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Research

Plant-to-plant defence induction in cotton is mediated by delayed release of volatiles upon herbivory

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Summary

- Caterpillar feeding immediately triggers the release of volatile compounds stored in the leaves of cotton plants. Additionally, after 1 d of herbivory, the leaves release other newly synthesised volatiles. We investigated whether these volatiles affect chemical defences in neighbouring plants and whether such temporal shifts in emissions matter for signalling between plants.
- Undamaged receiver plants were exposed to volatiles from plants infested with *Spodoptera* caterpillars. For receiver plants, we measured changes in defence-related traits such as volatile emissions, secondary metabolites, phytohormones, gene expression, and caterpillar feeding preference. Then, we compared the effects of volatiles emitted before and after 24 h of damage on neighbouring plant defences.
- Genes that were upregulated in receiver plants following exposure to volatiles from damaged plants were the same as those activated directly by herbivory on a plant. Only volatiles emitted after 24 h of damage, including newly produced volatiles, were found to increase phytohormone levels, upregulate defence genes, and enhance resistance to caterpillars.
- These results indicate that the defence induction by volatiles is a specific response to *de novo* synthesised volatiles, suggesting that these compounds are honest signals of herbivore attack. These findings point to an adaptive origin of airborne signalling between plants.

Introduction

Plants produce a wide range of secondary metabolites that enable them to defend themselves against antagonists, such as herbivores and pathogens. These compounds can function as toxins that directly reduce herbivore survival or reproductive success (e.g. quinones, alkaloids, anthocyanins, and terpenoids), or, as in the case of volatile organic compounds (VOCs), serve as indirect defence signals (Pichersky & Lewinsohn, 2011; Mithöfer & Boland, 2012; Kessler & Kalske, 2018; Pichersky & Raguso, 2018). These VOCs can be stored and emitted constitutively (Gershenzon, 1994, 2000; Clancy *et al.*, 2016), or induced and synthesised *de novo* following herbivory (Paré & Tumlinson, 1997). Importantly, these herbivore-induced changes include shifts in the composition and relative ratios of compounds within a volatile blend released by a plant (Turlings & Erb, 2018), which contain ecologically relevant cues of risk of attack. Herbivore-induced plant volatiles (HIPVs) may repel herbivores and attract their enemies; they can also serve as signals between different parts of an individual plant (within-plant signalling) to activate preventive systemic defences (Heil & Silva Bueno, 2007; Meents & Mithöfer, 2020), and may be used by neighbouring plants to prepare for future attacks (Morrell & Kessler, 2017; Schuman, 2023).

Initial discoveries demonstrating volatile-mediated interactions between plants in response to herbivore attack (Baldwin & Schultz, 1983; Farmer & Ryan, 1990; Bruin *et al.*, 1992) were met with some scepticism but are now widely accepted as being both common and ecologically relevant (Heil & Karban, 2010; Ninkovic *et al.*, 2019; Kessler *et al.*, 2023). Numerous studies

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have reported on the role of signalling between plants mediated by HIPVs (Baldwin & Schultz, 1983; Dolch & Tscharntke, 2000; Karban *et al.*, 2003; Heil & Silva Bueno, 2007), with field studies revealing specificity in the volatile cues involved (Karban *et al.*, 2004; Moreira *et al.*, 2016; Kalske *et al.*, 2019). Herbivore-induced plant volatiles reported to act as potential signalling cues include jasmonates (Farmer & Ryan, 1990), green leaf volatiles (Engelberth & Engelberth, 2019), and aromatic compounds (Erb *et al.*, 2015). These HIPVs from a damaged plant can reach an undamaged neighbouring plant, which can then enter a so-called 'primed' state (Ton *et al.*, 2007; Mauch-Mani *et al.*, 2017). Although defences in primed plants are sometimes not expressed or only at low levels, these plants exhibit greatly enhanced induction of defence compounds after being attacked (Conrath *et al.*, 2006; Martinez-Medina *et al.*, 2016). In addition, undamaged plants exposed to HIPVs may also immediately upregulate their defences without the need of a direct contact with the attacker; these induced defences will be present before herbivore attack (Karban *et al.*, 2003; Waterman *et al.*, 2024).

Gossypium hirsutum L. (Malvaceae), known as upland cotton, is cultivated world-wide primarily for the production of textile fibres. It is heavily attacked by pests and requires high amounts of pesticide application, accounting for a substantial portion of world-wide pesticide use (Coupe & Capel, 2016; Huang *et al.*, 2021). While the use of these chemicals has resulted in increased crop yields, it has also had extremely negative impacts on the environment (Van Der Werf, 1996; Aktar *et al.*, 2009), particularly in soil and water pollution. More benign pest control strategies are sought, including the enhancement of the plants' natural defences (Llandres *et al.*, 2018). *Gossypium hirsutum* is known to respond to insect herbivory by altering its volatile emission profile both quantitatively and qualitatively, as well as increasing its content of nonvolatile defensive terpenoid aldehydes, such as gossypol and heliocides (Loughrin *et al.*, 1994; McCall *et al.*, 1994; Röse *et al.*, 1996; McAuslane *et al.*, 1997; Arce *et al.*, 2021). Interestingly, the volatile blends emitted by damaged plants also change over time from herbivory onset, with stored volatile compounds being released immediately after damage (such as the terpenes α-pinene and caryophyllene), and *de novo* synthesised compounds being emitted in high quantities after at least 24 h of attack onset (Loughrin *et al.*, 1994; Paré & Tumlinson, 1997). The latter compounds include terpenes such as β-ocimene and β-farnesene and the aromatic indole, emitted in very low amounts or not at all from undamaged or freshly damaged plants. Thus, two pools of volatiles are released after herbivory, hereafter named fresh damage volatiles and old damage volatiles.

It is known that cotton plants attacked by herbivorous mites are more resistant to new colonisation by mites than undamaged plants in both laboratory and field conditions (Karban, 1985, 1986). Similarly, cotton is more resistant to *Spodoptera* caterpillars when damaged by mites (Karban, 1988), and it has also been found that *Spodoptera* caterpillars are deterred from feeding when plants have been previously damaged by caterpillars (Alborn *et al.*, 1996). These findings indicate that induction by herbivores is a broad response that protects cotton against future attacks, although its importance for plant fitness has been difficult to test given that cotton is perennial (Karban, 1993). Plant–plant signalling by cotton volatiles was first studied by Bruin *et al.* (1992), who found that cotton seedlings infested with herbivorous mites produced VOCs that caused a decrease in oviposition by herbivorous mites on neighbouring plants, which were also more attractive to predatory mites. More recently, Zakir *et al.* (2013) showed, both in laboratory and field, a significant reduction in oviposition by *Spodoptera littoralis* (Lepidoptera: Noctuidae) moths on undamaged cotton plants previously exposed to damaged neighbouring cotton plants. In addition, using wild cotton plants, Briones-May *et al.* (2023) found that exposure to HIPVs from neighbouring plants primes the induction of extrafloral nectar of receiver plants under glasshouse conditions. Field studies performed in Mali afford additional evidence for VOC-mediated signalling, by showing that topping (i.e. manual removal of the apical part of flowering cotton plants) resulted in reduced infestation by the cotton bollworm (*Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)) on the topped plant, as well as on intact neighbouring plants (Llandres *et al.*, 2018). Similar signalling effects have been recently found for infestation by *Aphis gossypii* Glover (Llandres *et al.*, 2023). Despite progress made thus far, there is no precise information about how and which direct defences are triggered by HIPV exposure in cotton (Quijano-Medina *et al.*, 2024). The signalling effects of different pools of volatile compounds that are released at distinct time points after damage onset have so far been ignored, although it is likely that they do not convey the same information about herbivory risk. Cotton is ideal for addressing this question and to test the functional role and adaptive significance of volatiles in plant signalling. As *de novo* synthesised volatiles released after herbivory can be expected to carry the most reliable information, we hypothesised that specifically this pool of volatiles would trigger responses in neighbouring plants.

In this study, we investigated the effects of exposure to HIPVs from emitter plants on undamaged receiver plants by measuring chemical profiles (including volatiles and direct defence metabolites, namely gossypol and heliocides, as well as phytohormones), associated gene expression (to get at subtler responses associated with priming), and caterpillar feeding preference as proxy of downstream consequences for plant resistance. To do this, we exposed undamaged *G. hirsutum* plants to airborne VOCs emitted by plants infested with *Spodoptera* caterpillars. We also assessed the impact of the timing of herbivory by exposing receiver plants to HIPVs from plants at two contrasting time points since herbivory onset, namely 0–24 h since damage onset vs 24–48 h since damage onset. This allowed us to determine whether the chronological changes in volatile emissions are relevant for plant signalling between cotton plants. By taking into account the temporal dynamics of volatile emissions, we were able to separate the effects of the two different pools of volatiles released by damaged plants. This separation helped to elucidate how each pool activates defensive cascades in neighbouring plants that prepares them for incoming attacks. The results presented here indicate that the volatiles released after 24 h after damage,

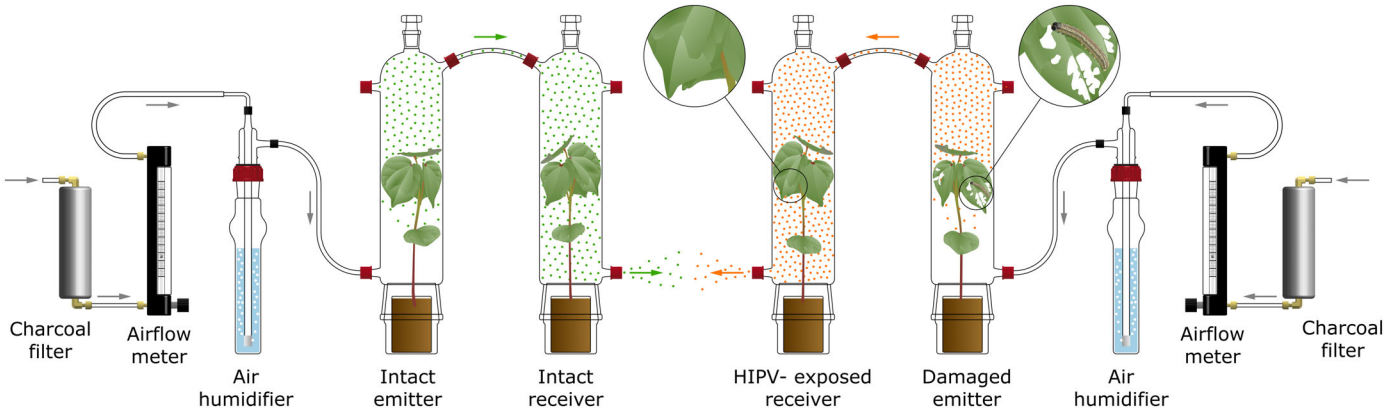

Fig. 1 Schematic representation of the setup for volatile exposure. The actual setup consisted of 12 pairs of bottles.

which most reliably indicate an attack by caterpillars, are the most relevant for plant-to-plant information conveyance.

Materials and Methods

Plants

Two *Gossypium hirsutum* L. seed sources were used. One was a cultivated variety (STAM 59A; commonly cultivated in Africa) provided by CIRAD (French Agriculture Research Centre for International Development, France) and IER (Institut d'Etudes Rurales, Mali), and the other consisted of feral cotton seeds that were collected in Puerto Escondido, Oaxaca, Mexico (15°53′00.8″N 97°06′29.3″W). This feral cotton descends from ancestral domesticated varieties grown in the region hundreds of years ago (local communications) and have readapted to wild conditions. These two genotypes were used to include defence responses from plants with different domestication histories. To enhance germination rates, cultivated seeds were soaked in tap water at 27°C in the dark for 24 h before germination; for feral seeds, seed coats were scratched and pierced delicately with a nail file and a puncher, then seeds were placed on moist cotton wool at 28°C in the dark for 48 h. Pregerminated seeds were planted individually in plastic pots (height: 8.5 cm, diameter: 6 cm) filled with commercial potting soil (Profi Substrat, Einheitserde, Germany). Seedlings were grown in phytotrons (GroBanks CLF Plant Climatics, Germany) under the following conditions: 16 h : 8 h, 28°C : 25°C, light : dark and 65 µmol m^{-2} s^{-1}. Plants were grown until the fourth true leaf had fully developed.

Insects

Two *Spodoptera* (Lepidoptera: Noctuidae) species were used for experiments, namely *Spodoptera frugiperda* (JE Smith) and *S. exigua* (Hübner), both reared under quarantine conditions at the University of Neuchâtel, Switzerland. They were reared on wheat-germ-based diet (Frontier Scientific Services, Newark, USA) at 25 ± 2°C, 60% relative humidity, 16 h : 8 h, light : dark. Late first- and early second-instar larvae were used for experiments.

Experimental protocol

To assess the effect of HIPVs exposure on the defensive responses of neighbouring plants, we performed two separate experiments. First, we exposed noninfested plants (receiver plants) to volatiles from caterpillar-infested plants (emitter plants) for 48 h. Caterpillars were left on the emitter plants during the experiment to ensure a constant volatile release and induction. Although the effect of volatiles released by caterpillars and their frass on receiver plants cannot be fully excluded, it seems unlikely that they play a role in this context; caterpillars and their frass were present in all damage treatments and previous volatile collections found no differences between volatiles released from damaged plants with caterpillars and damaged plants after removing caterpillars and frass. Second, in a follow-up experiment aimed at gaining mechanistic insight into effects of different volatile pools based on time since herbivory onset (fresh and old damaged volatiles), we exposed noninfested plants to HIPVs from either plants with fresh damage (0 h to 24 h) or plants with older damage (24 h to 48 h). Cotton seedlings were individually placed in glass bottles (Verre & Quartz Technique SA, Neuchâtel, Switzerland). Pairs of emitter and receiver plants were connected using Teflon tubing. A continuous stream of purified and humidified air was pushed through the system, exposing receiver plants to VOCs from emitter plants (Fig. 1).

Experiment 1: Defence induction by volatiles from caterpillar-damaged plants Emitter plants were infested with 10 second-instar *Spodoptera* spp. larvae. Either *S. exigua* or *S. frugiperda* were used in different experimental blocks, to compare the responses to attack by each insect species. Receiver plants were exposed for 48 h to volatiles from either undamaged emitters (control) or from caterpillar-infested plants (Fig. 1). Half of the plant pairs were the commercial variety, and the other half were feral plants (N = [5, 6] per treatment, caterpillar species, and variety combination). After the exposure period, the first and

fourth true leaves (counting from bottom to top of the plant, not counting the cotyledon) were collected, flash-frozen, and stored at −80°C until further processing (see Materials and Methods section ahead). In receiver plants, we measured gossypol and heliocides levels, as well as expression levels of genes known to be involved in the biosynthesis of gossypol and volatiles (Sunilkumar *et al.*, 2006; Tian *et al.*, 2018; Huang *et al.*, 2021).

We repeated the above procedure with a separate batch of plants, and with the same methodology but using *S. exigua*. After exposure, the second and fourth true leaves of receiver plants (n = 9 per treatment, variety combination) were carefully excised at the base and promptly used in preference assays with *S. exigua* caterpillars. VOCs were collected from a subset of intact receiver plants from both treatments (n = 6 per treatment, variety combination) immediately following the 48-h exposure. They were subsequently infested with 10 second-instar *S. exigua* caterpillars for 24 h, after which VOCs were collected again.

Experiment 2: Defence induction upon exposure to volatiles from fresh or old damage First, we collected volatiles from plants infested with 10 second-instar *S. exigua* caterpillars at different time points after herbivory onset to measure temporal changes in VOC emissions. Collections were carried out 4 h, 22 h, 28 h, and 46 h after onset of damage on different plants per each time point (n = 18, n = 24, n = 18, n = 24; respectively). Only *S. exigua* was used for this experiment as results from Experiment 1 did not indicate large differences in the effects on receiver plants.

We performed another volatile exposure experiment where receiver plants were exposed for 48 h to one of three odour types (Supporting Information Fig. S1). The first group involved emitters for which caterpillars had just been placed on plants (emissions dominated by stored volatiles, i.e. 'fresh damage'). After 24 h of exposure, emitter plants were replaced by a new set of plants that we had just infested with caterpillars. The second treatment group involved emitter plants with 24 to 48 h since herbivory onset (emissions dominated by *de novo* synthesised volatiles, i.e. 'old damage'). Analogous to the first group, after 24 h of exposure, emitter plants were replaced by a new set of plants with 24 h since damage onset. Thus, in both treatments, emitter plants were replaced once to ensure continuous exposure to only fresh or only old damage volatiles for 48 h (Fig. S1). In all cases, emitter plants were infested with 10 second-instar *S. exigua* caterpillars, and emitter bottles were changed for clean ones after the first 24 h of damage. Finally, the third group consisted in control plants that were exposed to clean airflow for 48 h as a proxy of basal defence levels for comparison to receivers in the other two groups. All plants used in this experiment were the commercial variety (STAM 59A). Receiver plants were processed immediately following the exposure treatment (n = 16 for each treatment). In all cases, the third true leaf was harvested (as the fourth leaf was used for caterpillar preference tests), flash-frozen, and stored at −80°C until further processing. Responses measured in receiver plants were as follows: VOC emissions, gossypol and heliocides levels, phytohormones levels, and expression levels of genes involved in the biosynthesis of gossypol and volatiles. In addition, the fourth true leaf of receiver plants was carefully excised at the base and promptly used in preference assays with *S. exigua* caterpillars.

Volatile collection and analysis

Purified and humidified air was pushed through the system at a rate of 1.2 l min^{-1}. VOCs were collected on filters containing 25 mg of 80/100 mesh Haysep-Q adsorbent (Sigma) that were coupled to air drawn from the system at a rate of 0.7 l min^{-1} (Turlings *et al.*, 1998; Arce *et al.*, 2021) for 2 h. Filters were eluted with 100 µl dichloromethane (Honeywell, Riedel-de Haën, Germany), and the extract was spiked with 10 µl internal standard (n-octane and n-nonyl acetate, 20 ng $µl^{-1}$ each (Turlings *et al.*, 2000)). Samples were stored at −80°C until further use.

VOC samples were analysed using gas chromatography – mass spectrometry (GC-MS; GC: 6890 N, MS: 5973 MSD for Experiment 1; GC: 7890B, MS: 5977B MSD, for Experiment 2; both from Agilent Technologies, Santa Clara, CA, USA). A 2 µl or a 1.5 µl aliquot of each sample from Experiment 1 or 2, respectively, was injected onto a HP-5MS column (30 m × 250 µm × 0.25 µm, Agilent Technologies) in splitless mode. We used a constant flow rate of 1.1 ml min^{-1} He, with a temperature program of 40°C for 3 min, increased to 100°C at a rate of 8°C min^{-1}, followed by ramping at 5°C min^{-1} to 200°C, followed by a postrun period of 250°C for 3 min. Identification and quantification of compounds were performed through comparison to the mass spectra of authentic commercial standards and NIST 17 library spectra.

Gossypol and heliocides extraction and analysis

Frozen leaves were ground into a fine powder under liquid nitrogen. 50 ± 5 mg frozen leaf powder was extracted with 80 µl acetonitrile, and samples were homogenised using 4–6 glass beads (1.25–1.65 mm diameter) in a mixer mill (TissueLyser II; Qiagen). The samples were then centrifuged for 5 min at 17 500 ***g***. Recovered supernatant was centrifuged a second time to ensure a fully limpid solution, and then transferred to an amber glass vial.

Samples were analysed using ultra high-performance liquid chromatography, coupled to a diode array detector set at 288 ± 2 nm (HP1100; Agilent Technologies), for Experiment 1 or UHPLC-DAD; Ultimate 3000 Dionex, Thermo Fischer Scientific, MA, USA, for Experiment 2. Each sample was injected onto an Extent-C18 column (2.1 × 150 mm, 5 µm; Agilent Technologies) for Experiment 1 or an ACQUITY BEH C18 (2.1 × 100 mm, 1.7 µm; Waters, MA, USA) for Experiment 2. The following mobile phases were used at a constant flow rate of 0.5 ml min^{-1} for Experiment 1 or 0.45 ml min^{-1} for Experiment 2: solvent A (0.05% formic acid in water) and solvent B (0.05% formic acid in acetonitrile). After injection (injection volume 1.5 µl or 5 µl for Experiment 1 or Experiment 2, respectively), the following gradient was used: for Experiment 1, solvent B increased from 35 to 90% in 20 min, then to 100% in 1 min, was held at 100% for 3 min, followed by re-equilibration at 35% solvent B for 6 min; for Experiment 2, solvent B

increased from 45 to 90% in 8 min, then to 100% in 0.5 min, was held at 100% for 2.5 min, followed by re-equilibration at 45% solvent B for 3.5 min. Quantification was based on comparison to standard reference compounds.

Plant hormone profiling

The levels of plant defence hormones jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile), salicylic acid (SA), and abscisic acid (ABA) were measured as described previously (Glauser *et al.*, 2014). Briefly, frozen leaf material was ground to a powder in liquid nitrogen; 100 mg of leaf powder was extracted with 990 µl ethyl acetate and formic acid (99.5 : 0.5, v/v). Isotopically labelled hormones were added as internal standards (d_5-JA, $^{13}C_6$-JA-Ile, d_6-SA, and d_6-ABA, 1 ng in 10 µl). Samples were homogenised as described above. Supernatant was recovered, and the pellets were re-extracted with 500 µl of solvent (as described previously). The supernatants were combined and dried, and then resuspended in 200 µl methanol and water (70 : 30, v/v). Phytohormones were analysed using an Acquity UPLC (Waters AG, Baden-Dättwil, Switzerland) coupled to a QTRAP 6500+, (Sciex, Framingham, MA, USA). Analyst v.1.7.1 was used to control the instrument and for data processing. Quantification was performed based on internal standardisation using labelled internal standards at a concentration of 5 ng ml^{-1} both in the final extracts and in the calibration points.

RNA isolation and quantitative real-time PCR analysis for gene expression

Frozen leaf material was ground to a fine powder in liquid nitrogen, and used to measure the transcription levels of several critical genes involved in the biosynthesis of gossypol and volatiles. Total RNA was extracted using the GeneJET Plant Purification Mini Kit (Thermo Fischer Scientific, Baltics UAB, Vilnius, Lithuania) according to the manufacturer's instructions. The complete DNA removal was performed using the RNase-Free DNase Set (Qiagen). Each total RNA sample (500 ng) was reverse transcribed using the GoScript™ Reverse Transcription System (Promega). Quantitative real-time PCR (qPCR) was performed with the Rotor-Gene™ 6000 (Corbett Research, Hilden, Germany) using GoTaq® qPCR Master Mix (Promega). Primers used for quantitative polymerase chain reaction are listed in Table S1. For the expression analysis of each gene, samples from control plants were designated as calibrator. Relative expression levels of each gene were normalised with *GhACT4* (GenBank accession no.: AY305726) and *Histone3* (GenBank accession no.: AF024716) and calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Preference assays with *S. exigua* larvae

For Experiment 1, the second and fourth true leaves that were excised from both control and HIPV-exposed plants were, respectively, paired (i.e. second leaf *vs* second leaf, fourth leaf *vs* fourth leaf), and placed on top of moistened filter paper in individual Petri dishes. We only used *S. exigua* due to sample limitation. Three first-instar *S. exigua* caterpillars were released at the same distance from the two leaves and were allowed to move freely and feed on the leaves. The position of the caterpillars was recorded at six time points (10 min, 60 min, 180 min, 240 min, 300 min, and 1440 min) after caterpillar release. The experiment was performed three times with three different batches of plants ($n = 9$). Leaf area consumption was also measured by taking photographs of the leaves 24 h after caterpillar release and quantifying using Adobe Photoshop.

For Experiment 2, at the end of the 48-h exposure, leaf discs (diameter: 2.8 cm) were cut from the 4th leaf of each plant (from control, fresh HIPVs, and old HIPVs exposure treatments) and placed together equidistant from each other in five to six Petri dishes (diameter: 15 cm) depending on plant availability, and we repeated the experiment three times with different sets of plants. Because of space constraints within the Petri dish, we used leaf discs. This offered the caterpillars tissue of different treatments with a standardised size, and ensured that the three treatments could be tested at the same time. One *S. exigua* caterpillar (2^{nd}–3^{rd} instar) was placed in the middle of the three leaf discs and its movement was monitored. We recorded the first choice (i.e. the first leaf disc touched) plus hourly time points up to 4 h after caterpillar release to record on which leaf disc the caterpillar was feeding.

Statistical analyses

All analyses were performed with the software R (v.4.3.2) and the packages lme4 (Bates *et al.*, 2015) for linear models, *vegan* for multivariate analyses, and *emmeans* for *post hoc* tests. To analyse the effects of the volatile treatment on measured variables of both experiments, we used linear models (normal distribution of residuals) and generalised linear models (GLMs, gamma distribution of residuals with log link function) depending on the type of distribution, except when indicated otherwise.

Experiment 1. Induction by volatiles from damaged emitters - For gossypol, heliocides, and gene expression in receivers from Experiment 1, we used emitter volatile treatment (control volatiles or herbivore-induced volatiles), variety (cultivated or feral), caterpillar species (*S. exigua* or *S. frugiperda*), leaf (Leaf 4 or Leaf 1), and experimental block as explanatory fixed factors. Plant identity was included as a random factor to account for nonindependence between the two sampled leaves per plant. For VOCs (total and individual compounds) of receiver plants before and after damage (same plants used for the preference test), we tested the effect of volatile treatment, variety, and experimental block as explanatory fixed variables. In all models, the two-way interactions with the volatile treatment were tested, except for the interaction with experimental block. Effects on caterpillar preference were tested with a generalised linear mixed model with binomial distribution in separated tests for plant variety and leaf number. First, we analysed the preference across all time points, using Petri dish as a random factor to account for the repeated measurements. Then, we included time as a fixed factor in the model

to test differences in preference for each time point. Differences in consumed leaf area were analysed using a normal distribution with volatile treatment as a fixed factor and Petri dish as a random factor.

Experiment 2. Induction by volatiles from emitters with contrasting timing of damage We analysed the emitter VOCs (total and individual compounds) using time point after damage onset as an explanatory factor. A redundancy analysis (RDA) was performed on the proportions of emitter volatiles to analyse differences in composition of the volatile blend. Before performing the RDA, the data were centre log ratio-transformed as VOCs matrices are compositional data (Aitchison & Egozcue, 2005; Holliday *et al.*, 2009). For analysing gossypol, heliocides, gene expression, and receiver VOCs, we used volatile treatment (clean air, fresh damage, or old damage) as the explanatory factor. Caterpillar preference for leaves exposed to control and fresh and old damage volatiles across all time points was tested with a Fisher's exact test and then we tested the preference for each time point using an exact multinomial test (package RSTATIX).

Results

Experiment 1: Defence induction by volatiles from caterpillar-damaged plants

Plants exposed to damaged emitter plants (HIPV exposure) contained marginally significantly higher levels of gossypol than plants exposed to undamaged emitter plants ($\chi^2_{(1)} = 3.56$, $P = 0.059$; Fig. 2a), whereas heliocide levels were not significantly different between both treatments ($\chi^2_{(1)} = 0.47$, $P = 0.49$; Fig. 2b). Leaf 4 accumulated more of both terpenoid aldehydes than Leaf 1 (Table S2). Caterpillar species, cotton variety, and their interactions with exposure treatment did not significantly influence levels of gossypol or heliocides (Table S2).

Undamaged receiver plants exposed to damaged emitters released more total volatiles than receiver plants exposed to undamaged ones (Fig. 2c; Table S2). Both constitutive (monoterpene, aromatics, and GLVs) and inducible VOCs (homoterpenes) in undamaged receiver plants were increased with exposure to damaged emitters (Fig. 3a,b, respectively). Most of the individual compounds showed a similar trend, but only 4,8-dimethyl-1,3,7-nonatriene (DMNT) was significantly higher in receiver plants exposed to damaged emitters than those exposed to undamaged emitters (Fig. 3c; Table S2). Total emissions increased more than 10-fold after 24 h of damage by *S. exigua*, with no significant differences between treatments (Fig. 2d; Table S2).

All tested genes from the terpenoid aldehyde (Fig. 4a) and volatile terpene pathways (Fig. 4b) were significantly more expressed in leaves of receivers exposed to damaged emitters than those exposed to undamaged emitters (Table S2). Plant variety was important in explaining gene expression for several genes (Table S2), with feral plants exhibiting higher expression levels. Caterpillar species was also relevant for some genes involved in terpenoid aldehyde synthesis (Table S2), namely *Cad1A*, *Cdn1C3 and CYP706b* were more expressed in plants exposed to

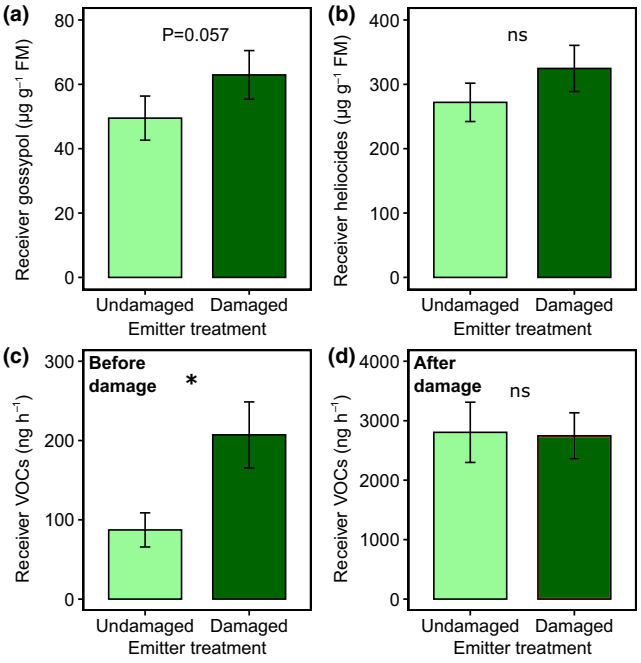

Fig. 2 Chemical traits of receiver *Gossypium hirsutum* plants exposed for 48 h to volatiles of undamaged or damaged emitter plants. Mean concentrations of foliar gossypol (a) and total heliocides (b) in undamaged receiver plants that were exposed to the two types of volatiles. Total amounts of volatiles emitted by receiver plants before (c) and after 24 h of being damaged by *Spodoptera exigua* caterpillars (d). Data for (a, b) were pooled for the following factors: variety (cultivated or feral cotton), caterpillar species (*Spodoptera frugiperda* or *S. exigua*) and leaf (Leaf 1 or Leaf 4 from bottom to top). Data for (c, d) was pooled for variety (cultivated or feral cotton). Generalised linear (mixed) models were performed to assess differences between factor levels. Asterisk indicates a $P < 0.05$. Shown are variable means ±SE. ns indicates no statistically significant difference between treatments.

damaged emitters from plants attacked by *S. frugiperda* than those attacked by *S. exigua*. In addition, Leaf 4 expressed higher levels of *CYP706b* than Leaf 3, and higher levels of terpene associated genes in Leaf 3 than in Leaf 4 (Table S2). Further, the interaction between leaf and volatile exposure treatment was statistically significant for the gene *GhTPS14* (Table S2), with lower expression induction by damaged emitters in Leaf 4 than in Leaf 3. All other factors and interactions involving emitter treatments did not significantly explain gene expression levels (Table S2).

Caterpillars tended to prefer leaves from plants exposed to undamaged emitters compared to leaves exposed to damaged emitters, but for cultivated plants, this trend was overall not significant ($\chi^2_{(1)} = 1.49$, $P = 0.22$; $\chi^2_{(1)} = 1.38$, $P = 0.24$; for the 2nd and 4th leaf, respectively) and only significant for one observation time point for the 2nd leaf (Fig. 5). In feral plants, we observed a significant preference for leaves exposed to undamaged emitters across all time points ($\chi^2_{(1)} = 8.45$, $P = 0.0036$; $\chi^2_{(1)} = 29.5$, $P < 0.0001$; for the 2nd and 4th leaf, respectively). This preference was clear for two time points for the 2nd leaf and in most of the time points for the 4th leaf (Fig. 5). The leaf area consumed in the choice tests was not statistically different

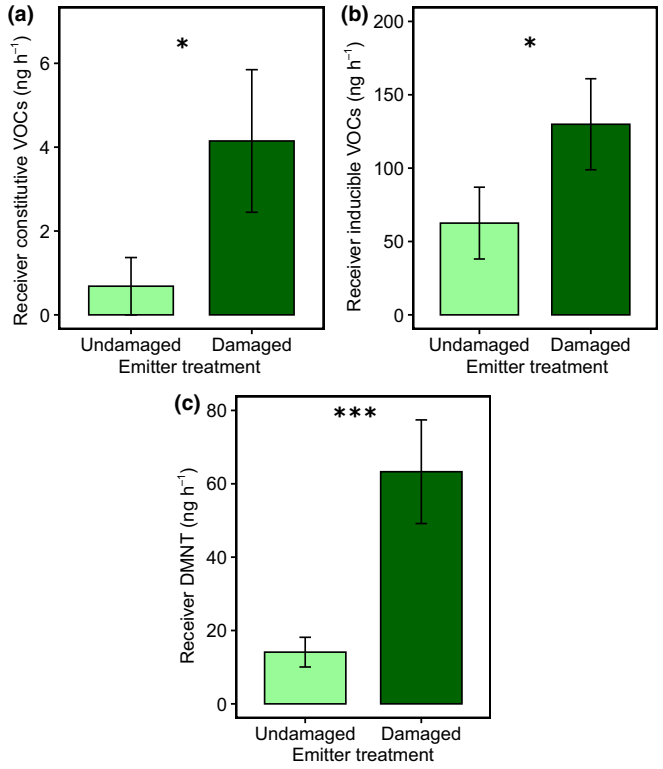

Fig. 3 Volatile emissions from intact receiver *Gossypium hirsutum* plants exposed for 48 h to volatiles of undamaged or damaged emitter plants. Comparison of (a) constitutive volatiles (monoterpenes, aromatics, and GLVs) and (b) inducible volatiles (indole and homoterpenes) from receiver plants. 4,8-Dimethyl-1,3,7-nonatriene (c) was more emitted by receiver plants following exposure to volatiles from damaged emitter plants. Data were pooled for variety (cultivated or feral cotton). Generalised linear models were performed to assess differences between factor levels. Asterisks indicates significance level (*, $P < 0.05$; ***, $P < 0.001$). Shown are variable means ±SE.

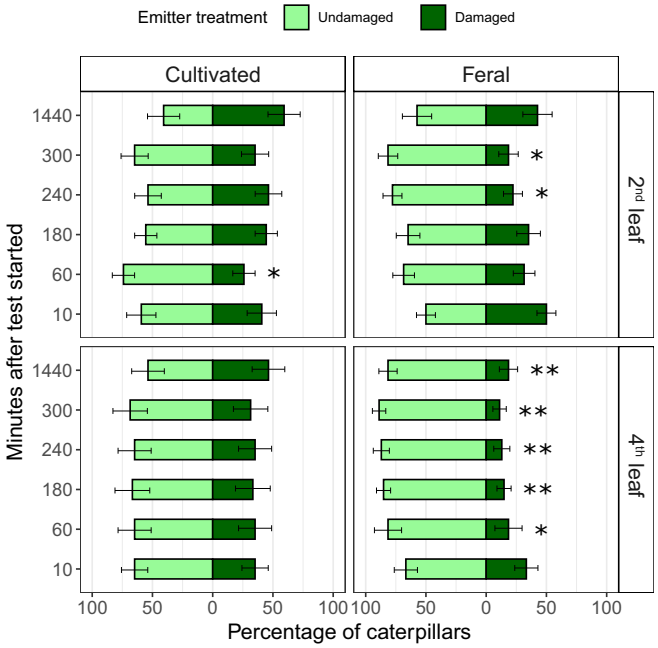

Fig. 4 Preferences of *Spodoptera exigua* caterpillars in two-choice tests between leaves from intact *Gossypium hirsutum* plants exposed to undamaged or damaged emitter plants. The mean percentage of caterpillars found feeding on each of both leaves is shown for the 2nd and 4th leaf of cultivated and feral plants at different time points. Generalised linear models were performed to assess deviation from the expected distribution by chance of 50% of preference. Asterisks indicate a significant preference (*, $P < 0.05$; **, $P < 0.01$). Shown are variable means ±SE.

between leaves exposed to undamaged and damaged emitters for the 2nd and 4th leaf of cultivated plants (respectively, $\chi^2_{(1)} = 0.24$, $P = 0.63$; $\chi^2_{(1)} = 0.027$, $P = 0.87$; Fig. S2) and for the 2nd leaf of feral plants ($\chi^2_{(1)} = 2.16$, $P = 0.14$; Fig. S2). For the 4th leaf of feral plants, caterpillars consumed more leaf area of plants exposed to undamaged emitters than those exposed to damaged emitters ($\chi^2_{(1)} = 23.04$, $P < 0.0001$; Fig. S2).

Experiment 2: Defence induction upon exposure to volatiles from fresh or old damage

Emitter VOCs Herbivore damage by *S. exigua* increased the emission of volatiles in emitter plants. In addition, total emissions ($\chi^2_{(3)} = 87.8$, $P < 0.0001$; Fig. 6a) as well as volatile composition changed through time (RDA: $\chi^2_{(3)} = 367.5$, $P < 0.0001$, constrained proportion = 0.40; Fig. 6b). Fresh damage volatiles (4 h and 22 h) were dominated by constitutively stored compounds such as benzaldehyde and α-pinene, whereas old damage volatiles (28 h and 46 h) were dominated by inducible compounds such as β-ocimene and indole. Emissions after 28 h of damage were more characterised by α-farnesene and hexenyl acetate and after 46 h by TMTT and β-farnesene. All the tested individual compound emissions were affected by time after damage (Table S3), but the temporal dynamics of constitutive and inducible compounds were contrasting. Stored compounds were emitted from freshly damaged plants and increased slightly with time after damage, or even decreased in the case of benzaldehyde (Fig. 6c). Inducible volatiles, on the other hand, were almost absent from freshly damaged plants and increased dramatically after 1 or 2 d of damage (Fig. 6d).

Receiver responses The terpene aldehydes, gossypol (Fig. S3A) and heliocides (Fig. S3B), were not significantly different among receiver plants exposed to clean air, fresh damage volatiles (stored volatiles released from 0 h to 24 h since damage onset), or old damage volatiles (stored plus *de novo* synthesised volatiles released from 24 h to 48 h since damage onset; $\chi^2_{(2)} = 1.02$, $P = 0.30$; $\chi^2_{(2)} = 0.28$, $P = 0.87$, respectively). The total VOCs emitted from receiver plants were also not significantly different between exposure to fresh and old damage volatiles ($\chi^2_{(1)} = 2.13$, $P = 0.14$; Fig. S3C). However, the levels of the phytohormones JA (Fig. 7a) and JA-Ile (Fig. 7b) were higher for plants exposed to emitters with old damage than to clean air or emitters with fresh damage ($\chi^2_{(2)} = 1.02$, $P =$; $\chi^2_{(2)} = 36.43$, $P < 0.0001$; $\chi^2_{(2)} = 14.83$, $P = 0.0006$, respectively). There was no such

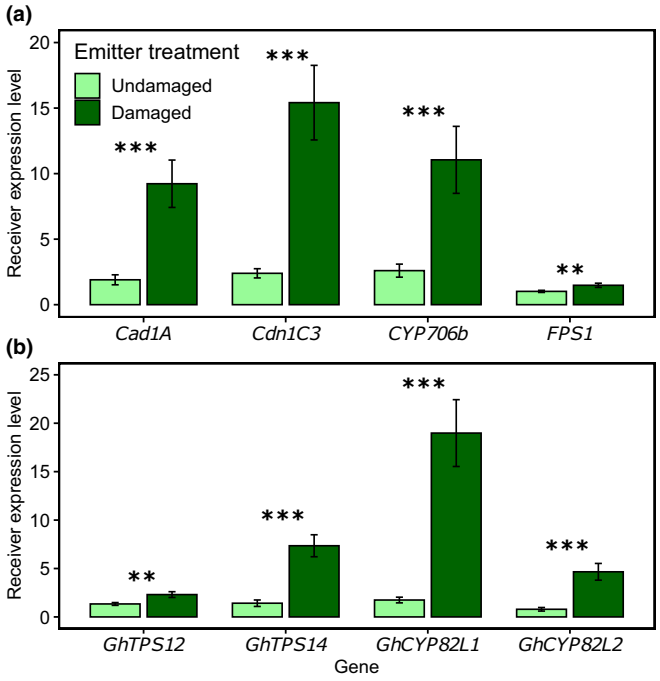

Fig. 5 Mean levels of gene expression in leaves of undamaged receiver *Gossypium hirsutum* plants exposed to undamaged and damaged emitter plants. Several genes were tested that are associated with the gossypol biosynthetic pathway (a), and the terpene biosynthetic pathway (b). Data were pooled for the following factors: variety (cultivated or feral cotton), caterpillar species (*Spodoptera frugiperda* or *S. exigua*), and leaf (Leaf 1 or Leaf 4 from bottom to top). Generalised linear mixed models were performed to assess differences between factor levels. Asterisks indicate significance level (**, $P < 0.01$; ***, $P < 0.001$). Shown are variable means ±SE.

effect on salicylic acid (SA, Fig. 7c) or ABA (Fig. 7d) ($\chi^2_{(2)} = 4.55$, $P = 0.10$; $\chi^2_{(2)} = 0.93$, $P = 0.63$, respectively). In all comparisons, plants exposed to control and fresh damage volatiles did not show significant differences (JA: $P = 0.80$; JA-Ile: $P = 0.69$; Fig. 7).

Genes associated with the synthesis of terpene aldehydes were differentially expressed in receiver plants among the different volatile exposure treatments (Fig. 8a; Table S3). Plants exposed to old damage volatiles showed higher expression of *Cad1A* and *CdnC3* than plants exposed to clean air, and higher expression of *Cad1A* than plants exposed to fresh damage volatiles. *CYP706b* showed a similar trend although not statistically significant (Fig. 8a; Table S4). None of these three genes showed differences in expression between the exposure to clean air and fresh damage volatiles. Genes associated with the synthesis of volatile terpenes showed a different pattern (Fig. 8b; Table S4). Gene *GhCYP82L1* was not differentially expressed among volatiles exposure treatments. Gene *GhCYP82L2* was more expressed in plants exposed to fresh and old damage volatiles than in plants exposed to clean air. Curiously, *GhTPS14* was expressed more in plants exposed to clean air than in plants exposed to fresh and old damage volatiles.

In the preference bioassay, caterpillars consistently avoided leaf discs previously exposed to old damage volatiles across all time points (Fisher's exact test: $P = 0.019$). Specifically, the first choice of caterpillars was not different among leaf discs exposed to clean air, fresh or old damage volatiles ($P = 0.859$; Fig. 9). However, after 1 h, there were more caterpillars on the clean air treatment ($P = 0.0297$), and after 2, 3, and 4 h, there were no longer any caterpillars feeding on leaves exposed to old damage volatiles ($P = 0.00584$, $P = 0.0189$, $P = 0.0565$, respectively; Fig. 9).

Discussion

Airborne signalling between plants has been recognised as a mechanism wherein plants receive information contained in volatile emissions released by neighbouring plants under herbivore attack, and in turn prepare their defensive arsenal for future herbivore attacks. Although this phenomenon has been described in cotton, so far little is known about the defences activated by volatiles and the specificity of signalling compounds. In this study, we showed that chemical defences in cotton can be induced directly by exposure to HIPVs from neighbouring plants and that this induction can have consequences for herbivore preference. Interestingly, the genes that were activated by HIPVs from neighbouring plants are the same genes that are activated by herbivory on the same plant, as shown in previous studies (Zebelo *et al.*, 2017). We also showed that only HIPVs released 1 d after the onset of damage are responsible for this activation, suggesting that the response of cotton plants to HIPVs is a specific response to certain plant volatiles that are synthesised *de novo* in damaged plants, which can be considered reliable indicators of herbivore attack (Paré & Tumlinson, 1997). Previous studies have explored the effects of varying exposure duration to volatiles (Girón-Calva *et al.*, 2012; Moreira *et al.*, 2021), but ours appears to be the first study to look at the separate effects of differently timed pools of volatile released by damaged plants on neighbouring conspecifics. If other plant species also release such distinctly timed pools of volatiles after insect damage, it could suggest a common signalling strategy to convey and receive honest and reliable signals that so far has been largely overlooked.

Signalling by HIPVs has been reported for several plant species, sometimes resulting in direct defence induction and sometimes in defence priming (Heil & Karban, 2010; Ninkovic *et al.*, 2019). Here, we found evidence of direct induction of plant defences by neighbouring HIPVs. Although induction and priming refer to different concepts, they are interrelated. In the case of priming, it has been proposed that a transient small defence response can be detected right after the priming signal (Martinez-Medina *et al.*, 2016). Whether this initial response can be considered an actual induced defence or not depends on the degree of activation. We found an increase in gossypol production in leaves after exposure to HIPVs (*c.* 30%; Fig. 2), a small response compared with the levels of gossypol induction when plants are damaged themselves (> 100%; Bezemer *et al.*, 2004). By contrast, the expression of genes associated with the synthesis of terpenes and terpenoids was highly enhanced, as it occurs when plants are subjected to direct damage (Zebelo *et al.*, 2017). This indicates that the increase in gossypol was limited, but the

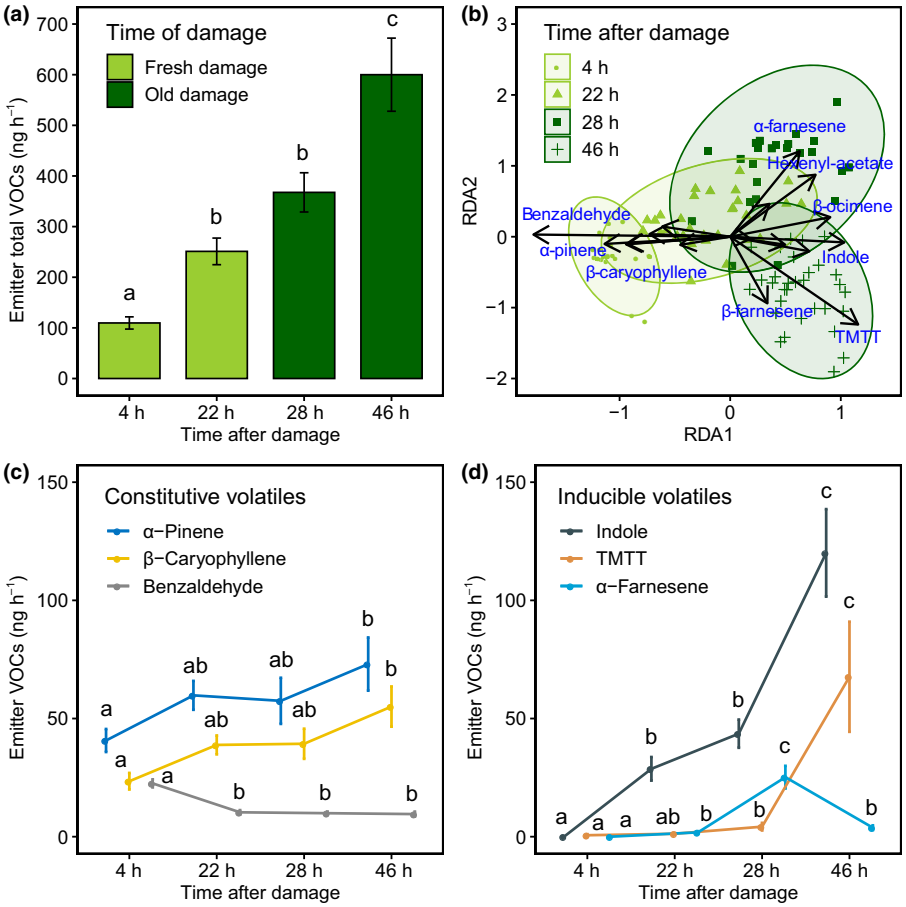

Fig. 6 Changes in plant volatile emissions through time in *Gossypium hirsutum* plants damaged by *Spodoptera exigua* caterpillars. (a) Mean total volatile emission in plants after 4, 22, 28, and 24 h of being damaged by caterpillars. (b) Redundancy analysis (RDA) plot of plant volatiles depending on the time after damage for 4 h (pale green dots), 22 h (pale green triangles), 28 h (dark green squares), and 46 h (dark green crosses). Arrows indicate the loading vectors for the most influential compounds. Ellipses indicate a 95% confidence of distribution of observations within a group. The mean emissions of constitutive and inducible volatiles across time that had highest influence according to the RDA are shown in (c, d), respectively. Generalised linear models were performed to assess differences among factor levels. Shown are variable means ±SE. Different letters indicate significant difference between times within the same compound.

associated genes were already highly activated in response to the volatile signal. Volatiles emitted by undamaged receiver plants were also significantly increased after exposure to HIPVs (Fig. 3). That caterpillars avoided leaves exposed to HIPVs (Figs 5, 9) suggests that this set of responses resulted in a significant protection of leaves exposed to HIPVs; thus, they can be considered as induced defences. Whether the plants induced by HIPVs are also primed for future attacks remains to be tested. In wild cotton, there is evidence that HIPVs can prime the systemic induction of extrafloral nectar in response to direct herbivore damage (Briones-May *et al.*, 2023) indicating that a mix of responses can be induced at different times after exposure to volatiles.

Cotton volatiles are induced by herbivory in a time-dependent manner (Loughrin *et al.*, 1994), whereby initiated leaf damage results in the release of constitutively stored monoterpenes such as α-pinene and limonene, and several sesquiterpenes such as β-caryophyllene and α-humulene. We also observed increases in the production of constitutive compounds in receiver plants that had been exposed to HIPVs from damaged emitters (Fig. 3), which is likely due to the general increase in the expression of genes involved in terpenoid production (Fig. 4). After 1 d of damage, the volatile profile changes dramatically and includes, in addition, inducible compounds that are *de novo* synthesised (Paré & Tumlinson, 1997) such as the monoterpene β-ocimene and the sesquiterpene α-farnesene, the homoterpenes DMNT, 8,12-trimethyl-l,3,7,1 l-tridecatetraene (TMTT), and the aromatic volatile indole. Moreover, several green leaf volatiles are released from the damaged sites of leaves with fresh and old damage (McCall *et al.*, 1994). Only one of these is also *de novo* synthesised upon attack, (*Z*)-3-hexenyl acetate, and is also systemically released from undamaged leaves (Röse *et al.*, 1996). In this study, we confirm the temporal emission patterns in emitter damaged plants, and in addition, we show for the first time that exposure to the volatiles released the first day of damage is not enough to induce changes in gene expression and phytohormones in neighbouring undamaged plants (Figs 7, 8). Only volatiles emitted after 24 h of damage activated the induction in receiver plants. This shows a differential role for stored and *de novo* synthesised volatiles pools in signalling between plants. It is important to note that after 1 d of damage, emitter plants released volatiles at higher concentrations than freshly damaged plants. Thus, both the composition and concentration of HIPVs changed with time, and either or both signals could be necessary to induce receiver plants, suggesting that the reception of volatiles by cotton plants is specific, and only some compounds in precise concentrations trigger the induced responses.

Old damage volatiles activated defence gene expression but did not significantly increase gossypol contents or volatile emissions in receiver plants (Figs 8, S3). This might be due to the sequential nature of plant defence events, where the induction of gene

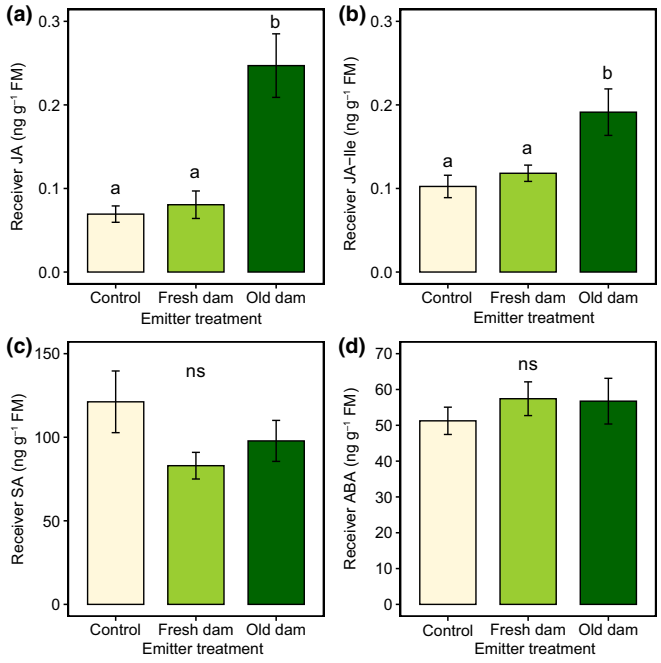

Fig. 7 Mean levels of phytohormones in leaves of *Gossypium hirsutum* plants exposed to clean air (Control) or emitter plants with fresh damage (Fresh dam – 0 h to 24 h after damage started) or old damage (Old dam – 24 h to 48 h after damaged started). Emitter plants were damaged by *Spodoptera exigua* caterpillars. The measured phytohormones were jasmonic acid (a), jasmonic acid-isoleucine (b), salicylic acid (c), and abscisic acid (d) are shown. Generalised linear models were performed to assess differences among factor levels. Different letters indicate significant differences between treatments. Shown are variable means ±SE. ns indicates no statistically significant difference between treatments.

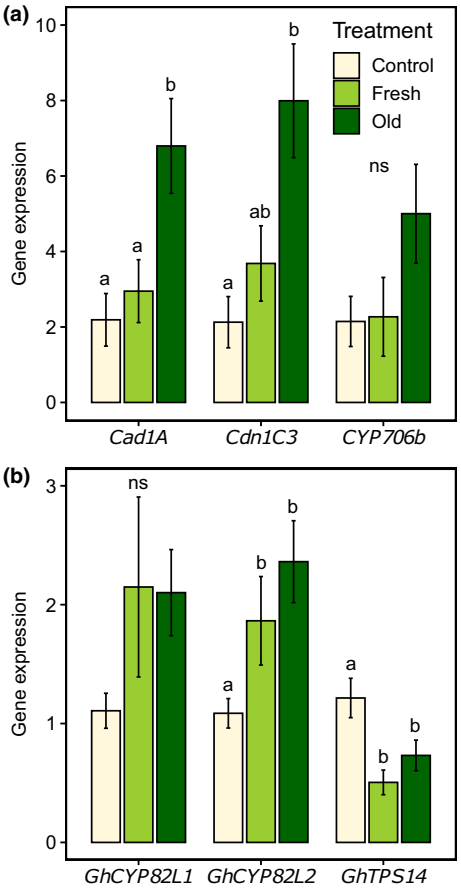

Fig. 8 Gene expression levels in receiver *Gossypium hirsutum* plants exposed to clean air (Control) or volatiles from emitters with fresh damage (Fresh – 0 h to 24 h after damage onset), or old damage (Old – 24 h to 48 h after damage onset). Emitter plants were damaged by *Spodoptera exigua* caterpillars. Several genes were tested that are associated with the gossypol biosynthetic pathway (a), and the terpene biosynthetic pathway (b). Generalised linear models were performed to assess differences among factor levels. Different letters indicate significant differences between treatments within the same gene. Shown are variable means ±SE. ns indicates no statistically significant difference between treatments.

expression precedes metabolites accumulation (Engelberth *et al.*, 2004; Brosset & Blande, 2022). More detailed temporal and spatial measurements of transcriptional and metabolic changes will help to accurately determine the dynamic plant defence responses to VOC exposure. Only phytohormones related to chewing damage such as JA and Ile-JA increased with the exposure to old damage volatiles, whereas ABA and SA did not show any trend (Fig. 7). This indicates that the response to HIPV exposure is also particular to defence and not a general stress signal. Cotton volatiles released during the first day of damage, such as α-pinene and caryophyllene, are also released upon mechanical damage (Paré & Tumlinson, 1997). Since the release of *de novo* synthesised HIPVs after 1 d of damage is activated by specific herbivore elicitors (Paré & Tumlinson, 1997; Röse & Tumlinson, 2005; Arce *et al.*, 2021), old damage volatiles are honest and highly reliable signals of herbivore activity. This strongly suggests that plant volatile reception is a mechanism that evolved as an adaptive trait in response to herbivory.

In our experiments, caterpillars also avoided eating leaves exposed to newly produced volatiles; when given the choice, they preferred to feed on leaves exposed to volatiles of undamaged plants or freshly damaged plants (Figs 5, 9). This supports the idea that cotton plants are better defended after they perceived that neighbours are attacked by herbivores. Cotton plants induced by mite and caterpillar damage are better protected against caterpillars (Karban, 1988; Alborn *et al.*, 1996). Induction by volatiles is potentially equivalent to actual herbivore induction in terms of plant protection (Llandres *et al.*, 2018). This is in line with the induction of phytohormone and terpenoid genes that have been previously associated with chemical defences against herbivores (Sunilkumar *et al.*, 2006; Campos *et al.*, 2014; Zebelo *et al.*, 2017). Proper tests comparing plants induced by volatiles and herbivores would give more information about these patterns. Gossypol and heliocides in leaf 3 did not increase after exposure to old damage volatiles; however, we could not analyse these defensive compounds in Leaf 4 because it was used for the caterpillar test. It is likely that Leaf 4 shows differences in these terpenoids, since younger leaves are more inducible and show higher concentrations of defensive compounds (Bezemer *et al.*, 2004). While direct induced defences protected the leaves from herbivores, we cannot rule out the possibility of

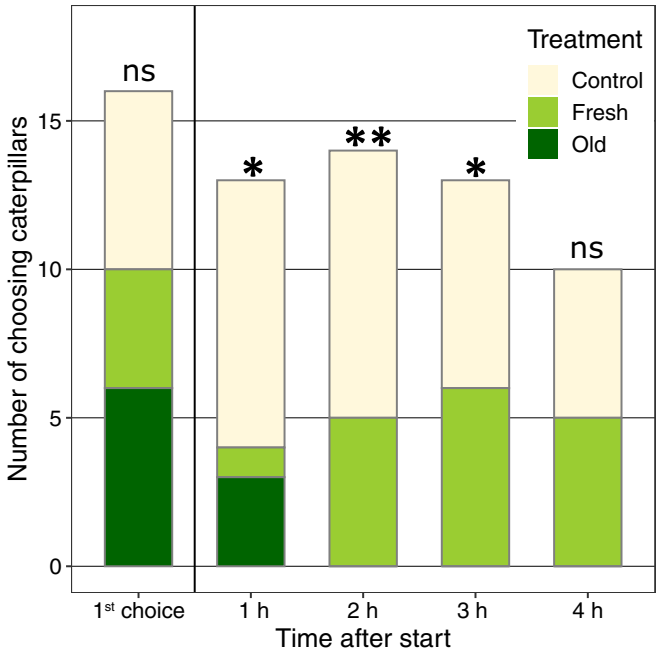

Fig. 9 Preferences of caterpillars in a three-choice test with leaf discs from *Gossypium hirsutum* plants exposed to clean air (Control), fresh damage volatiles (Fresh – 0 h to 24 h after damage onset), or old damage volatiles (Old – 24 h to 48 h after damage onset) from emitter plants damaged by *Spodoptera exigua* caterpillars. The total number of caterpillars found feeding in each of the treatment is shown for several time points across 16 tests. Asterisks indicate a significant difference between the observed responses and the expected responses if there was no preference among treatments according to an exact multinomial test (*, $P < 0.05$; **, $P < 0.01$). ns indicates no statistically significant difference between treatments.

passive adsorption of HIPVs onto undamaged leaves. For instance, in sagebrush, undamaged plants can absorb and reemit volatiles from damaged plants than can help them to repel herbivores (Grof-Tisza *et al.*, 2022). However, undamaged plants exposed to those with old damage did not show higher volatile emissions compared to those exposed to clean air or freshly damaged, suggesting minimal significant adsorption of HIPVs.

The plants used for the first experiment were of different origins and domestication status. The cultivated plants came from a common line of cultivated cotton widely used in Mali; the feral plants came from seeds collected from plants in the south coast of Mexico. The latter are likely descendants of ancient cultivated cotton, which have been growing under wild conditions for a couple of hundred years (local communications). Therefore, both plant types are unrelated genotypes but showed very similar defence induction by HIPVs in terms of terpenoid aldehyde production, volatile emission, and gene expression. This implies that the signalling between cotton plants is highly conserved within the species. However, the feral plants exhibited higher levels of defences and were more effective at repelling caterpillars in the preference test (Fig. 5). This is in line with the expectation that domestication has decreased the levels of defences, as plants are protected from insects by other means such as insecticides and that human-controlled selection focussed on productive traits that can trade-off with resistance (Chen *et al.*, 2015). Our results not only support the domestication-defence theory but also show that plant signalling may not have been diminished in cultivated cotton.

The findings presented in this study shed light onto the intricate mechanisms underlying airborne signalling between plants and the consequent induction of defences against herbivores. We demonstrate that neighbouring plants can directly activate chemical defences, thereby influencing herbivore preference. This is not only shown for the activation of shared defence pathways in response to both direct herbivory and HIPV exposure but also in terms of specificity and timing of these responses. Previous research has demonstrated that the pool of *de novo* synthesised volatiles induced by caterpillar feeding carries specific information about the cause of the damage (Loughrin *et al.*, 1994; Paré & Tumlinson, 1997; Arce *et al.*, 2021). We here show that neighbouring plants use this information to initiate their own defensive responses against potential incoming attacks. Overall, these findings contribute to a deeper understanding of plant defence strategies and emphasise the ecological significance of plant signalling in shaping herbivore–plant interactions. Further research in this area holds promise for elucidating the broader ecological implications and evolutionary underpinnings of plant signalling networks. In addition, it will be important to consider the wider impact for sustainable management in cotton cultivation, for example in the use of defence elicitors in integrated pest management.

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Competing interests

None declared.

Author contributions

TCJT, LG, BB, LAR and TB designed the research. LG, WY, AV and GG performed the research. CBS and LG analysed the data. CBS, MVC and WY wrote the first draft of the manuscript with substantial contributions from all authors. LG, WY, and MVC contributed equally to this work.

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Data availability

The data that support the findings of this study are openly available in Zenodo at doi: 10.5281/zenodo.12706960.

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Supporting Information

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

Fig. S1 Experimental design for Experiment 2.

Fig. S2 Consumed leaf area by *Spodoptera exigua* on cotton leaves in Experiment 1.

Fig. S3 Effects of emitter volatiles on chemical defences of receiver undamaged plants.

Table S1 Primers used for quantitative polymerase chain reaction.

Table S2 Results of statistical models of plant traits in Experiment 1.

Table S3 Results from univariate tests of volatiles from emitter plants in Experiment 2.

Table S4 Results from statistical models of gene expression in receiver plants in Experiment 2.

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