV produced by *H. virescens* damaged plants (Figure 2; Allmann & Baldwin, 2010). *H. virescens* damaged plants produced very little (*E*)-2-hexenal. These differences in HIPVs provide the foundation for our research question—whether herbivore-specific HIPVs prime herbivore-specific or non-specific defenses in neighboring undamaged plants.

Undamaged *N. benthamiana* plants did not emit measurable amounts of volatiles (Figure **S2**). *N. benthamiana* plants damaged by actual/simulated herbivory and mere mechanical wounding emitted a distinct blend of volatiles. Sesquiterpenes and aldoxime were only detectable after herbivory whereas GLVs, monoterpenes, and indole were also emitted after mechanical wounding (Figure **S3**) albeit in lower quantities. Although the magnitude of GLVs, monoterpenes, and indole in herbivore-fed and mechanically damaged plants were different, the presence or absence of sesquiterpenes and aldoxime was specific to actual and simulated herbivory. This presence or absence of whole classes of volatiles after actual/simulated herbivory makes *N. benthamiana* an ideal model plant for plant-insect interaction studies.

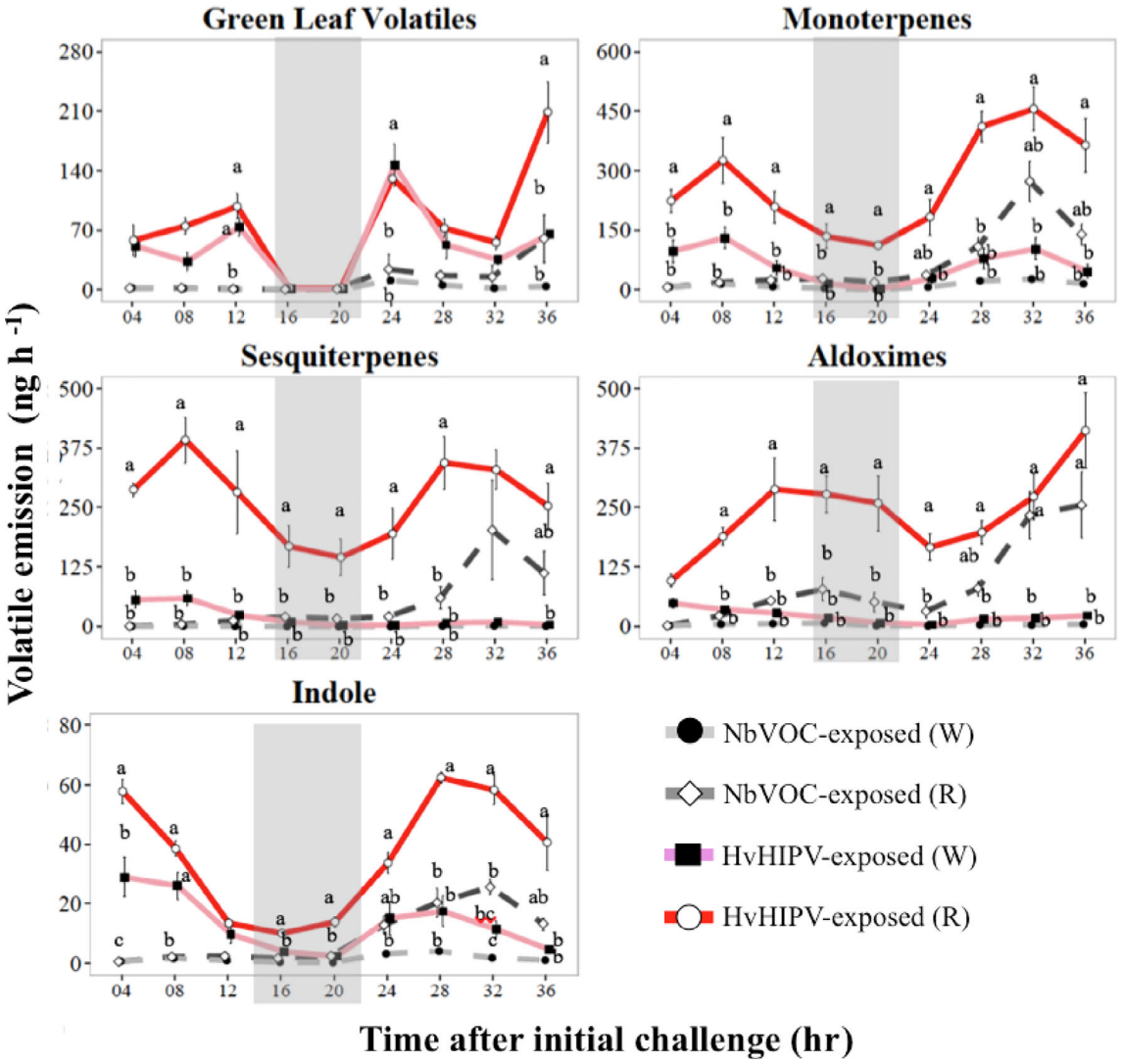

FIGURE 4 Exposure to HvHIPVs induces priming in conspecific neighboring plants for simulated *H. virescens* feeding. *N. benthamiana* plants were exposed to volatiles from *H. virescens* damaged (HvHIPV-exposed) or undamaged (NbVOC-exposed) plants for 48 hr. After 48 hr of exposure to volatiles, the receiver plants were transferred to individual clean bell jars and challenged repeatedly by mechanical wounding followed by either *H. virescens* regurgitant (diluted with water, 1:1 v/v) application (R) or water application (W). The graph shows total amounts of five major families of volatiles for HvHIPV-exposed (solid line) and NbVOC-exposed plants (dotted line) with or without regurgitant application at different time intervals after initial challenge: green leaf volatiles, monoterpenes, sesquiterpenes, aldoximes, and indole. Values represent means ± *SE* (n = 3). Data were analyzed with a mixed model for repeated measures. Different letters indicate significant (p<.05) differences between treatments within time points, with Bonferroni's correction for multiple comparisons. Shaded areas indicate night time volatile collections [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Exposure to HIPVs enhances volatiles and JA induction in challenged neighboring plants

First, we tested whether intact, unchallenged receiver plants change their volatile profile after exposure to insect-damaged emitter plants. Receiver plants were exposed to the volatiles emitted by either herbivore-damaged or undamaged emitter plants for 48 hr. After 48 hr of exposure to volatiles, individual receiver plants were transferred to a clean bell jar and volatiles were collected for 36 hr in 4-hr intervals. The plants exposed to *M. sexta* damaged emitter plants (hereafter MsHIPV-exposed plants) emitted significantly higher amounts of volatiles, except GLVs, than the plants exposed to constitutive volatiles from undamaged plants (hereafter NbVOC-exposed plants) for the first 8 hr (two collection intervals) (Figure 3) and then dropped to base levels by the end of the first day. On the second day, MsHIPV-exposed plants released significantly higher amounts of monoterpenes (at 28-hr collection) and indole (at 24 and 28 hr collections). However, sesquiterpene, aldoxime, and GLV emission remained at base levels (Figure 3). We found a similar result when receiver plants were exposed to *H. virescens* damaged emitter plants (hereafter HvHIPV-exposed plants, Figure **S4**).

In a separate experiment, we found that HIPV exposure did not induce JA production in undamaged receiver plants. We exposed receiver plants to either *M. sexta* damaged (MsHIPVs) or undamaged

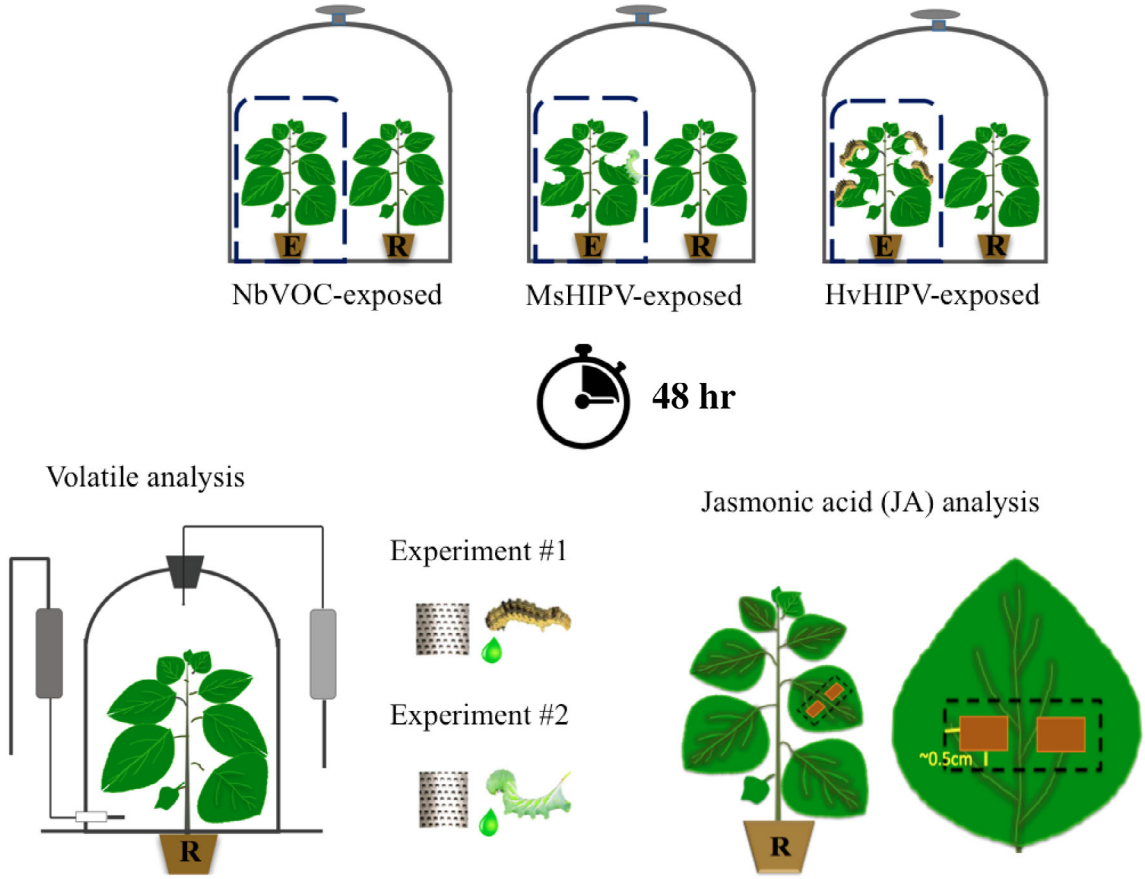

FIGURE 5 Cross-priming experiment design. Cross-priming experiments were conducted to assess the herbivore-specificity in HIPV mediated plant defense priming. Receiver plants (R) and emitter plants (E) in these experiments were placed next to one another under a bell jar. All the emitter plants were confined inside wire cases to prevent caterpillar escape. Emitter plants were damaged by *H. virescens* (Hv) or *M. sexta* (Ms). Hence, receiver plants were either HvHIPV or MsHIPV exposed. Receiver plants, which shared the same bell jar as undamaged plants (Nb), are called NbVOC exposed. After 48 hr of exposure to volatiles, all the receiver plants (R) were transferred to individual clean bell jars and treated similarly. Volatile analysis: In the first experiment, all the receiver plants were challenged every four hours during day time by mechanical wounding followed by *H. virescens* regurgitant application. In the second experiment, they were challenged every four hours during day time by mechanical wounding followed by *M. sexta* regurgitant application. For JA analysis, all the receiver plants were challenged only once either by *H. virescens* regurgitant application or *M. sexta* regurgitant application in separate experiments. The damaged portion of the leaf including leaf material immediately surrounding the damage site was harvested 30 min after challenge [Colour figure can be viewed at wileyonlinelibrary.com]

(NbVOCs) emitter plants for 48 hr. Half of the MsHIPV-exposed and NbVOC-exposed plants were then mechanically wounded and *M. sexta* regurgitant was applied to the wounds. The damaged portion of the leaf was harvested 30 min after challenge for JA analysis. JA produced by MsHIPV-exposed and NbVOC-exposed intact plants was below the detection limit of the GC-MS. However, upon simulated herbivory, MsHIPV-exposed plants produced significantly higher amounts of JA than NbVOC-exposed plants (Figure **S5**).

\We then examined whether volatiles from herbivore-damaged emitter plants induce volatiles and JA priming in conspecific receiver plants challenged by the same herbivore. We carried out separate experiments for each individual herbivore, *M. sexta* and *H. virescens*. For both herbivore species, the release of all five groups of volatile (GLVs, monoterpenes, sesquiterpenes, aldoximes, and indole) was significantly enhanced in HIPV-exposed plants challenged by conspecific herbivore regurgitant applied to wounds (Figure 4 and Figure **S6**). NbVOC-exposed plants challenged by herbivore regurgitant produced higher amounts of volatiles than HIPV-exposed and NbVOC-exposed plants challenged by mere wounding on the second day. Moreover, HIPV-exposed plants challenged by mere wounding produced significantly less volatiles, except for GLVs, than both HIPV-exposed and NbVOC-exposed plants challenged by herbivore regurgitant (Figure 4 and Figure **S6**). However, GLV emission by HIPV-exposed plants followed a different pattern compared to other groups of volatile. Unlike other groups of volatile, GLV emissions were significantly higher in HIPV-exposed plants after challenge by either conspecific herbivore regurgitant or mere wounding on the first day and the first 4 h of the second day of damage. During later collections on the second day, both HIPV-exposed plants and NbVOC-exposed plants emitted similar amounts of GLVs.

In both of the priming experiments, HIPV-exposed plants accumulated 4-5 times more JA at the damage site than NbVOC-exposed plants if conspecific herbivore regurgitant was applied to the wounds (Figure **S7**). However, HIPV exposure did not result in similar augmentation of JA production after wounding alone.

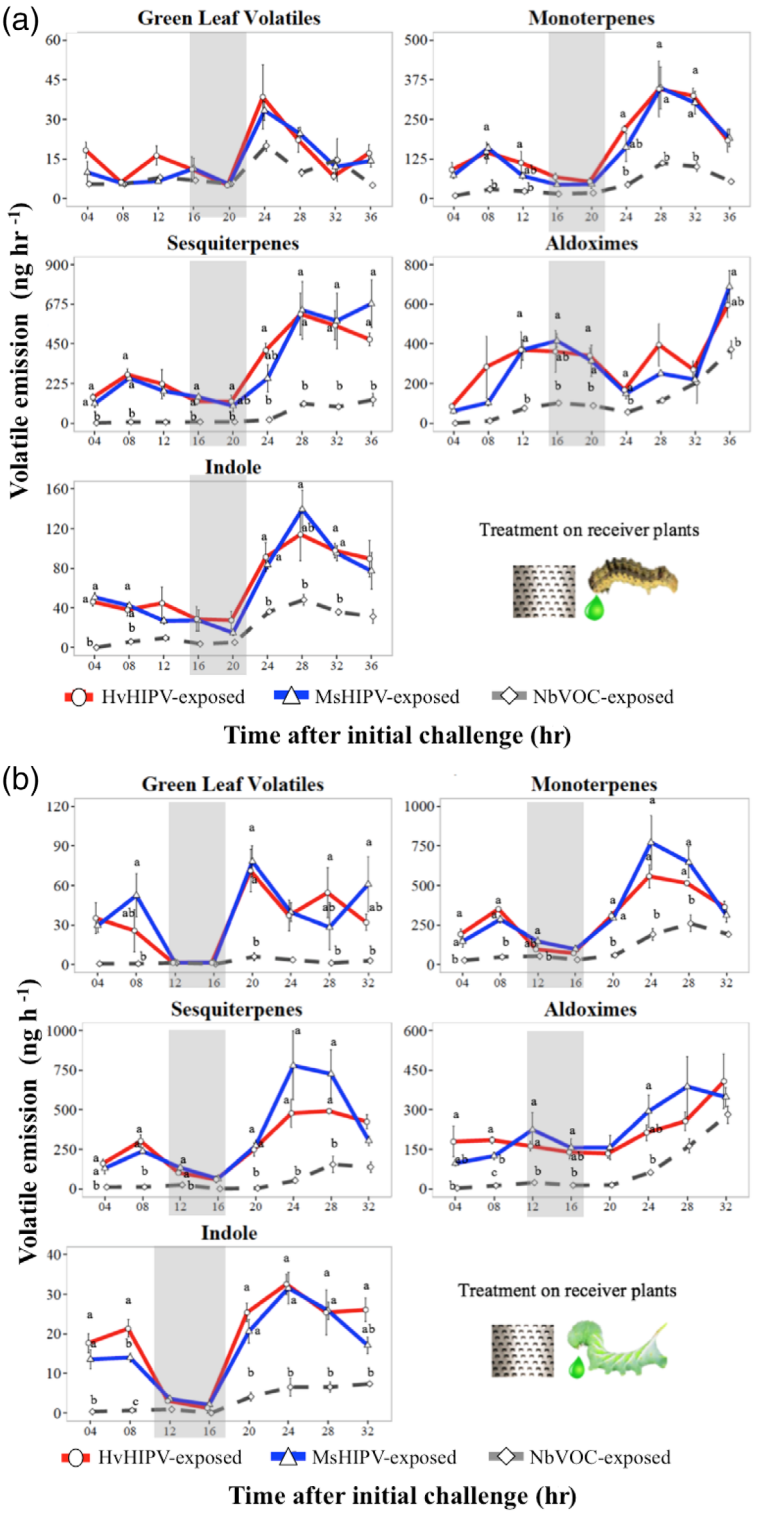

FIGURE 6 Both generalist (*H. virescens*) and specialists (*M. sexta*) herbivore-infested plants prime neighboring plants for general defense. The receiver *N. benthamiana* plants were exposed to volatiles from either *H. virescens* damaged (HvHIPV-exposed, red solid line), or *M. sexta* damaged (MsHIPV-exposed, blue solid line) or undamaged (NbVOC-exposed, grey dotted line) plants for 48 hr. After 48 hr of exposure to volatiles, the receiver plants were transferred to individual clean bell jars and challenged repeatedly by mechanical wounding followed by (a) *H. virescens* regurgitant application or (b) *M. sexta* regurgitant application. The graph shows total amounts of five major families of volatiles for HvHIPV exposed, MsHIPV exposed, and NbVOC exposed receiver plants at different time intervals after initial challenge: green leaf volatiles, monoterpenes, sesquiterpenes, aldoximes and indole. Values represent means ± *SE* (n = 4). Data were analyzed with a mixed model for repeated measures. Different letters indicate significant (p <.05) differences between treatments within time points, with Bonferroni's correction for multiple comparisons. Shaded areas indicate night time volatile collections [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Previous HIPV exposure results in a heightened defense response to subsequent insect feeding independent of the identity of the subsequent herbivore species

In cross-priming experiments, we tested whether plants respond to herbivore-damage differently after exposure to plant volatiles induced by conspecific or heterospecific herbivores. We exposed receiver plants to *M. sexta* feeding induced HIPVs (MsHIPVs) or *H. virescens* feeding induced HIPVs (HvHIPVs) or constitutive volatiles (NbVOCs). After 48 hr of exposure to volatiles, all the receiver plants were challenged by *H. virescens* regurgitant in one experiment and *M. sexta* regurgitant in another (Figure 5). Volatile emissions by *H. virescens* regurgitant challenged receiver plants exposed to either HvHIPVs or

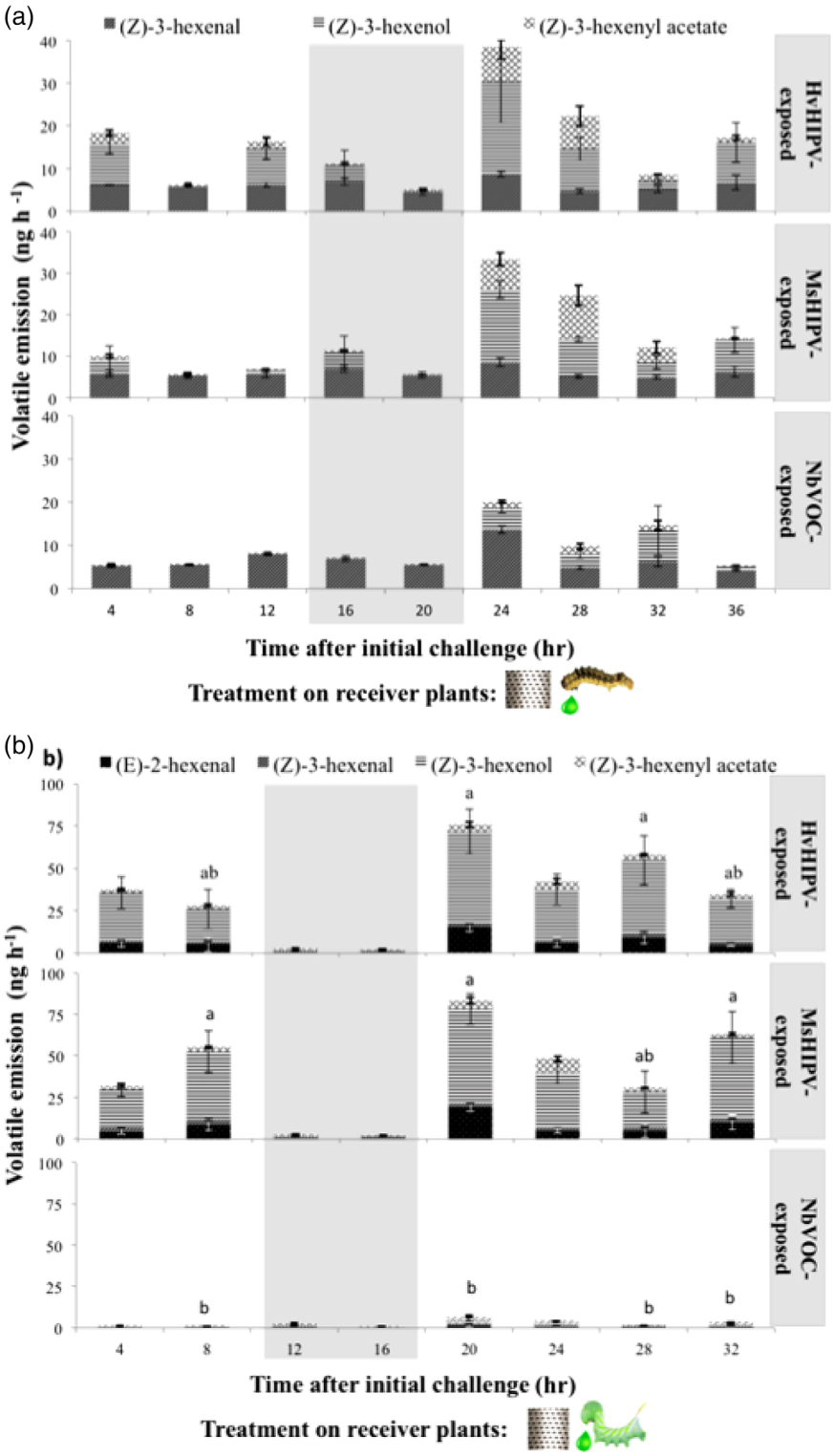

FIGURE 7 Primed plants produce GLVs specific to the attacking herbivore. Receiver *N. benthamiana* plants were exposed to volatiles from either *H. virescens* damaged (HvHIPV exposed) or *M. sexta* damaged (MsHIPV exposed) or undamaged (NbVOC exposed) plants for 48 hr. After 48 hr of exposure to volatiles, the receiver plants were transferred to individual clean bell jars and challenged repeatedly by mechanical wounding followed by (a) *H. virescens* regurgitant application and (b) *M. sexta* regurgitant application. The graph shows different GLVs for HvHIPV-exposed, MsHIPV-exposed, and NbVOC-exposed receiver at different time intervals after initial challenge: (*Z*)-3-hxenal, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate plants. Values represent means ± *SE* (*n* = 4). Data were analyzed with a mixed model for repeated measures. Different letters indicate significant (p <.05) differences in total GLV emission between treatments within time points, with Bonferroni's correction for multiple comparisons. Shaded areas indicate night time volatile collections [Colour figure can be viewed at wileyonlinelibrary.com]

MsHIPVs were not significantly different. However, both HvHIPV- and MsHIPV-exposed receiver plants produced significantly higher amounts of volatiles than the NbVOC-exposed receiver plants after challenge by *H. virescens* regurgitant (Figure 6**a**). We found a similar result in the corresponding experiment where the receiver plants were challenged by *M. sexta* regurgitant (Figure 6**b**). Both HvHIPV- and MsHIPV-exposed receiver plants released similar but significantly higher amounts of volatiles than NbVOC-exposed receiver plants after challenge by *M. sexta* regurgitant.

In both of the experiments, the HIPV profiles emitted by the challenged receiver plants were specific to the challenging herbivore rather than the damaging herbivore used to induce the emitter plants. The major difference between *M. sexta* and *H. virescens* induced volatile profiles other than amounts was the prevalence of (*E*)-2-hexenal

in *M. sexta* induced volatile emissions (Figure 2). Independent of which herbivore had been used to induce volatiles in the emitter plants, *H. virescens* regurgitant challenged plants produced only the 3 GLVs, (*Z*)-3-hexenal, (*Z*)-3-hexenol, and (*Z*)-3-hexenyl acetate (Figure 7**a**), while in *M. sexta* regurgitant challenged plants (*E*)-2-hexenal was the major GLV (Figure 7**b**). These results demonstrate that previous exposure to HIPVs only increases the amount, but not the composition of the volatiles induced by the challenging insect. We did not see other obvious differences in the composition of other groups of volatiles (-Figures **S8** and **S9**)

Similarly, JA accumulation at the damaged site of the receiver plants was primed after HIPV exposure, but it was not affected by the identity of the inducing herbivore. The receiver plants exposed to HIPVs (both HvHIPVs and MsHIPVs) produced significantly higher amounts of JA than the plants exposed to constitutive volatiles (NbVOCs) after challenge by *H. virescens* regurgitant (Figure **S10a**). Similarly, in another experiment, HvHIPV-exposed plants produced JA amounts comparable to MsHIPV-exposed plants, but significantly higher than NbVOC-exposed plants after challenge by *M. sexta* regurgitant (Figure **S10b**). HvHIPV-exposed plants produced four times more JA than NbVOC-exposed plants though the values were not significantly different.

Taken together, these cross-priming results show that exposure to both conspecific and heterospecific HIPVs resulted in the plant's primed augmented response to the attacking herbivore. But the response itself was specific to the attacking caterpillar, rather than dependent on the previously perceived HIPVs.

4 | DISCUSSION

Several studies have shown that HIPVs provide herbivore-specific information to parasitoids and predators of the herbivore (Allmann & Baldwin, 2010; McCormick et al., 2012). Yet, whether herbivore-specific HIPVs prime neighboring receiver plants for a specific herbivore or a broad range of herbivores is not clear. Priming for a specific herbivore could mean that the primed plant only mounts an augmented defense response when the same species of herbivore attacks it. The defense response to any other herbivore should then be "naïve," meaning the response should be of the same amount and with the same timing as any previously unexposed plant. Our study demonstrates that the unique HIPVs induced in the emitter plants by either generalist *H. virescens* or specialist *M. sexta* enhances the induction of defensive HIPVs and the plant hormone JA in challenged receiver plants independent of whether herbivory was simulated with regurgitant from the priming or "novel" herbivore. In other words, although the induced amounts of HIPVs in the receiver plants increased significantly, the provenance of the priming HIPVs did not affect the induced amounts or timings. The same was true for the primed volatile profiles: the composition of the induced HIPV profile was specific to the herbivore attacking the receiver plant. Altogether, our study suggests that in our system the HIPVs of the emitter plants only conveyed the information of herbivore-inflicted damage since the receiver plant responded indiscriminately with an increase in HIPVs specific to the subsequently attacking caterpillar. While we cannot exclude an induction of a defensive metabolite specific to the priming caterpillar species, these results suggest that, in this study system, there is no herbivore specificity of plant-plant communication with respect to primed HIPV and JA production.

In this study, both caterpillar species induced the same groups of volatile organic compounds—GLVs, monoterpenes, sesquiterpenes, indole, and aldoximes—in their host plant, *N. benthamiana*, yet the volatile profiles differed in the overall amounts and the GLV composition. In particular, while feeding on *N. benthamiana*, *H. virescens* induced large amounts of (*Z*)-3-hexenal and proportionally small amounts of (*E*)-2-hexenal. In other words, the ratio of (*Z*)-3-hexenal to (*E*)-2-hexenal was high, whereas *M. sexta* feeding increased (*E*)-2-hexenal and, therefore, the ratio of (*Z*)-3-hexenal to (*E*)-2-hexenal was considerably lower (Allmann & Baldwin, 2010). Although this change in GLV ratio provides herbivore-specific information to egg predators of the herbivore (Allmann & Baldwin, 2010), according to our results, it does not translate in neighboring receiver plants to a differential induction of defensive volatiles and JA (Figure 6 and Figure S10).

H. virescens and *M. sexta* herbivory induces plant volatiles (this study) and defense responses differentially in *N. attenuata* (Voelckel & Baldwin, 2004). One likely explanation is the different composition of elicitors present in their regurgitant. *M. sexta* regurgitant contains volicitin, glutamine fatty acid conjugate (FACs), and glutamic FACs, whereas *H. virescens* regurgitant contains only volicitin and glutamine FACs (Alborn, Brennan, & Tumlinson, 2003; Yoshinaga et al., 2014). It is also conceivable that the feeding location and feeding styles of these two herbivores explain their unique volatile profiles (Bingham & Agrawal, 2010; Halitschke, Kessler, Kahl, Lorenz, & Baldwin, 2000; Zhang, Fu, Wang, & Yang, 2016), especially since these herbivores deposit different amounts of regurgitant on wounded sites (Peiffer & Felton, 2009). The herbivore regurgitant is the mixture of herbivore oral secretions, salivary secretions, and gut contents. Therefore, this difference in volatile emissions could also, in part, be explained by the amount and type of salivary secretions and degraded plant molecules deposited on the feeding site. Some herbivore salivary secretions elicit defenses, while others suppress it (Huang et al., 2019; Tian et al., 2012). Similarly, extracellular plant molecules released or deposited at the wounded site act as damage-associated molecular patterns (DAMPs) and could differentially trigger defense responses (Duran-Flores & Heil, 2016, 2018).

Previous studies have reported the induction of defense priming in response to different types of priming cues directly associated with herbivory. This includes priming by HIPVs (Engelberth et al., 2004; Paschold et al., 2006), oviposition-associated elicitors deposited on the oviposition site (Bandoly & Steppuhn, 2016; Drok, Bandoly, Stelzer, Lortzing, & Steppuhn, 2018; Pashalidou, Lucas-Barbosa, van Loon, Dicke, & Fatouros, 2013), and herbivore-derived olfactory cues such as sex attractants (Helms, De Moraes, Tooker, & Mescher, 2013). Many priming studies measured defense responses in HIPV-exposed neighboring plants after subsequent damage by a conspecific herbivore. Whether the subsequent damage by a heterospecific herbivore

affects the extent of induction of defense priming received less attention. In the present study, we focused on herbivore mediated specificity in priming by HIPVs.

HIPVs induced by either generalist *H. virescens* or specialist *M. sexta* primed *N. benthamiana* plants for enhanced production of defensive volatiles and the plant hormone JA after subsequent challenge with a conspecific herbivore's regurgitant. To examine whether this priming response of *N. benthamiana* is herbivore species-specific, we carried out cross-priming experiments. Although we only looked for a limited primed response, we could not detect a species-specific priming effect; volatiles induced by either of the two herbivore species primed the receiver plant for JA production and higher amounts of volatile emission, with the response being specific to the challenging herbivore. Yet, it seems that there is no general pattern of species-specific priming. Similar to our findings, Helms et al. (2013) reported that the sex attractant of the gall-inducing fly *Eurosta solidaginis* primed a direct defense response in receiver *Solidago sp*. affecting the chewing herbivore *Trirhabda virgata*, thus suggesting nonspecific defense priming in this system. On the other hand, there are studies documenting herbivore-specific induction of defense priming for volatiles or oviposition-mediated priming of plants (Bandoly, Grichnik, Hilker, & Steppuhn, 2016; Drok et al., 2018; Moreira et al., 2018b). In *Baccharis salicifolia* plants, the reproductive rate of either *Uroleucon macolai* (specialist aphid) or *Aphis gossypii* (generalist aphid) was only significantly affected or reduced when emitter and receiver plants were damaged by the same aphid species, thus suggesting herbivore-specific direct defense priming in receiver plants (Moreira et al., 2018b). Drok et al. (2018) found that oviposition by generalist *Spodoptera exigua* and specialist *M. sexta* primed *N. attenuata* plants in a species-specific manner for induced transcriptional and phytohormone response after larval feeding. In addition, oviposition itself primed *N. attenuata* plants for subsequent caterpillar attack. The performance of *M. sexta* larvae was not affected by previous oviposition by the same or by a different herbivore on host plants whereas *S. exigua* larvae suffered from increased mortality and gained less weight on oviposition primed plants (Bandoly et al., 2016).

Although our study tested only two herbivores, it suggests that HIPVs have non-specific effects in plant defense priming. Here, we focused mainly on volatile production by HIPV-exposed and constitutive volatile-exposed receiver plants and showed enhanced production of defensive volatiles by HIPV-exposed receiver plants after simulated herbivory (wounding + regurgitant application). Augmented volatile concentrations could provide a better indirect defense to the receiver plant by increasing the ability of parasitoids' and predators' to locate their host or prey as well as attracting them from long distances (Aljbory & Chen, 2018; Joo et al., 2018). Since we only looked for primed volatiles and JA, there is still the possibility that receiver plants recognized the identity of herbivores by perceiving herbivore-specific HIPVs (Choh, Ozawa, & Takabayashi, 2013) and primed direct defenses that could affect the target and non-target herbivore differentially by affecting growth, development, and reproduction of conspecific herbivores as described for *A. gossypii* and *U. macolai* in *B. salicifolia* plants (Moreira et al., 2018b). This needs to be tested by herbivore performance on the receiver plant. A global gene expression analysis to investigate whether herbivore-specific HIPVs differentially prime for induced direct defense genes might also provide additional information.

The question remains as to how likely outbreaks of one pest species are at the exclusion of all other pests. It seems that only in this scenario, specific priming would be advantageous. Looking at priming from a different angle, herbivores are not uniformly distributed over a location, therefore an attack by any herbivore could be a "sampling test" for the herbivore density and distribution in the field. Hence, any herbivore attack in the vicinity is a strong predictive of an impending attack. A plant primed to increase the specific defensive volatile profile of its actual attacker will be better defended than a naïve neighboring plant. This hypothesis remains to be tested in the field.

Exposure to HIPVs from neighboring plants led to adsorption. We observed that HIPV-exposed, intact receiver plants emitted considerable amounts of volatiles on the first day of collection, even sesquiterpenes and aldoximes, which are only emitted from plants damaged by actual and simulated herbivory (Figure 3 and Figure S4). Exposure to HIPVs alone might not be sufficient to induce volatiles given the fact that production of HIPVs, except for GLVs, is regulated by JA signaling in tobacco (Halitschke & Baldwin, 2003) and HIPV exposure alone did not induce JA in undamaged HIPV-exposed plants (Figure **S5**). Therefore, it seems like the volatiles, except GLVs, released by herbivore-damaged emitter plants adhered to and were subsequently re-released from the receiver plant on the first day as shown by Choh, Shimoda, Ozawa, Dicke, and Takabayashi (2004). The adsorption of volatiles depends on several factors such as thickness of cuticular wax layers of leaves, characteristics of volatiles (volatility and polarity), and environmental factors such as temperature, light, humidity, and wind velocity (Niinemets, Loreto, & Reichstein, 2004). Semi-volatile compounds with low vapor pressures can persist on the leaf surface (Niinemets et al., 2004) and alter the volatile profile of receiver plants (Himanen et al., 2010). There is evidence that adsorbed volatiles increase the fitness of receiver plants by deterring herbivores (Himanen et al., 2010) and attracting natural enemies of herbivores (Choh et al., 2004).

The current study revealed that exposure to herbivore-specific HIPVs can shape plant defense responses to multiple chewing herbivores. The ability of plants to perceive and respond to HIPVs may have evolved to protect plant parts that lack vascular connections. At the same time, the ability to detect and react to plant volatiles enables plants to eavesdrop on HIPVs emitted by herbivore-damaged neighbors as a predictor of future threats. However, herbivore attack on neighboring plants does not necessarily mean a guaranteed attack on receiver plants. Hence, it may be advantageous for HIPV-exposed plants to prepare their defenses for future attack with minimal associated costs compared to direct activation of defense (van Hulten et al., 2006). Faster and stronger defense specific to the subsequently attacking herbivore could even maximize the benefit. Yet, in most systems in nature there are several different herbivore species present. In this scenario priming only for a particular herbivore might not be the most effective defense. Rather, a two-pronged defense consisting of primed JA-dependent general defenses and species-specific defenses specifically induced by a particular herbivore might be more successful.

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CONFLICTS OF INTEREST

The authors declare no potential conflict of interest.

AUTHORS' CONTRIBUTION

B.P.T., I.S.A., and J.H.T. conceived the idea for the research and participated in the planning and designing of the experiments and writing of the manuscript, B.P.T. performed the experiments and collected and analyzed the data.

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SUPPORTING INFORMATION

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

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