Received: 20 October 2022 | Revised: 6 December 2022 | Accepted: 12 December 2022

DOI: 10.1111/pce.14516

ORIGINAL ARTICLE

Plant, Cell & Environment WILEY

Insect exuviae as soil amendment affect flower reflectance and increase flower production and plant volatile emission

Katherine Y. Barragán-Fonseca[1,2] | Quint Rusman[1] | Daan Mertens[3] | Berhane T. Weldegergis[1] | Joseph Peller[4] | Gerrit Polder[4] | Joop J. A. van Loon[1] | Marcel Dicke[1]

[1]Laboratory of Entomology, Wageningen University & Research, Wageningen, The Netherlands

[2]Grupo en Conservación y Manejo de Vida Silvestre, Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá, Colombia

[3]Department of Entomology and Nematology, University of California, Davis, California, USA

[4]Greenhouse Horticulture, Wageningen University & Research, Wageningen, The Netherlands

Correspondence

Marcel Dicke, Laboratory of Entomology, Wageningen University & Research, Wageningen, The Netherlands.
Email: marcel.dicke@wur.nl

Funding information

Departamento Administrativo de Ciencia, Tecnología e Innovación (COLCIENCIAS)

Abstract

Soil composition and herbivory are two environmental factors that can affect plant traits including flower traits, thus potentially affecting plant–pollinator interactions. Importantly, soil composition and herbivory may interact in these effects, with consequences for plant fitness. We assessed the main effects of aboveground insect herbivory and soil amendment with exuviae of three different insect species on visual and olfactory traits of *Brassica nigra* plants, including interactive effects. We combined various methodological approaches including gas chromatography/mass spectrometry, spectroscopy and machine learning to evaluate changes in flower morphology, colour and the emission of volatile organic compounds (VOCs). Soil amended with insect exuviae increased the total number of flowers per plant and VOC emission, whereas herbivory reduced petal area and VOC emission. Soil amendment and herbivory interacted in their effect on the floral reflectance spectrum of the base part of petals and the emission of 10 VOCs. These findings demonstrate the effects of insect exuviae as soil amendment on plant traits involved in reproduction, with a potential for enhanced reproductive success by increasing the strength of signals attracting pollinators and by mitigating the negative effects of herbivory.

KEYWORDS

Brassica nigra, floral traits, herbivory, insect residual streams, plant phenotype, plant–pollinator interactions

1 | INTRODUCTION

As a plant's reproductive organs, flowers are valuable plant structures that interact with pollinating animals to ensure reproduction. Plants have evolved a wide diversity of floral traits such as colour, size, pattern and scent to attract flower visitors (Weiss, 1991). In addition to these flower-specific traits, the number of flowers on a plant plays a crucial role in pollinator attraction, with larger floral displays attracting more flower visitors (Ohashi & Yahara, 2002; Salisbury et al., 2015). Although different floral traits separately offer specific information to flower visitors, visual and olfactory cues are often used in combination to simultaneously assess presence, location, quantity and reward quality of flowers. This improves pollinator foraging performance, strauss potentially enhancing pollination and plant fitness (Barragán-Fonseca et al., 2020; Leonard et al., 2011; Raguso & Willis, 2002; A. L. Russell et al., 2018; Schiestl, 2015). Hence, a greater number of larger flowers enhanced visual and olfactory cues, generally contributing to maximizing plant reproduction.

Environmental factors such as soil composition and foliar herbivory can affect optical and chemical flower traits and thereby influence plant reproduction (Elle & Hare, 2002). Such effects are relatively well documented for herbivory (A. Kessler & Chautá, 2020; Rusman, Poelman, et al., 2019). For instance, leaf herbivory by different insect species resulted in changes in flower morphology, petal colour and the composition of the volatile blend of flowering *Brassica nigra* plants (Rusman, Poelman, et al., 2019). Herbivory may also reduce the amount of volatiles emitted by flowers (Schiestl, 2015; Schiestl et al., 2014). Consequently, the changes in floral traits in response to herbivore attack may compromise flower attractiveness and lead to a species-specific disruption of plant–pollinator interactions (Rusman, Lucas-Barbosa, et al., 2019; Rusman, Poelman, et al., 2019; Schiestl et al., 2014). In addition, herbivore-induced changes in floral traits can reduce floral constancy and pollination effectiveness by interfering with honest signalling (Rusman, Lucas-Barbosa, et al., 2019). By inducing changes in flower traits herbivory influences plant reproduction.

Soil composition and nutrient availability can also alter floral traits (Caruso et al., 2019; Strauss & Whittall, 2006). Soil nutrient enrichment and changes in soil water levels influence the quality and quantity of available resources, allowing the plant to increase its investment in flower production (Carvalheiro et al., 2021). This leads to an enhanced flower display, increasing the probability of pollination (David et al., 2019). Thus, enriching the soil with organic substances may positively impact floral traits through changes in soil nutrients and organic matter content (Parađiković et al., 2019; Rowen et al., 2019). Soil amendments can also alter the soil microbial community (Rowen et al., 2019). Beneficial soil microorganisms can enhance flower abundance (Göre & Altin, 2006), colour (Flores et al., 2007) and size (Kumari et al., 2016), either through direct interactions with the root microbiome, or by increasing the availability of nutrients in the rhizosphere (De Tender et al., 2019; Heinen et al., 2018; Todeschini et al., 2018).

Insect exuviae are a novel type of soil amendment that may be used to promote plant growth and reproduction (Barragán-Fonseca et al., 2022). Insect exuviae contain chitin, a substance yielding macronutrients and micronutrients to stimulate beneficial rhizobacteria (De Tender et al., 2019). In addition to chitin, insect exuviae upon degradation in the soil also provide other nutrients such as nitrogen that stimulate the abundance and diversity of beneficial rhizobacteria promoting plant growth (Nurfikari & de Boer, 2021). Thus, insect exuviae have the potential to affect floral traits by increasing the availability of nutrients, changing nutrient uptake through stimulation of native soil rhizobacteria and by the changes these microbes induce in plants (Barragán-Fonseca et al., 2022). Furthermore, chitin elicits systemic defence responses in plants against diseases (Parada et al., 2018). However, the indirect interactions between belowground and aboveground organisms via changes in floral traits are understudied (Barber & Soper Gorden, 2014). Recently, we showed that amending soil with *Hermetia illucens* exuviae can alter plant traits, such as plant size and flower abundance, with consequences for insect pollinators and seed production (Barragán-Fonseca et al., 2023). However, it remains unexplored whether soil amendment with insect residual streams affects specific floral traits that are used by insect pollinators when foraging for floral resources. Floral traits such as colour and odour attract animals that pollinate outcrossing plant species (Barragán-Fonseca et al., 2020; Galizia et al., 2004; Raguso, 2008). Although our knowledge of the effects of herbivory on plant traits has grown, there is a paucity of research linking soil amendments and herbivory with the plant traits that influence pollinators.

Because plants are exposed to soil composition and herbivory simultaneously, these factors may interact in their effect on the abundance, morphology or chemical traits of the flowers. Plant resistance to insect herbivores is influenced by physical, chemical and biological soil properties (Altieri & Nicholls, 2003). Thus, the type and amount of nutrients supplied to a plant may affect the performance and abundance of herbivores attacking the plant (Rowen et al., 2019; Stafford et al., 2012). An increased soil nutrient availability might also support plant development, even when exposed to herbivory (Meyer, 2000), and minimize herbivory-mediated changes in floral traits. Alternatively, increased soil nutrient levels may make the plant more susceptible to insect herbivore attack due to an increased nitrogen-to-carbon ratio in leaves, which enhances herbivore performance (Altieri & Nicholls, 2003) and potentially amplifies herbivore-induced effects on flower traits. A deeper understanding of how herbivory may interact with soil amendment in terms of their effects on plant traits would help to expand knowledge of the combined effects of biotic and abiotic factors above- and belowground.

Here, we evaluate the main and interactive effects of herbivory and soil amendment with exuviae of three different insect species on visual and volatile chemical traits of flowers of *B. nigra* plants. We tested the following hypotheses: 1. Herbivory affects visual flower traits such as flower number or petal size and alters olfactory cues from flowering plants by reducing the emission rate of certain volatile compounds or changing the volatile blend composition. 2. Soil amendments increase flower number, petal size and volatile emission rate. 3. The effect of soil amendment interacts with herbivory, with positive effects of soil amendments on visual and chemical floral traits affecting the potential negative effects of herbivory. By combining various methodological approaches including gas chromatography/mass spectrometry, spectroscopy and machine learning, we provide novel insights into the effects of chitin-based soil amendments on floral traits.

2 | MATERIALS AND METHODS

2.1 | Plants and insects

Black mustard (*B. nigra* (L.) W.D.J. Koch, Accession CGN06619) seeds were obtained from the Centre for Genetic Resources, propagated by natural pollination and exposed to natural conditions in an experimental field near Wageningen University. In a greenhouse (22 ± 1°C, 50%–70% RH, L16:D8), seeds were sown in pots (Ø 17 cm, 2 L), in which the plants grew for the entire duration of the experiment. Plants were individually watered every day to maintain a high level of soil moisture in the pots.

Two specialist herbivorous insect species were used in the experiment, *Pieris brassicae* L. (Lepidoptera: Pieridae) and *Brevicoryne brassicae* L. (Hemiptera: Aphididae). The insects were reared on Brussels sprout plants (*Brassica oleracea* L. var. *Gemmifera*) at 22 ± 1°C, 50%–70% RH, and L16:D8.

2.2 | Herbivore infestation

Five neonate caterpillars and 10 aphids were inoculated on one true leaf per plant. To prevent aphids from spreading to neighbouring plants, we enclosed the leaf in a mesh bag, and closed it around the petiole by wrapping cotton wool around it. Infestation lasted for 7 days. Untreated control plants went through the same procedure, except for the infestation with the herbivores. The bagged leaves were cut from all plants before assessment of plant traits.

2.3 | Soil amendment

Soil amendments were made by adding exuviae of either of three insect species: black soldier fly (*H. illucens* L., Diptera: Stratiomyidae; hereafter BSF), house cricket (*Acheta domesticus* L., Orthoptera: Gryllidae) and yellow mealworm (*Tenebrio molitor* L., Coleoptera: Tenebrionidae) (Figure 1). Insect exuviae were provided by Bestico, Fair Insects and Nijenkamp, respectively. Exuviae were oven-dried at 60°C for 24–48 h until a constant weight was obtained. Samples were pulverized into a fine powder using a TissueLyser II stainless steel ball mill (QIAGEN). Soil was collected from the experimental fields of Wageningen University and sieved (Ø 200 mm, 4 mm) to remove pebbles. Soil was amended by mixing 2 g of pulverized insect exuviae per kg of soil. To mix the soil and

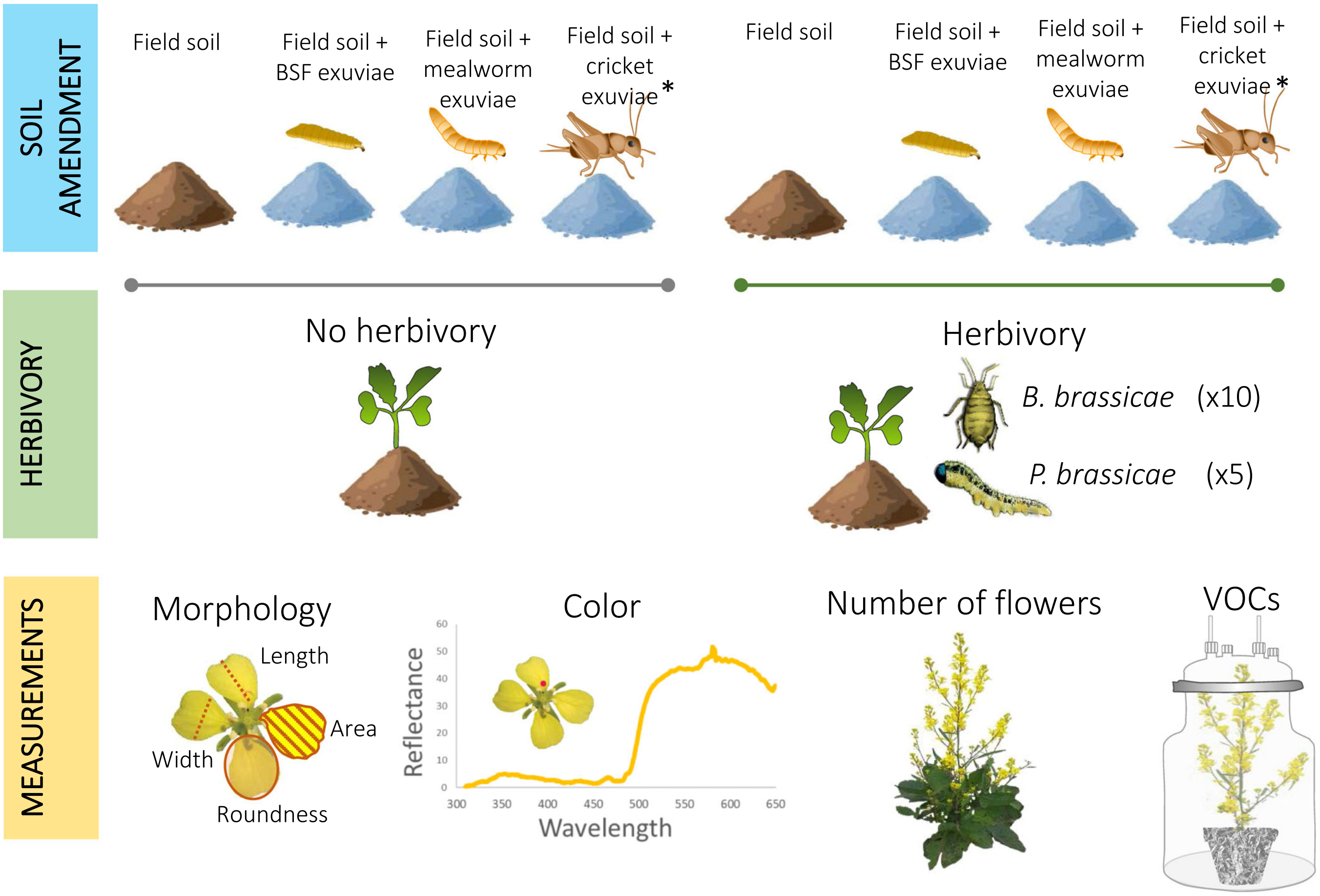


FIGURE 1 Overview of the experimental design. Seeds of *Brassica nigra* were sown in field soil or in field soil amended with BSF exuviae, yellow mealworm exuviae or house cricket exuviae. After 4 weeks (morphology and colour analysis) and 5 weeks (VOC analysis), half of the plants of each treatment were infested with the herbivores *Pieris brassicae* and *Brevicoryne brassicae*. Measurements were taken after 1 week of herbivore exposure. *For flower morphology and colour assays the cricket treatment was not assessed because of a limited supply of house cricket exuviae. BSF, black soldier fly; VOC, volatile organic compound.

exuviae, 20 g of powdered exuviae was mixed thoroughly by hand into 10 kg bags of dry soil. The same procedure was followed for control soil, except that no powdered exuviae was added.

2.4 | Plant treatments

B. nigra plants were randomly assigned to receive neither, one, or both plant treatments, that is, herbivore infestation and soil amendment with insect exuviae. This resulted in eight groups: no treatment (control, C), soil amendment with yellow mealworm exuviae (MW), BSF exuviae (BSF) or house cricket exuviae (CK), non-amended soil plus herbivore infestation (H) or soil amendment with yellow mealworm exuviae plus herbivory (MW + H), soil amendment with BSF exuviae plus herbivory (BSF + H) or soil amendment with house cricket exuviae plus herbivory (CK + H) (Figure 1). For flower size, morphology, and colour assays the CK and CK + H treatments were not included because of a limited supply of house cricket exuviae.

2.5 | Assessing flower size, morphology, colour and abundance

We investigated the effect of the different treatments on flower size, morphology and colour. Whole flowers (single open flowers) were mounted on a platform made of cork (Ø 2.3 cm), located 11.4 cm under a UV camera (IDS UI-1240LE; resolution 1.31 Mp; lens = IDS-10M11-C2514, 25 mm, 1.1″). A UV ring lamp was mounted surrounding the camera providing even illumination of the object. After a top-view picture was made of the whole flower, the petals were separated from the rest of the flower, and a top-view picture was taken from the four petals together. After image capture, the cork platform containing the petals was transferred to a spectroscopy set-up (Rusman, Poelman, et al., 2019). For two petals, we measured the top (centred, 0.5 mm below the distal edge of the petal) and the base (centred, 0.5 mm above the point where the petal narrows and bends downwards) with a spectrometer (SD2000; Ocean Optics) using a fibre optic reflection probe and a deuterium-halogen light source (DH2000-FHS; Ocean Optics). The spectrometer was calibrated with a white reference (WS-2, TOP Sensory Systems) and a black reference (by covering the input fibre). Six flowers of the distal inflorescence of two flowering branches (three flowers per inflorescence) were analysed for each plant, and flowers were taken from 10 plants per treatment. To retrieve flower size and morphological measurements, we processed whole flower and petal images by using a classification model created by using images of flowers and petals for separating flower and background. All other images were segmented by this trained classification model. For UV, we segmented the images using an intensity thresholding method. A mask was created of the image by taking the brightest 5% of pixels and darkest 5% in the image. The mask was then denoised and all small pixel areas were removed. Due to the absorption properties of the flowers, the largest area in this mask was the flower. This was done in MATLAB (Version R2021a) with the perClass toolbox (perClass Enterprise 5.4; PR Sys Design). As a manual check, we inspected each image after automated segmentation and discarded any segmentations where the mask did not directly overlap the flower's area due to glare or shadows. Following segmentation, 30 flower images per treatment were analysed for pixel count (area), length, width, ratio of fitting ellipse (major and minor chord length) of each petal and petal eccentricity (shape of fitting ellipse). We analysed four petals per six flowers per plant. We assessed flower abundance in response to soil amendment and herbivory treatment by counting the number of flowers per plant. Flower abundance, morphology, size and colour were assessed 7 days after the first flower had opened (Figure 1).

2.6 | Plant volatile analysis

To investigate the effect of treatments on the chemical composition of the headspace of *B. nigra*, 10 to 14 replicate plants per treatment were prepared as described above. Dynamic headspace sampling of volatiles was carried out in a climate-controlled room (20 ± 1°C). The pot with the soil and plant roots were carefully wrapped in aluminium foil. The plants were then individually placed into 30 L glass jars connected to an air flow (Figure 1). Synthetic air (Linde Gas Air Synthetic 4.0 Monitoring) was supplied at 230 ml min^{-1} to each jar. Plants were left for 30 min to acclimatize before headspace volatile collection began. Volatile collection started by drawing air out of the glass jars at 200 ml min^{-1} through a stainless steel tube filled with 200 mg Tenax TA adsorbent (20/35 mesh; CAMSCO) for 1.5 h.

To prevent any contribution from the collection set-up, the adsorbent material, and the analytical system to the plant volatile profile and to make the necessary corrections, volatiles from potted soil without a plant and wrapped in aluminium foil were collected at regular intervals and used as background samples. The Tenax TA adsorbent with headspace samples was dry-purged under a stream of nitrogen (50 ml min^{-1}) for 15 min at ambient temperature to remove moisture.

The collected volatiles were then thermally released from the Tenax TA adsorbent using an Ultra 50:50 thermal desorption unit (Markes) at 250°C for 10 min under a helium flow of 20 ml min^{-1}, while simultaneously recollecting the volatiles in a thermally cooled universal solvent trap (Unity; Markes) at 0°C. Once the desorption process was completed, volatile compounds were released from the cold trap by ballistic heating at 40°C sec^{-1} to 280°C, which was then kept for 10 min, while all volatiles were transferred to a ZB-5MS analytical column (30 m long × 0.25 mm ID × 1 µm F.T. with 10 m built-in guard column (Phenomenex), placed inside a Thermo Trace Ultra gas chromatograph (GC) oven (Thermo Fisher Scientific), for further separation of plant volatiles. The GC oven temperature was initially held at 40°C for 2 min and was then raised at 6°C min^{-1} to a final temperature of 280°C, where it was maintained for 4 min under a constant helium flow of 1 ml min^{-1}. For the detection of volatiles, a Thermo Trace DSQ quadrupole mass spectrometer (MS) (Thermo Fisher Scientific), coupled to the GC was operated in an electron impact ionization (EI) mode at 70 eV in a full scan with a mass range of 35–400 amu at 4.70 scans sec^{-1}. The MS transfer line and ion source were set at 275°C and 250°C, respectively.

Automated baseline correction, peak selection (S/N > 3) and alignments of all extracted mass signals of the raw data were processed following an untargeted metabolomic workflow using MetAlign software, producing detailed information on the relative abundance of mass signals representing the available metabolites (Lommen, 2009). This was followed by the reconstruction of the extracted mass features into potential compounds using the MSClust software through data reduction by means of unsupervised clustering and extraction of putative metabolite mass spectra (Tikunov et al., 2012). Tentative identification of volatile metabolites was based on a comparison of the reconstructed mass spectra with those in the NIST 2014 and Wageningen Mass Spectral Database of Natural Products MS libraries, as well as experimentally obtained linear retention indices.

2.7 | Statistical analysis

Analyses of the number of flowers, flower and petal morphology, flower colour (relative reflectance of yellow and UV) and emission of individual volatile compounds were performed by first selecting the best model from a set of candidate models based on the Akaike Information Criterion (Burnham, 2002). All candidate models included the type of soil amendment, herbivory and their interaction as explanatory variables, and where relevant included the identity of the plant and the flower as random intercepts to account for the dependency of observations, with flower nested in plant when both were included. Candidate models differed in terms of their probability distributions. We compared models with a Gaussian and gamma distribution, and where relevant Poisson and negative binomial distributions using (generalized) linear (mixed) models ((G)L(M)Ms). L(M)Ms were further extended to account for the potential heterogeneity of variance by fitting generalized least-squares (GLS) models (Zuur et al., 2009). We used diagnostic plots to verify that model assumptions were met (Zuur et al., 2009). We estimated the effect size and significance of fixed factors using type II Wald χ^2-tests. Pairwise post hoc comparisons were evaluated by Tukey's honest significant difference test and contrasts were considered significantly different at $p \leq 0.05$. Statistical analyses were done using the emmeans (L. Russell, 2018), glmmTMB (Brooks et al., 2017), lme4 (Bates et al., 2015), car (Fox & Weisberg, 2019) and nlme (Pinheiro et al., 2021) packages in R v.4.0.0 (R Core Team, 2018), and R studio v. 1.4.1106.

We analysed reflectance spectra with permutational multivariate analyses of variance (PERMANOVAs). In a first analysis, we included the type of soil amendment, herbivory, their interaction, petal part (top or base), plant and flower identity. Because we were mostly interested in the effect of type of soil amendment and herbivory, and because the overall analysis identified a large difference in reflectance between the top and base part of petals, we performed separate analyses per petal part (Rusman, Poelman, et al., 2019). The final models were performed with the type of soil amendment, herbivory and their interaction as explanatory variables. Because plant and flower identity explained a significant part of the variation, we corrected for dependency of observations by performing stratified permutations with flower identity nested in plant identity. To ensure equal groups, we removed plants with incomplete sampling. We then randomly drew seven plants for treatments with more than seven replicates remaining and performed the PERMANOVA. We repeated the random draw of seven plants followed by PERMANOVA 100 times and present the median, 1st and 3rd quartiles, and 5th and 95th percentiles of the 100 R^2 and p-values we obtained. Although PERMANOVA is robust to heterogeneity of multivariate dispersion (Anderson, 2017; Anderson & Walsh, 2013), we performed homogeneity of variance tests for type of soil amendment and herbivory. We used the vegan package for these analyses (Oksanen et al., 2007).

To investigate which soil amendment types and herbivory combination differed in their spectrum, we performed support vector machine (SVM) analysis. An SVM is a classification algorithm based on machine learning that separates the different treatments by finding a vector that gives the maximum separation between the classes in multiple dimensions. In two dimensions this can be thought of as a line, in three dimensions as a plane, and it can be applied to n-dimensional data sets such as spectra where each wavelength is a dimension (Noble, 2006). We used 80% of the data set to train the SVM model and the remaining 20% of the data to test how well the classification performed. To assess the performance of the classification model we used confusion matrices. A confusion matrix shows the breakdown of the different treatments, and how many of them were correctly classified. From the confusion matrices, we calculated the recall and the precision. The recall is the number of true positives divided by the sum of true positives and false negatives. The precision is the number of true positives divided by the sum of true positives and false positives. The overall accuracy was calculated as the number of correct classifications divided by the sum of correct and incorrect classifications. Statistical analysis was performed using MATLAB (Version R2021) with the perClass toolbox (perClass Enterprise 5.4; PR Sys Design).

Peak heights of emitted volatiles were used as relative quantitative measurements. Since there was no direct correlation with the aboveground plant mass (low R^2), the obtained peak heights were imported into SIMCA-P 17 statistical software (Umetrics) without correcting for the plant mass, followed by log-transformation, mean-centreing and unit-variance scaling before subjecting the data to multivariate data analysis. Supervised orthogonal partial least squares-discriminant analysis (OPLS-DA) was used as a multivariate tool to compare and correlate treatment groups. The results are visualized as score plots revealing the sample structure according to the model components and loading plots showing the contribution of variables to the components as well as relationship among the variables. R^2 and Q^2 metrics, which describe the explained variation within the data set and the predictive power of the model, respectively, were calculated based on the averages of the sevenfold cross-validation. Compounds with variable importance in the projection (VIP) with score values ≥1 were considered potentially relevant and subjected to analyses to test for significant differences between treatments. For each of these compounds, we selected the best model from a group of candidate LM and GLS models. These models included the type of soil amendment, herbivory, and their interaction as explanatory variables and differed in terms of their variance functions. We selected the best model by AIC comparison, validated the model assumptions and estimated the effect sizes and significance of explanatory variables as explained above.

3 | RESULTS

3.1 | Flower abundance, morphology and colour

The type of soil amendment is a significant predictor of the number of flowers produced in *B. nigra* plants, independent of herbivory treatment ($\chi^2 = 81.62$; df = 3; $p < 0.0001$). Soil-amendment-treated plants had 49% more flowers than plants grown in soil without amendment. Although plants exposed to herbivory had 13% fewer flowers than uninfested plants, this effect was not significant ($\chi^2 = 3.74$; df = 1; $p = 0.0530$; Figure 2).

Herbivory affected flower size characteristics, such as flower area, petal area, petal length and petal width. Herbivory-treated plants produced flowers with 17% smaller area ($\chi^2 = 12.08$; df = 1; $p < 0.0001$), 26% smaller petal area ($\chi^2 = 13.74$; df = 1; $p < 0.0001$; Figure 3a), 12% smaller petal length ($\chi^2 = 11.58$; df = 1; $p < 0.0001$; Supporting Information: Figure S1a) and 14% smaller petal width ($\chi^2 = 12.41$; df = 1; $p < 0.0001$; Supporting Information: Figure S1b) than plants of the control treatment. Herbivory did not affect petal circularity ($\chi^2 = 0.065$; df = 1; $p = 0.7993$; Figure 3b). Soil amendment did not influence any petal morphology characteristic.

The spectral profile of the top and base parts of petals differed significantly (PERMANOVA: $R^2 = 51.4$, df = 1, $p = 0.001$; Figure 4). Therefore, we performed separate analyses for the top and base parts. The colour of top and base parts of *B. nigra* petals was affected by the type of the soil amendment and herbivory (Figure 4a,b). The high accuracy of the SVM models shows the ability to discriminate and identify the individual treatments based on the top and base spectra (Figure 4c,d). In other words, the SVM models indicate that the spectra of individual treatments differ substantially.

Spectral profiles of *B. nigra* petals contained two wavelength regions of interest: the yellow/orange region (570–650 nm) and the UV region (310–370 nm). The relative diffuse reflectance (RDR) of yellow/orange of the base part of petals was affected by herbivory ($\chi^2 = 3.91$; df = 1; $p = 0.048$, Figure 5b). Petals of plants infested with herbivores reflected less yellow/orange than did uninfested plants. The RDR of yellow/orange ($\chi^2 = 2.18$; df = 1; $p = 0.140$, Figure 5a) and UV ($\chi^2 = 1.60$; df = 1; $p = 0.207$, Figure 5c) of the top part of petals was not affected by herbivory, and neither was the ratio RDR yellow/RDR UV ($\chi^2 = 0.15$; df = 1; $p = 0.699$, Figure 5d). Soil amendment did

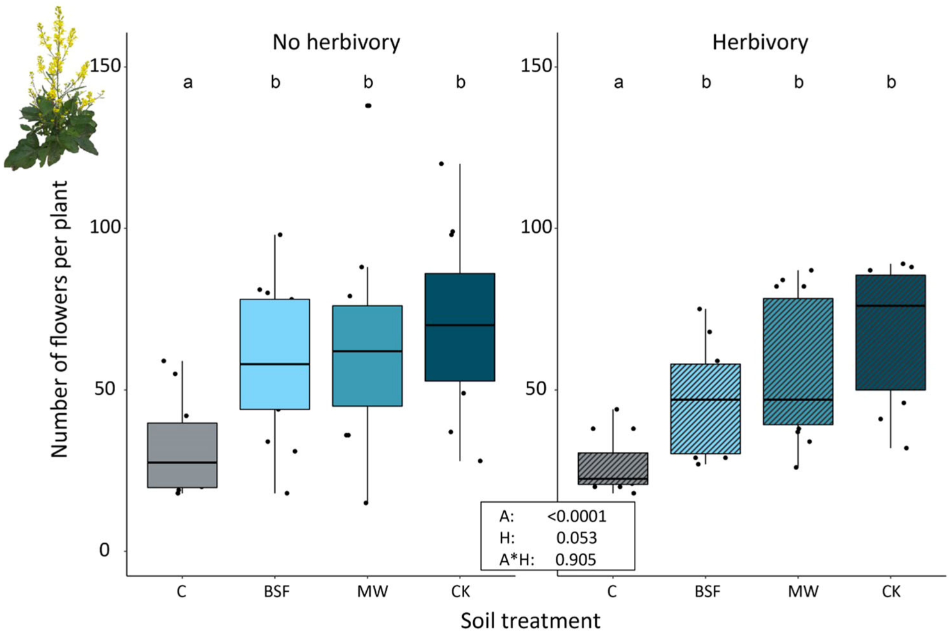


FIGURE 2 Number of flowers developed by *Brassica nigra* plants growing for 5 weeks in soil amended with yellow mealworm exuviae (MW), BSF exuviae (BSF) or house cricket exuviae (CK), with or without infestation with both *Pieris brassicae* caterpillars and *Brevicoryne brassicae* aphids. Control plants (C) grew in soil without soil amendment. Boxplots show median (horizontal bold line), first and third quartiles, and minimum and maximum. Dots represent the samples that were not in the 25%–75% interval. Flowers were counted after 7 days of herbivory. Boxes within each panel that have no letters in common are significantly different (Tukey's post hoc test; $\alpha = 0.05$). Number of replicates per treatment varied between 10 and 12 plants. A is the type of soil amendment, H is herbivory and A*H is the interaction between type of soil amendment and herbivory, followed by *p*-values. BSF, black soldier fly. [Color figure can be viewed at wileyonlinelibrary.com]

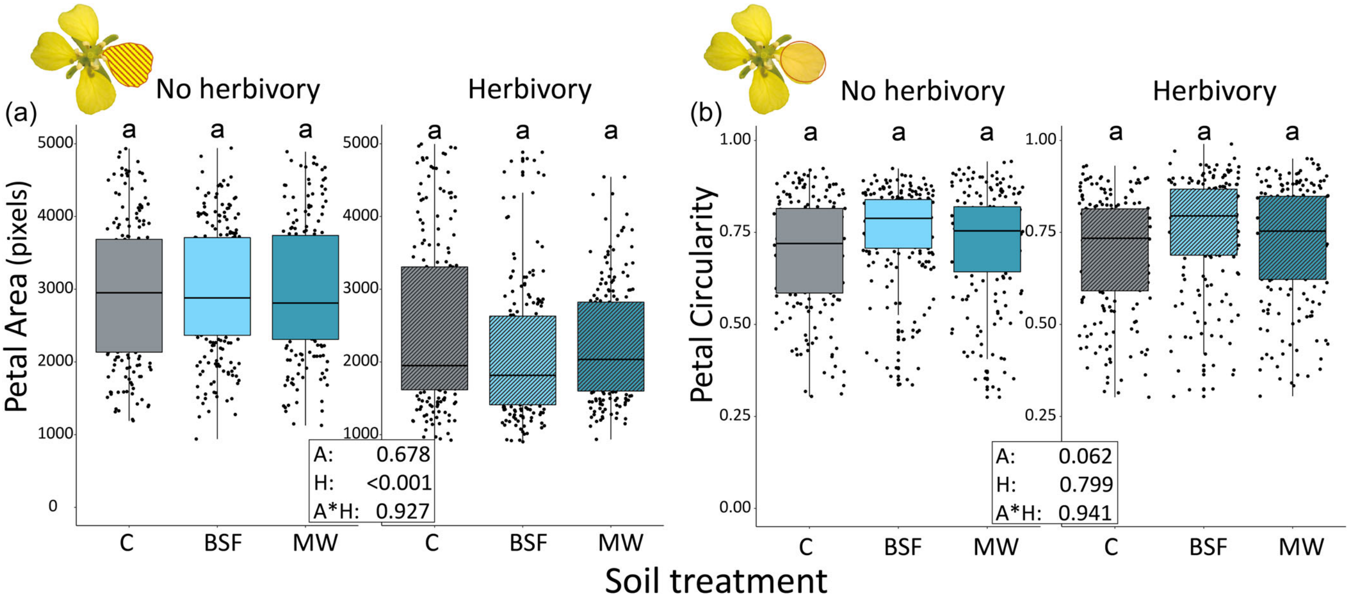


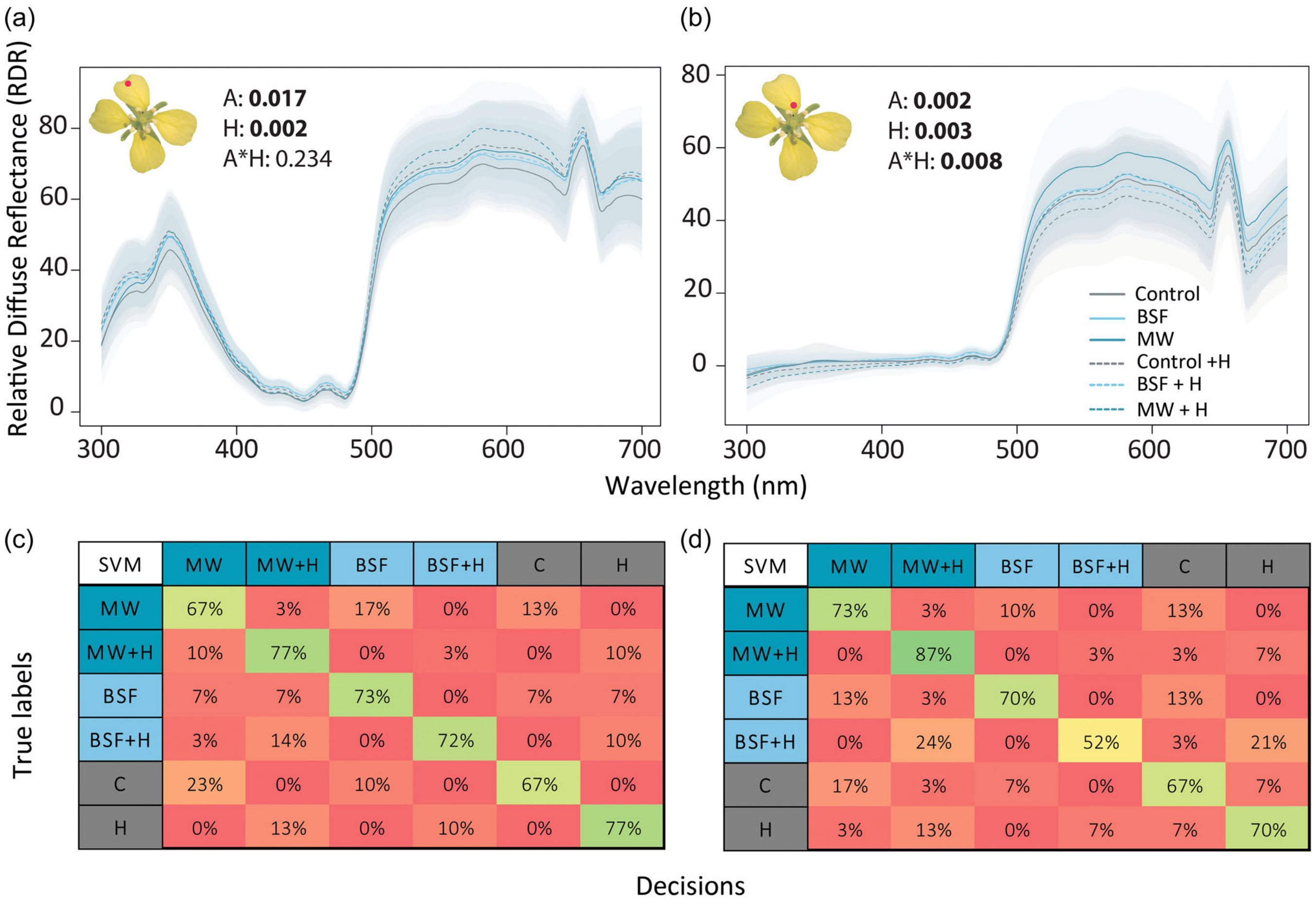
FIGURE 3 Morphometry for flowers of *Brassica nigra* plants grown in soil amended with yellow mealworm exuviae (MW) or black soldier fly exuviae (BSF) with or without herbivore infestation with *Pieris brassicae* caterpillars and *Brevicoryne brassicae* aphids. Control plants (C) grew in soil without amendment. We measured (a) petal area and (b) roundness. Boxplots show median (line), first and third quartiles, and 95% interval. Dots are observations outside this interval. Measurements were taken after 7 days of herbivory feeding. Six flowers of the distal inflorescence of two flowering branches (three flowers per inflorescence) were analysed for each plant, and flowers were taken from 10 plants per treatment. Number of samples per treatment varied between 185 and 225 petals. A is the type of soil amendment, H is herbivory and A*H is the interaction between type of soil amendment and herbivory, followed by *p*-values. Boxes within each panel that have no letters in common are significantly different (Tukey's post hoc test; $\alpha = 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

not influence the RDR of the yellow/orange and UV regions of the top and base part of the petals.

3.2 | Plant volatile emission

Across the eight treatments, 63 different volatile organic compounds (VOCs) were detected in the headspace of *B. nigra* plants. The list of VOCs detected and quantified comprised different classes including carbonyls (4.8%), monoterpenes (52.4%), sesquiterpenes (6.3%), homoterpenes (3.2%), benzenoids or/and phenylpropanoids (6.3%) and nitrogen and/or sulphur containing compounds (20.6%) (Supporting Information: Table S2). The monoterpenes α-pinene, limonene, allo-ocimene and verbenone were among the most abundant VOCs in the blends, that also contributed to the separation between the treatments together with the benzenoids benzyl alcohol and 4-methoxybenzaldehyde (VIP score values ≥ 1, Supporting Information: Table S2). OPLS-DA of all the treatments resulted in a weak ($R^2X = 0.394$, $R^2Y = 0.285$, $Q^2 = 0.055$) but significant (ANOVA of the cross-validated residuals [CV-ANOVA] $p = 0.048$) model with two predictive principal components explaining 31.9% and 7.5% of the total variance, respectively (Figure 6a). The first principal component separates the control treatments from the exuviae-amendment treatments. The OPLS-DA score plot (Figure 6b) shows the effect of the treatments on plants grown in amended soil (colour-coded symbols) clustering together, apart from the non-amended soil treatments (non-coloured open symbols). The separation of the control plants from the exuviae-amended plants along the first ordination axis, especially in relation to the MW and CK treated plants, is indicative of the influence of the soil amendment on VOC emission, which is also supported by the loading plot (Figure 6b). The second principal component separates the samples based on the presence of insect herbivory (Figure 6). The emission of 31 compounds was influenced by the type of soil amendment (Supporting Information: Table S2). These include 14 compounds, whose significance was attributed mainly to the type of soil amendment, and not to herbivory (Supporting Information: Table S2). Exposure of plants to herbivory resulted in quantitative differences in 21 compounds, of which 3 were affected by herbivory alone and not by soil amendment (Supporting Information: Table S2). The effects of soil amendment and herbivory significantly interacted for the emission of six volatile compounds, all of which are monoterpenes (Figure 7). Ten headspace components were affected by both factors but without interaction effect (Supporting Information: Table S2).

When investigating differences among the types of soil amendments used in terms of their effects on VOC levels of *B. nigra* plants, we found no difference in total VOC blend among plants without herbivory grown in soil amended with the different exuviae types (CV-ANOVA, $p = 1$). Likewise, we found no significant difference among plants grown in amended soil induced by herbivory (CV-ANOVA, $p = 1$). Hence, due to the lack of differences, we decided to treat the soil-amendment treatments as one class while keeping their



(c)

SVM	MW	MW+H	BSF	BSF+H	C	H
MW	67%	3%	17%	0%	13%	0%
MW+H	10%	77%	0%	3%	0%	10%
BSF	7%	7%	73%	0%	7%	7%
BSF+H	3%	14%	0%	72%	0%	10%
C	23%	0%	10%	0%	67%	0%
H	0%	13%	0%	10%	0%	77%

(d)

SVM	MW	MW+H	BSF	BSF+H	C	H
MW	73%	3%	10%	0%	13%	0%
MW+H	0%	87%	0%	3%	3%	7%
BSF	13%	3%	70%	0%	13%	0%
BSF+H	0%	24%	0%	52%	3%	21%
C	17%	3%	7%	0%	67%	7%
H	3%	13%	0%	7%	7%	70%

FIGURE 4 Reflectance spectra with relative diffuse reflectance of wavelengths (300–700 nm) of the top part (a) and base part (b) of petals of *Brassica nigra* plants grown in soil amended with yellow mealworm exuviae (MW) or BSF exuviae (BSF), herbivore infestation with both *Pieris brassicae* caterpillars and *Brevicoryne brassicae* aphids (H) or soil amendment with yellow mealworm exuviae plus herbivory (MW + H) or soil amendment with black soldier fly exuviae plus herbivory (BSF + H). Control plants (C) grew in soil without amendment. Shaded areas indicate the standard deviation of the reflectance. The red dot in the figure of the flower indicates where the measurements were taken. Six flowers of the distal inflorescence of two flowering branches (three flowers per inflorescence) were analysed for each plant, and flowers were taken from ten plants per treatment. Number of samples per treatment varied between 185 and 225 petals. A is the type of soil amendment, H is herbivory and A*H is the interaction between type of soil amendment and herbivory, followed by *P*-values. Bottom panels show confusion matrices of support vector machine classifiers for the reflectance spectra of (c) the top (F score: 84%, Precision: 86%, Recall: 82%) and (d) the basal part of petals (F score: 82%, Precision: 83%, Recall: 81%). [Color figure can be viewed at wileyonlinelibrary.com]

colour codes for individual treatments and compare them against control samples. The OPLS-DA ($R^2X = 0.405$, $R^2Y = 1$, $Q^2 = 0.227$) used to assess differences in headspace composition among plants grown in control soil and those grown in amended soil without herbivory generated a significant model (CV-ANOVA, $p = 0.021$) with one predictive and one orthogonal component (Supporting Information: Figure S2a). Control plants grown on unamended soil are separated from those grown on exuviae-amended soils (Supporting Information: Figure S2a). A strong correlation between soil amendment treatments and VOC blend is also supported by the loading plot (Supporting Information: Figure S2b). In total, 31 compounds showed to be important in the separation, with VIP score values ≥1, of which 71% were monoterpenes (Supporting Information: Table S2).

In a similar fashion, we evaluated whether soil amendment with insect exuviae impacted the headspace composition of plants exposed to herbivore attack. OPLS-DA ($R^2X = 0.509$, $R^2Y = 1$, $Q^2 = 0.361$) on herbivory-treated plants generated a significant model (CV-ANOVA, $p = 0.027$) with one predictive and three orthogonal components (Supporting Information: Figure S2c). The separation of control plants exposed to herbivory from plants grown on amended soil and exposed to herbivory shown in Figure S2c compared to what we observed for intact plants (Supporting Information: Figure S2a) suggests that the effect of soil amendment contributes more to the separation of the samples than the effect of herbivory. In the analysis, 25 VOCs appeared to be important in the separation of control plants under herbivory from the plants grown on amended soil under herbivory, with VIP score values ≥1 (Supporting Information: Table S2). Again, the separation was clearly influenced by terpenoid emission, where 84% of VOCs with VIP values ≥1 were monoterpenoids (Supporting Information: Table S2). It is important to note

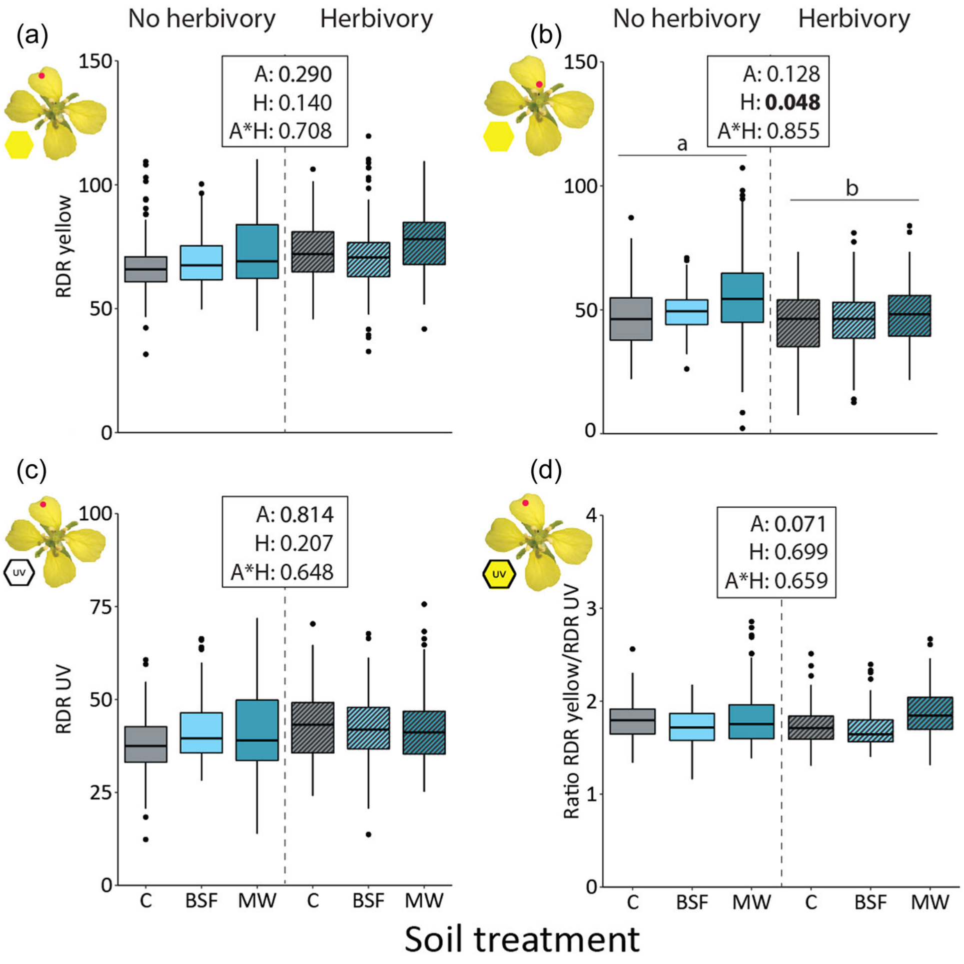


FIGURE 5 Relative diffuse reflectance (RDR) of yellow (570–650 nm) and UV (310–370 nm) wavelengths by petals of *Brassica nigra* plants grown in soil amended with yellow mealworm exuviae (MW) or black soldier fly exuviae (BSF) with or without herbivore infestation with both *Pieris brassicae* caterpillars and *Brevicoryne brassicae* aphids. Control plants (C) grew in soil without amendment. (a) RDR of yellow of top parts of petals. (b) RDR of yellow of base parts of petals. (c) RDR of UV of top parts of petals. (d) Ratio RDR yellow/RDR UV of top parts of petals. Boxplots show median (bold horizontal line), first and third quartiles, and 95% interval. Dots are observations outside this interval. The red dot on the flower images indicates where measurements were taken (top or base). Six flowers of the distal inflorescence of two flowering branches (three flowers per inflorescence) were analysed for each plant, and flowers were taken from ten plants per treatment. Number of samples per treatment varied between 185 and 225 petals. From each plant, six flowers were used, of which two petals were measured, both top and base parts. A is the type of soil amendment, H is herbivory and A*H is the interaction between type of soil amendment and herbivory, followed by p-values. [Color figure can be viewed at wileyonlinelibrary.com]

that the two identified and quantified isomers of the sesquiterpene α-farnesene were also among the important VOCs for the separation of the groups with a VIP value ≥1. The correlation between the emission rates of VOCs and soil amendment is visible in the loading plot (Supporting Information: Figure S2d).

4 | DISCUSSION

Our study provides evidence that both visual and olfactory plant traits are affected by amending soil with insect exuviae, as well as by exposing the plants to insect herbivory. Overall, soil amendment increased flower display and amount of VOC emission of *B. nigra* plants, while insect attack reduced petal size and volatile emission. Specifically, the number of flowers per plant was greater for plants grown in amended soil, whereas petal area was reduced upon herbivory by a combination of caterpillars and aphids. In addition, flower colour spectra and volatile emission were affected by both factors, and for some of the components of the volatile blend, we found significant interactions between the type of soil amendment and herbivory. Taken together, these results demonstrate that insect exuviae as soil amendment can enhance floral display and VOC emission and can counteract the negative effects of herbivory on these plant traits. Importantly, our study provides the first evidence of the individual as well as the interaction effects of soil amendment and herbivory on visual and olfactory plant traits involved in plant-pollinator interactions.

Changes in flower traits in response to environmental factors may influence plant reproductive success. The positive effect of soil

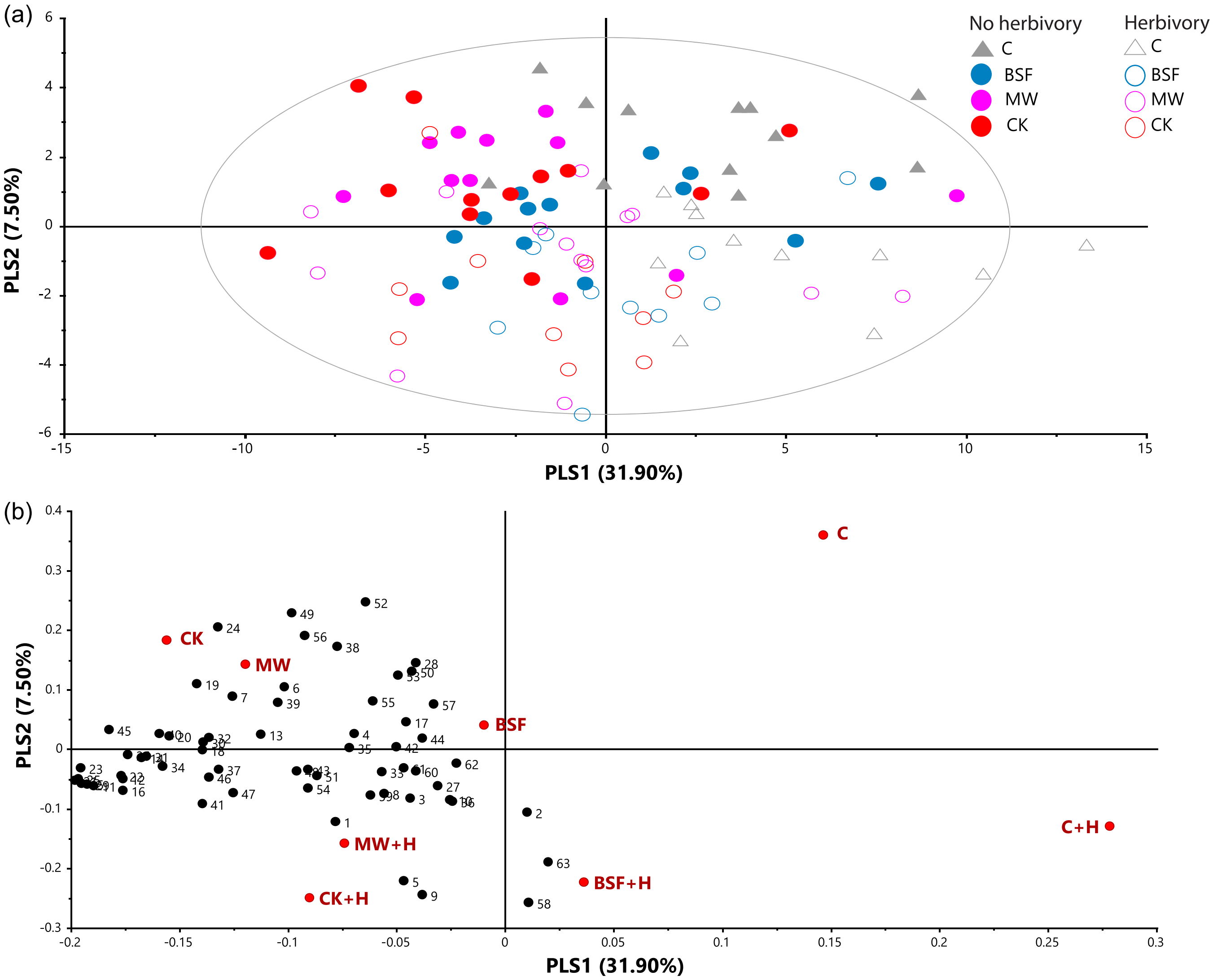


FIGURE 6 Effects of soil amendment and herbivory on the headspace composition of *Brassica nigra* plants. Plants were grown in field soil (C, $n = 12$) or in field soil amended with yellow mealworm exuviae (MW, $n = 12$), black soldier fly exuviae (BSF, $n = 13$) or house cricket exuviae (CK, $n = 12$). Four additional treatments were infested with *Pieris brassicae* caterpillars and *Brevicoryne brassicae* aphids: control plants (C + H, $n = 11$); plants grown in soil amended with yellow mealworm exuviae plus herbivory (MW + H, $n = 14$), amended with BSF exuviae plus herbivory (BSF + H, $n = 10$) or amended with house cricket exuviae plus herbivory (CK + H, $n = 10$). For the OPLS-DA, the three soil amendments were included as a single treatment, that is, soil amendment with exuviae. (a) OPLS-DA score plot of the headspace composition of different treatment groups depicted as a two-dimensional score plot using the first two PLS components. The ellipse represents the 95% confidence interval using Hotelling's T^2 statistic. (b) Loading plot displaying the contribution of each volatile compound to the separation between the two treatment groups, that is, control versus soil amendment. For compound identities indicated by numbers in the loading plot, please refer to Supporting Information: Table S2. OPLS-DA, orthogonal partial least squares-discriminant analysis. [Color figure can be viewed at wileyonlinelibrary.com]

amendment with insect exuviae on the number of flowers (Figure 2) indicates that plants grown on amended soil invested more resources in reproductive tissues than control plants. Insect exuviae as soil amendment may influence soil texture. Moreover, soil amendment with exuviae may influence flower traits through the supply of chitin and nutrients (e.g., nitrogen) that promote growth and influence the development of flower traits. The use of chitin-based soil amendment has been shown to supply nitrogen and calcium to plants (De Tender et al., 2019; Shamshina et al., 2020). Furthermore, soil amendments containing chitin can stimulate populations of chitinolytic bacteria (Debode et al., 2016), that are known to degrade chitin, thus releasing nutrients that can be used by plants and microorganisms (De Tender et al., 2019). This increase in nutrient availability and population size of beneficial microorganisms has been shown to increase the number of flowers, corolla size and raceme display size of plants (Burkle & Irwin, 2009; Rebolleda-Gómez et al., 2019). These flower traits have been found to be good predictors for the number of visits by insect pollinators (Kuppler et al., 2021; Salisbury et al., 2015). By altering plant traits attractive to pollinators in response to soil amendment with insect exuviae, plants may alter pollinator

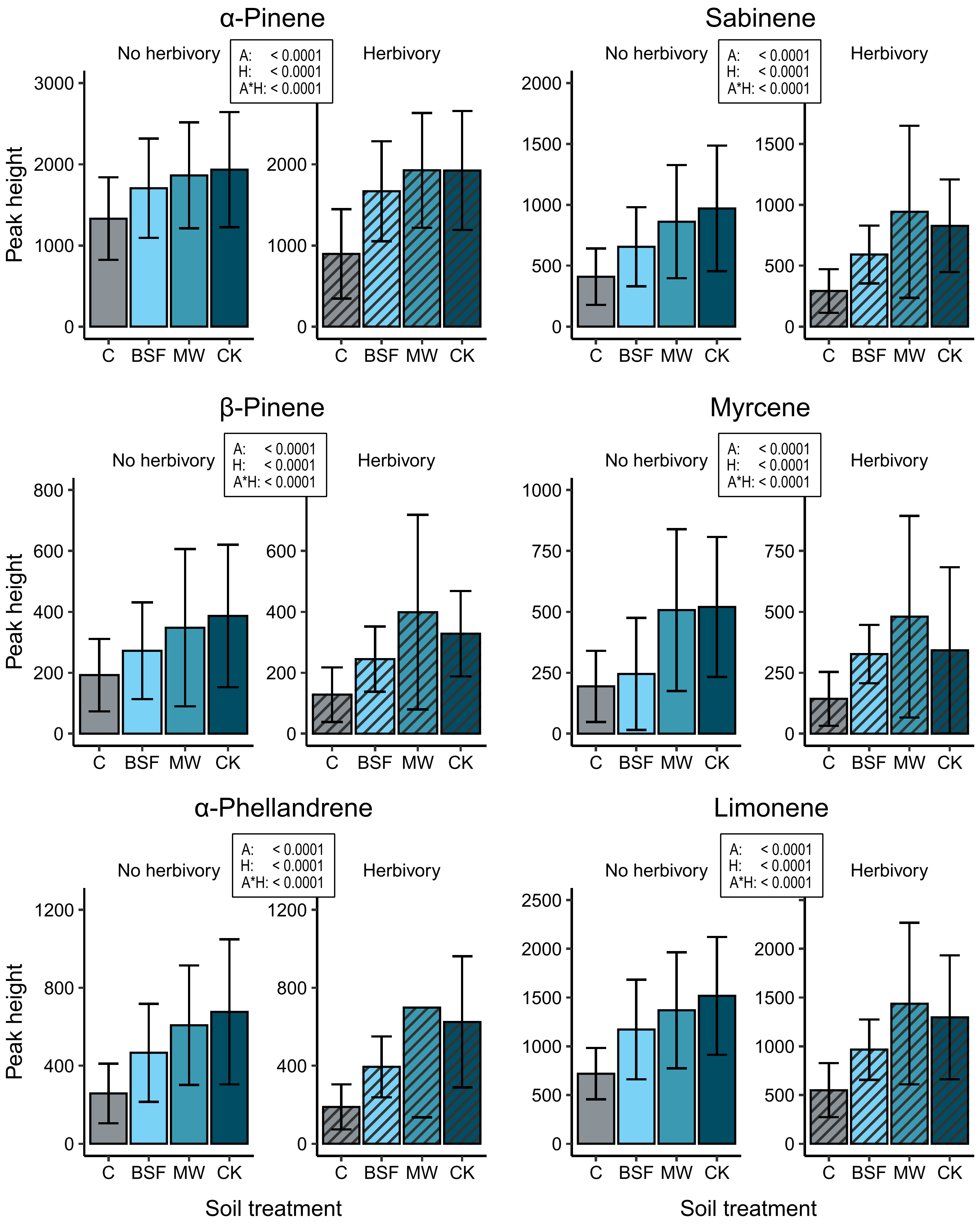


FIGURE 7 (See caption on next page)

attraction and subsequently impact seed production (Barragán-Fonseca et al., 2023).

Petal colour acts as a visual signal that is used by flower-visiting insects (Iriel & Lagorio, 2010; Van der Kooi et al., 2019) and can be affected by environmental factors like temperature, light and nutrition (Burchi et al., 2010). By increasing nutrient availability for plants, beneficial rhizobacteria can have important positive effects on flower colour, implying a beneficial effect of insect exuviae as soil amendments on plant–pollinator interactions (Barragán-Fonseca et al., 2022). For example, *Tagetes erecta* plants grown in soil inoculated with the plant-growth-promoting rhizobacterium *Bacillus subtilis*, produced more and heavier flowers with an increased colour intensity (Flores et al., 2007). Although in the present study the colour of top and base parts of *B. nigra* petals was affected by the type of soil amendment, we did not detect an increase in RDR of the light in the yellow and UV region. Therefore, the effects of the soil amendment-induced changes on pollinator attraction are hard to predict, and behavioural experiments have to be conducted to investigate the actual effects of insect exuviae as soil amendments on pollinator behaviour via changes in flower colour.

In addition to the effects on visual floral traits, soil amendment also altered the chemical traits of *B. nigra* plants. For plants grown in soil amended with insect exuviae, the amounts of volatiles emitted were higher than for control plants grown in unamended soil (Supporting Information: Table S2). Plant volatile emission can be affected by biotic (Todeschini et al., 2018) and abiotic environmental factors in the soil (Cna'ani et al., 2021). The nutrient concentrations in the soil greatly influenced the volatile emission from corn plants, with a lower emission rate of volatiles when plants were grown in low-nutrition conditions (Gouinguené & Turlings, 2002). Several studies showed that herbivory reduced the emission of floral VOCs (D. Kessler et al., 2010; Pareja et al., 2012; Schiestl et al., 2014). We found that the reduction in VOC emission by flowering *B. nigra* plants in response to herbivory was prominent for compounds like phenylacetaldehyde and benzyl alcohol, that are common flower volatiles (Farré-Armengol et al., 2020), known to elicit foraging responses in pollinating insects (Farré-Armengol et al., 2020; Mas et al., 2020; Schiestl et al., 2014). Furthermore, herbivory negatively affected all petal size traits that we quantified (Figure 3a, Supporting Information: Figure S1). Folivory is known to affect flower morphology (Rusman, Poelman, et al., 2019; Strauss & Whittall, 2006), abundance, colour (Lucas-Barbosa et al., 2016; Rusman, Poelman, et al., 2019) and plant VOC emission (A. Kessler et al., 2011; Lucas-Barbosa et al., 2016; Schiestl, 2015). As a consequence, changes in plant traits induced by foliar herbivory may affect the behaviour of flower visitors with effects on the structure of flower–visitor networks and, consequently, plant fitness (Hoffmeister et al., 2016; A. Kessler & Chautá, 2020; Lucas-Barbosa, 2016). Alterations in the soil environment caused by soil amendments influence plant traits that may affect the attraction of pollinators and pollination success (Carvalheiro et al., 2021; Strauss & Whittall, 2006). The negative effect of herbivory on chemical cues can reduce the attractiveness of flowers to pollinators (A. Kessler & Chautá, 2020; A. Kessler et al., 2011; Lehtilä & Strauss, 1999; Strauss, 1997). In a previous study, we observed that plants growing in soil amended with BSF exuviae were visited more frequently by insect pollinators compared to flowers of control plants. This effect was independent of herbivory (Barragán-Fonseca et al., 2023). In the present study, we also explored the effects of mealworm and house cricket exuviae, which showed different effects on the VOCs depending on the treatment (Figure 6, Supporting Information: Table S2).

Despite the effects of soil amendments and herbivory on specific traits of flowering plants, it is important to consider that insect pollinators respond to multiple plant traits. Visual and olfactory flower traits may act additively or synergistically in attracting pollinators (Junker & Parachnowitsch, 2015; Lucas-Barbosa et al., 2016). For example, pollinators can use flower colour as main cue, while additionally exploiting other floral traits such as shape and volatile emission (Garcia et al., 2019). Furthermore, the effects on traits of flowering plants can differentially influence pollinator taxa. First, because insect pollinator species have distinct visual systems (Briscoe & Chittka, 2001) and different olfactory receptors (De Bruyne & Baker, 2008). Second, because flower visitors can exploit different flower cues depending on the nutritional resources they require (Lucas-Barbosa et al., 2016). Additionally, even with a low number of pollinators, a large number of flowers can increase plant reproductive success by increasing the availability of pollen thereby enhancing pollen transfer efficiency (Motzke et al., 2015). In *B. nigra*, visual and chemical cues of flowering plants influence the behaviour of different pollinator species (Barragán-Fonseca et al., 2020). The syrphid fly *Episyrphus balteatus* and the butterfly *P. brassicae*, reported insect pollinators of brassicaceous species, exhibited a strong bias for visual cues over volatile chemical cues when both cues were offered separately; however, insect pollinators exploit visual and odour information simultaneously, thus visual and chemical plant traits both contribute to foraging pollinator preferences (Milet-Pinheiro et al., 2012; Raguso & Willis, 2002). *Pieris brassicae* can distinguish between flowers with different amounts of resources only

FIGURE 7 Effects of soil amendment and herbivory on the emission of compounds by *Brassica nigra* plants with significant interaction effects (A*H). Plants were grown in field soil (C, $n = 12$) or in field soil amended with yellow mealworm exuviae (MW, $n = 12$), black soldier fly exuviae (BSF, $n = 13$) or house cricket exuviae (CK, $n = 12$). Four additional treatments were infested with *Pieris brassicae* caterpillars and *Brevicoryne brassicae* aphids: control plants (C + H, $n = 11$); plants grown in soil amended with yellow mealworm exuviae plus herbivory (MW + H, $n = 14$), amended with BSF exuviae plus herbivory (BSF + H, $n = 10$) or amended with house cricket exuviae plus herbivory (CK + H, n = 10). A is the type of soil amendment, H is herbivory treatment and A*H is the interaction between type of soil amendment and herbivory, followed by *p*-values associated with the overall effect estimated in the statistical model. [Color figure can be viewed at wileyonlinelibrary.com] when visual and chemical cues were offered together (Barragán-Fonseca et al., 2020).

Our data suggest that the interactive effects of soil amendment and herbivory on flowering *B. nigra* plants may have important consequences for pollinator attraction. We show that VOC emission is affected by soil amendment and herbivory, with a stronger effect of soil amendment with exuviae than of herbivory (Supporting Information: Table S2; Figure 6; Supporting Information: Figure S2). There was a significant interaction between soil amendment and herbivory on the emission of several volatile compounds (Supporting Information: Table S2). Herbivory reduced the emission rate of VOCs, while soil amendment with insect exuviae resulted in increased volatile emission. Notably, plants growing in soil amended with insect exuviae exposed to herbivory showed a smaller reduction in the amount of specific volatiles emitted compared to plants grown in soil without amendment (Supporting Information: Table S2). This is the case for, for example, phenylacetadehyde, a compound used by insect pollinators of *Brassica rapa* as honest signal indicative of flower reward (Knauer & Schiestl, 2015). Significant interactions between soil amendment and herbivory were found mainly for monoterpenes (Supporting Information: Table S2). Emission of monoterpenes may protect plants against abiotic stress (Glenny et al., 2018) and biotic stress, such as herbivory (Heil, 2008). Plant defence against herbivores may be the main role of these terpenoids, followed by their effects on pollinator attraction (Schiestl, 2010). For example, limonene, produced in a larger amount by plants growing in soil amended with insect exuviae compared with control plants, has been implied to attract *Pieris* butterflies (Bruinsma et al., 2014) and reported to attract bumblebees (Byers et al., 2014), in the latter case implying a strong role of this compound on plant evolution and speciation in two species of monkeyflowers (*Mimulus*) (Byers et al., 2014).

Our study demonstrates that visual and chemical traits of flowers change in response to soil amendment with insect exuviae and herbivory, and that these variables in some cases interact. These effects are likely influencing the fitness of plant species such as *B. nigra*, which rely on insect pollinators for reproduction. Plants grown in amended soil had more flowers and an enhanced volatile emission. The significant interaction effect between soil amendment and herbivory on VOC emission showed that positive effects of soil amendment can compensate the negative effects of herbivory. As a result, plants growing in soil amended with insect exuviae can be more attractive for insect pollinators than control plants even when exposed to herbivory, and this might translate into higher plant fitness (Barragán-Fonseca et al., 2022). Incorporating effects of aboveground-belowground interactions as affected by environmental factors will increase our understanding of the evolution of flowers and their associated ecological networks (Carvalheiro et al., 2021; Dalrymple et al., 2020; Heinen et al., 2018).

AUTHOR CONTRIBUTIONS

Katherine Y. Barragán-Fonseca, Quint Rusman, Gerrit Polder, Joop J. A. van Loon and Marcel Dicke designed the study. Katherine Y. Barragán-Fonseca, Quint Rusman and Berhane T. Weldegergis collected the data. Katherine Y. Barragán-Fonseca, Quint Rusman, Daan Mertens, Joseph Peller and Berhane T. Weldegergis conducted the statistical analyses. Katherine Y. Barragán-Fonseca interpreted the results and wrote the manuscript and all authors commented on and approved the final version.

ACKNOWLEDGEMENT

K. Y. B.-F. was funded by the Colombian Department of Science, Technology and Innovation Colciencias (Convocatoria 783).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

ORCID

Katherine Y. Barragán-Fonseca http://orcid.org/0000-0003-3776-084X
Quint Rusman http://orcid.org/0000-0003-0285-7967
Daan Mertens https://orcid.org/0000-0003-4220-9075
Berhane T. Weldegergis http://orcid.org/0000-0001-8838-7876
Gerrit Polder http://orcid.org/0000-0003-4896-4776
Joop J. A. van Loon https://orcid.org/0000-0002-4260-0501
Marcel Dicke http://orcid.org/0000-0001-8565-8896

REFERENCES

Altieri, M.A. & Nicholls, C.I. (2003) Soil fertility management and insect pests: harmonizing soil and plant health in agroecosystems. *Soil and Tillage Research*, 72(2), 203–211.

Anderson, M.J. (2017) Permutational multivariate analysis of variance (PERMANOVA). In: Balakrishnan, N., Colton, T., Everitt, B., Piegorsch, W., Ruggeri, F. & Teugels, J.L. (Eds.) *Wiley statsref: statistics reference online [eBook]*. New York: John Wiley & Sons Ltd., pp. 1–15.

Anderson, M.J. & Walsh, D.C.I. (2013) PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing. *Ecological Monographs*, 83(4), 557–574.

Barber, N.A. & Soper Gorden, N.L. (2014) How do belowground organisms influence plant-pollinator interactions. *Journal of Plant Ecology*, 8(1), 1–11.

Barragán-Fonseca, K.Y., Greenberg, L.O., Gort, G., Dicke, M. & van Loon, J.J.A. (2023) Amending soil with insect exuviae improves herbivore tolerance, pollinator attraction and seed yield of *Brassica nigra* plants. *Agriculture, Ecosystems & Environment*, 342, 108219.

Barragán-Fonseca, K.Y., Loon, J.J.A., Dicke, M. & Lucas-Barbosa, D. (2020) Use of visual and olfactory cues of flowers of two brassicaceous species by insect pollinators. *Ecological Entomology*, 45(1), 45–55.

Barragán-Fonseca, K.Y., Nurfikari, A., Van De Zande, E.M., Wantulla, M., van Loon, J.J.A., De Boer, W. et al. (2022) Insect frass and exuviae to promote plant growth and health. *Trends in Plant Science*, 27(7), 646–654.

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67, 1–48.

Briscoe, A.D. & Chittka, L. (2001) The evolution of color vision in insects. *Annual Review of Entomology*, 46(1), 471–510.

Brooks, E., Kristensen, K., Benthem, J., Magnusson, A., Berg, W., Nielsen, A. et al. (2017) glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R Journal*, 9(2), 378–400.

Bruinsma, M., Lucas-Barbosa, D., ten Broeke, C.J.M., van Dam, N.M., van Beek, T.A., Dicke, M. et al. (2014) Folivory affects composition of nectar, floral odor and modifies pollinator behavior. *Journal of Chemical Ecology*, 40(1), 39–49.

De Bruyne, M. & Baker, T.C. (2008) Odor detection in insects: volatile codes. *Journal of Chemical Ecology*, 34(7), 882–897.

Burchi, G., Prisa, D., Ballarin, A. & Menesatti, P. (2010) Improvement of flower color by means of leaf treatments in lily. *Scientia Horticulturae*, 125(3), 456–460.

Burkle, L.A. & Irwin, R.E. (2009) The effects of nutrient addition on floral characters and pollination in two subalpine plants, *Ipomopsis aggregata* and *Linum lewisii*. *Plant Ecology*, 203(1), 83–98.

Burnham, K.P. (2002) Information and likelihood theory: a basis for model selection and inference. In: Burnham, K.P. (Ed.) *Model selection and multimodel inference: a practical information-theoretic approach*, 2nd edition. New York: Springer, pp. 49–97.

Byers, K.J., Bradshaw, H.D., Jr & Riffell, J.A. (2014) Three floral volatiles contribute to differential pollinator attraction in monkeyflowers (*Mimulus*). *The Journal of Experimental Biology*, 217(4), 614–623.

Caruso, C.M., Eisen, K.E., Martin, R.A. & Sletvold, N. (2019) A meta-analysis of the agents of selection on floral traits. *Evolution*, 73(1), 4–14.

Carvalheiro, L.G., Bartomeus, I., Rollin, O., Timóteo, S. & Tinoco, C.F. (2021) The role of soils on pollination and seed dispersal. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, 376(1834), 20200171.

Cna'ani, A., Dener, E., Ben-Zeev, E., Günther, J., Köllner, T.G., Tzin, V. et al. (2021) Phylogeny and abiotic conditions shape the diel floral emission patterns of desert Brassicaceae species. *Plant, Cell & Environment*, 44(8), 2656–2671.

Dalrymple, R.L., Kemp, D.J., Flores-Moreno, H., Laffan, S.W., White, T.E., Hemmings, F.A. et al. (2020) Macroecological patterns in flower colour are shaped by both biotic and abiotic factors. *New Phytologist*, 228(6), 1972–1985.

David, T.I., Storkey, J. & Stevens, C.J. (2019) Understanding how changing soil nitrogen affects plant–pollinator interactions. *Arthropod-Plant Interactions*, 13(5), 671–684.

Debode, J., De Tender, C., Soltaninejad, S., Van Malderghem, C., Haegeman, A., Van der Linden, I. et al. (2016) Chitin mixed in potting soil alters lettuce growth, the survival of zoonotic bacteria on the leaves and associated rhizosphere microbiology. *Frontiers in Microbiology*, 7, 565.

Elle, E. & Hare, J.D. (2002) Environmentally induced variation in floral traits affects the mating system in *Datura wrightii*. *Functional Ecology*, 16(1), 79–88.

Farré-Armengol, G., Fernández-Martínez, M., Filella, I., Junker, R.R. & Peñuelas, J. (2020) Deciphering the biotic and climatic factors that influence floral scents: a systematic review of floral volatile emissions. *Frontiers in Plant Science*, 11, 1154.

Flores, A.C., Luna, A.A.E. & Portugal, V.O. (2007) Yield and quality enhancement of marigold flowers by inoculation with *Bacillus subtilis* and *Glomus fasciculatum*. *Journal of Sustainable Agriculture*, 31(1), 21–31.

Fox, J. & Weisberg, S. (2019) *An R companion to applied regression*, 3rd edition. Thousands Oaks: SAGE Publications.

Galizia, C.G., Kunze, J., Gumbert, A., Borg-Karlson, A.-K., Sachse, S., Markl, C. et al. (2004) Relationship of visual and olfactory signal parameters in a food-deceptive flower mimicry system. *Behavioral Ecology*, 16(1), 159–168.

Garcia, J.E., Shrestha, M., Howard, S.R., Petersen, P. & Dyer, A.G. (2019) Signal or cue: the role of structural colors in flower pollination. *Current Zoology*, 65(4), 467–481.

Glenny, W.R., Runyon, J.B. & Burkle, L.A. (2018) Drought and increased CO2 alter floral visual and olfactory traits with context-dependent effects on pollinator visitation. *New Phytologist*, 220(3), 785–798.

Göre, M.E. & Altin, N. (2006) Growth promoting of some ornamental plants by root treatment with specific fluorescent pseudomonads. *Journal of Biological Sciences*, 6(3), 610–615.

Gouinguené, S.P. & Turlings, T.C.J. (2002) The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Physiology*, 129(3), 1296–1307.

Heil, M. (2008) Indirect defence—recent developments and open questions. In: Lüttge, U., Beyschlag, W. & Murata, J. (Eds.) *Progress in botany*. Berlin: Springer, pp. 359–396.

Heinen, R., Biere, A., Harvey, J.A. & Bezemer, T.M. (2018) Effects of soil organisms on aboveground plant-insect interactions in the field: patterns, mechanisms and the role of methodology. *Frontiers in Ecology and Evolution*, 6, 106.

Hoffmeister, M., Wittköpper, N. & Junker, R.R. (2016) Herbivore-induced changes in flower scent and morphology affect the structure of flower–visitor networks but not plant reproduction. *Oikos*, 125(9), 1241–1249.

Iriel, A. & Lagorio, M.G. (2010) Implications of reflectance and fluorescence of *Rhododendron indicum* flowers in biosignaling. *Photochemical & Photobiological Sciences*, 9(3), 342–348.

Junker, R.R. & Parachnowitsch, A.L. (2015) Working towards a holistic view on flower traits—how floral scents mediate plant–animal interactions in concert with other floral characters. *Journal of the Indian Institute of Science*, 95(1), 43–68.

Kessler, A. & Chautá, A. (2020) The ecological consequences of herbivore-induced plant responses on plant-pollinator interactions. *Emerging Topics in Life Sciences*, 4(1), 33–43.

Kessler, A., Halitschke, R. & Poveda, K. (2011) Herbivory-mediated pollinator limitation: negative impacts of induced volatiles on plant–pollinator interactions. *Ecology*, 92(9), 1769–1780.

Kessler, D., Diezel, C. & Baldwin, I.T. (2010) Changing pollinators as a means of escaping herbivores. *Current Biology*, 20(3), 237–242.

Knauer, A.C. & Schiestl, F.P. (2015) Bees use honest floral signals as indicators of reward when visiting flowers. *Ecology Letters*, 18(2), 135–143.

Van der Kooi, C.J., Dyer, A.G., Kevan, P.G. & Lunau, K. (2019) Functional significance of the optical properties of flowers for visual signalling. *Annals of Botany*, 123(2), 263–276.

Kumari, A., Goyal, R., Choudhary, M. & Sindhu, S. (2016) Effects of some plant growth promoting rhizobacteria (PGPR) strains on growth and flowering of *Chrysanthemum*. *Journal of Crop and Weed*, 12(1), 7–15.

Kuppler, J., Wieland, J., Junker, R.R. & Ayasse, M. (2021) Drought-induced reduction in flower size and abundance correlates with reduced flower visits by bumble bees. *AoB Plants*, 13(1), plab001.

Lehtilä, K. & Strauss, S.Y. (1999) Effects of foliar herbivory on male and female reproductive traits of wild radish, *Raphanus raphanistrum*. *Ecology*, 80(1), 116–124.

Leonard, A.S., Dornhaus, A. & Papaj, D.R. (2011) *Why are floral signals complex: an outline of functional hypotheses*. Cambridge: Cambridge University Press.

Lommen, A. (2009) MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Analytical Chemistry*, 81(8), 3079–3086.

Lucas-Barbosa, D. (2016) Integrating studies on plant–pollinator and plant–herbivore interactions. *Trends in Plant Science*, 21(2), 125–133.

Lucas-Barbosa, D., Sun, P., Hakman, A., Beek, T.A., Loon, J.J.A. & Dicke, M. (2016) Visual and odour cues: plant responses to pollination and herbivory affect the behaviour of flower visitors. *Functional Ecology*, 30(3), 431–441.

Mas, F., Horner, R.M., Brierley, S., Butler, R.C. & Suckling, D.M. (2020) Selection of key floral scent compounds from fruit and vegetable crops by honey bees depends on sensory capacity and experience. *Journal of Insect Physiology*, 121, 104002.

Meyer, G.A. (2000) Interactive effects of soil fertility and herbivory on *Brassica nigra*. *Oikos*, 88(2), 433–441.

Milet-Pinheiro, P., Ayasse, M., Schlindwein, C., Dobson, H.E.M. & Dötterl, S. (2012) Host location by visual and olfactory floral cues in an oligolectic bee: innate and learned behavior. *Behavioral Ecology*, 23(3), 531–538.

Motzke, I., Tscharntke, T., Wanger, T.C. & Klein, A.M. (2015) Pollination mitigates cucumber yield gaps more than pesticide and fertilizer use in tropical smallholder gardens. *Journal of Applied Ecology*, 52(1), 261–269.

Noble, W.S. (2006) What is a support vector machine. *Nature Biotechnology*, 24(12), 1565–1567.

Nurfikari, A. & de Boer, W. (2021) Chitin determination in residual streams derived from insect production by LC-ECD and LC-MS/MS methods. *Frontiers in Sustainable Food Systems*, 5, 795694.

Ohashi, K. & Yahara, T. (2002) Visit larger displays but probe proportionally fewer flowers: counterintuitive behaviour of nectar-collecting bumble bees achieves an ideal free distribution. *Functional Ecology*, 16(4), 492–503.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J. et al. (2007) The vegan package. *Community Ecology Package*, 10(631–637), 719.

Parada, R.Y., Egusa, M., Aklog, Y.F., Miura, C., Ifuku, S. & Kaminaka, H. (2018) Optimization of nanofibrillation degree of chitin for induction of plant disease resistance: elicitor activity and systemic resistance induced by chitin nanofiber in cabbage and strawberry. *International Journal of Biological Macromolecules*, 118, 2185–2192.

Pariđiković, N., Teklić, T., Zeljković, S., Lisjak, M. & Špoljarević, M. (2019) Biostimulants research in some horticultural plant species—a review. *Food and Energy Security*, 8(2), e00162.

Pareja, M., Qvarfordt, E., Webster, B., Mayon, P., Pickett, J., Birkett, M. et al. (2012) Herbivory by a phloem-feeding insect inhibits floral volatile production. *PLoS One*, 7(2), e31971.

Pinheiro, J., Bates, D., DebRoy, S. & Sarkar, D. (2021) *nlme: linear and nonlinear mixed effects models. R package version 3*. R Core Team (2021), pp. 1–152.

Raguso, R.A. (2008) Wake up and smell the roses: the ecology and evolution of floral scent. *Annual Review of Ecology, Evolution, and Systematics*, 39, 549–569.

Raguso, R.A. & Willis, M.A. (2002) Synergy between visual and olfactory cues in nectar feeding by naıve hawkmoths, *Manduca sexta*. *Animal Behaviour*, 64(5), 685–695.

R Core Team. (2018) R: a language and environment for statistical computing. In: Computing, R. F. f. S. (Ed.). Vienna.

Rebolleda-Gómez, M., Forrester, N.J., Russell, A.L., Wei, N., Fetters, A.M., Stephens, J.D. et al. (2019) Gazing into the anthosphere: considering how microbes influence floral evolution. *New Phytologist*, 224(3), 1012–1020.

Rowen, E., Tooker, J.F. & Blubaugh, C.K. (2019) Managing fertility with animal waste to promote arthropod pest suppression. *Biological Control*, 134, 130–140.

Rusman, Q., Lucas-Barbosa, D., Poelman, E.H. & Dicke, M. (2019) Ecology of plastic flowers. *Trends in Plant Science*, 24(8), 725–740.

Rusman, Q., Poelman, E.H., Nowrin, F., Polder, G. & Lucas-Barbosa, D. (2019) Floral plasticity: herbivore-species-specific-induced changes in flower traits with contrasting effects on pollinator visitation. *Plant, Cell & Environment*, 42(6), 1882–1896.

Russell, A.L., Mauerman, K.B., Golden, R.E. & Papaj, D.R. (2018) Linking components of complex signals to morphological part: the role of anther and corolla in the complex floral display. *Animal Behaviour*, 135, 223–236.

Russell, L. (2018) Emmeans: estimated marginal means, aka least-squares means. *R package version*, 1(2).

Salisbury, A., Armitage, J., Bostock, H., Perry, J., Tatchell, M. & Thompson, K. (2015) Editor's choice: enhancing gardens as habitats for flower-visiting aerial insects (pollinators): should we plant native or exotic species? *Journal of Applied Ecology*, 52(5), 1156–1164.

Schiestl, F.P. (2010) The evolution of floral scent and insect chemical communication. *Ecology Letters*, 13(5), 643–656.

Schiestl, F.P. (2015) Ecology and evolution of floral volatile-mediated information transfer in plants. *New Phytologist*, 206(2), 571–577.

Schiestl, F.P., Kirk, H., Bigler, L., Cozzolino, S. & Desurmont, G.A. (2014) Herbivory and floral signaling: phenotypic plasticity and tradeoffs between reproduction and indirect defense. *New Phytologist*, 203(1), 257–266.

Shamshina, J.L., Kelly, A., Oldham, T. & Rogers, R.D. (2020) Agricultural uses of chitin polymers. *Environmental Chemistry Letters*, 18(1), 53–60.

Stafford, D.B., Tariq, M., Wright, D.J., Rossiter, J.T., Kazana, E., Leather, S.R. et al. (2012) Opposing effects of organic and conventional fertilizers on the performance of a generalist and a specialist aphid species. *Agricultural and Forest Entomology*, 14(3), 270–275.

Strauss, S. & Whittall, J. (2006) Non-pollinator agents of selection on floral traits. In: Harder, L. & Barrett, S. (Eds.) *Ecology and evolution of flowers*. Oxford: University Press, pp. 120–138.

Strauss, S.Y. (1997) Floral characters link herbivores, pollinators, and plant fitness. *Ecology*, 78(6), 1640–1645.

De Tender, C., Mesuere, B., Van der Jeugt, F., Haegeman, A., Ruttink, T., Vandecasteele, B. et al. (2019) Peat substrate amended with chitin modulates the N-cycle, siderophore and chitinase responses in the lettuce rhizobiome. *Scientific Reports*, 9(1), 9890.

Tikunov, Y.M., Laptenok, S., Hall, R.D., Bovy, A. & De Vos, R.C.H. (2012) MSClust: a tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. *Metabolomics*, 8(4), 714–718.

Todeschini, V., AitLahmidi, N., Mazzucco, E., Marsano, F., Gosetti, F., Robotti, E. et al. (2018) Impact of beneficial microorganisms on strawberry growth, fruit production, nutritional quality, and volatilome. *Frontiers in Plant Science*, 9, 1611.

Weiss, M.R. (1991) Floral colour changes as cues for pollinators. *Nature*, 354(6350), 227–229.

Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A. & Smith, G.M. (2009) *Mixed effects models and extensions in ecology with R*. New York: Springer, p. 574.

SUPPORTING INFORMATION

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

How to cite this article: Barragán-Fonseca, K.Y., Rusman, Q., Mertens, D., Weldegergis, B.T., Peller, J., Polder, G. et al. (2023) Insect exuviae as soil amendment affect flower reflectance and increase flower production and plant volatile emission. *Plant, Cell & Environment*, 46, 931–945. https://doi.org/10.1111/pce.14516