

RESEARCH ARTICLE

Insect herbivory but not plant pathogen infection drive floral volatile-mediated indirect effects on pollinators and plant fitness in *Brassica rapa*

Xoaquín Moreira¹  | Luis Abdala-Roberts²  | Rieta Gols³  | Beatriz Lago-Núñez¹ | Sergio Rasman⁴  | Gregory Röder⁴  | Pilar Soengas¹ | Carla Vázquez-González^{1,5}  | María Elena Cartea¹

¹Misión Biológica de Galicia (MBG-CSIC), Pontevedra, Galicia, Spain

²Departamento de Ecología Tropical, Campus de Ciencias Biológicas y Agropecuarias, Universidad Autónoma de Yucatán, Mérida, Yucatán, Mexico

³Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands

⁴Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

⁵Department of Ecology and Evolutionary Biology, University of California, Irvine, California, USA

Correspondence

Xoaquín Moreira

Email: xmoreira1@gmail.com

Funding information

Spanish Ministry of Science, Innovation and Universities, Grant/Award Number: RTI2018-096591-BI00 and RTI2018-099322-B-I00; Xunta de Galicia, Grant/Award Number: IN607A 2021/03

Handling Editor: Anne Kempel

Abstract

1. Plant enemies can indirectly affect pollinators by modifying plant traits, but simultaneous tests of herbivore and pathogen effects are lacking, and the role of floral volatiles has seldom been assessed.
2. In this study, we tested for indirect effects of insect herbivores and pathogens on pollinator attraction via altered floral volatile emissions, and its consequences for plant fitness in *Brassica rapa*. Plants in the field were exposed to either no damage or damage by caterpillars (*Mamestra brassicae*), aphids (*Brevicoryne brassicae*), a leaf fungus (*Sclerotinia sclerotiorum*), or a bacterium (*Xanthomonas campestris* pv. *campestris*). We then recorded pollinator visits and measured floral traits (flower number, volatiles) and plant fitness-correlates. We additionally performed a greenhouse experiment with artificial floral emitters to test for effects of target volatiles on pollinator attraction.
3. In the field experiments, relative to controls, plants subjected to herbivory by the aphid *B. brassicae* (but not those exposed to the other enemies) exhibited a marked reduction in the emission of two volatile organic compounds (nonanal and 2-butyl-1-octanol), experienced lower pollinator visits and produced seeds of lower quality in terms of seed biomass and germination rate, while flower output itself was not affected. Consistently, artificial emitters with reduced amounts of these volatile organic compounds were less attractive to pollinators under greenhouse conditions.
4. *Synthesis.* These results provide strong evidence for volatile-mediated indirect interactions between plant enemies and pollinators ultimately impacting plant fitness, and further point at enemy and compound specificity in such effects.

KEYWORDS

2-butyl-1-octanol, *Brevicoryne brassicae*, *Mamestra brassicae*, nonanal, pollinator-mediated plant reproduction, *Sclerotinia sclerotiorum*, volatiles, *Xanthomonas campestris*

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2023 The Authors. *Journal of Ecology* published by John Wiley & Sons Ltd on behalf of British Ecological Society.

1 | INTRODUCTION

Herbivores negatively impact plant reproduction through direct consumptive effects (Janzen, 1970; Maron, 1998). However, herbivory can also have extended effects on other plant-associated interactions which can indirectly impact plant reproduction, and be equally important or even surpass direct effects of damage (Mothershead & Marquis, 2000; Ohgushi, 2005). For instance, herbivores have been shown to negatively affect pollinator visitation (e.g. visit frequency, time spent visiting flowers) or pollination efficiency (e.g. pollen deposition on flowers), which often lead to reduced reproductive output (Bronstein et al., 2007; Haas & Lortie, 2020; Jones & Agrawal, 2017; Rusman et al., 2020). Given the impact of these herbivore-mediated indirect effects on plant reproduction, many studies have focused on identifying plant traits and mechanisms underlying the outcome of these multi-species interactions, and on understanding the ultimate impacts of changes in these traits on plant-associated antagonistic and mutualistic interactions (Jacobsen & Raguso, 2018; Lucas-Barbosa, 2016; Moreira et al., 2019).

Two main mechanisms by which herbivory can negatively affect pollinator attraction have been proposed. First, herbivory can alter plant attractiveness to pollinators when the latter avoid encounters with herbivores feeding on the flowers (i.e. 'pollinator avoidance mechanisms'; Kessler et al., 2011). In this case, it has been proposed that herbivore presence hinders or interferes with pollinator access to flowers and/or signals increased risk of predation (Bronstein et al., 2007; Lohmann et al., 1996). Second, herbivory can alter attractiveness to pollinators by modifying plant traits that mediate pollinator attraction (i.e. 'plant-mediated mechanisms'; Lehtilä & Strauss, 1997; Mothershead & Marquis, 2000). For instance, herbivore-damaged plants produce fewer or smaller flowers, as well as a lower amount or quality of nectar and pollen, which render plants less attractive to pollinators (e.g. Mothershead & Marquis, 2000; Rusman, Karssemeijer, et al., 2019; Rusman, Poelman, et al., 2019; Strauss et al., 1996). One important floral trait affecting pollinator preference and thus successful pollination is the production of odour blends of volatile organic compounds (VOCs) (Lucas-Barbosa et al., 2011, 2016; Raguso, 2008; Ramos & Schiestl, 2019; Schiestl, 2015). A number of studies have shown that herbivory can alter the emission of floral VOCs, resulting in reduced reproductive output (Burkle & Runyon, 2016; Kessler et al., 2011; Schiestl et al., 2014). These studies have shown that addressing qualitative and quantitative changes in floral VOC emissions in response to herbivory, as well as exploring the role of specific compounds important for plant-pollinator interaction changes, can help to better understand and in some cases largely explain the outcome of plant-herbivore-pollinator interactions.

In addition to herbivory, pathogen impacts on plant fitness can be as important or more than those due to herbivory (Bagchi et al., 2014; Song & Corlett, 2022). Moreover, the molecular and physiological mechanisms underpinning pathogen effects on

plant phenotypes, in particular those associated with plant defence, have been well studied (Biere & Goverse, 2016; Cipollini & Heil, 2010). Recent studies have shown that pathogen-elicited changes in plant traits can affect the performance of insect herbivores (Fernández-Conradi et al., 2018; Moreira, Abdala-Roberts, et al., 2018) and their natural enemies (Desurmont et al., 2016; Ponzio et al., 2013). In addition, other studies have shown that plant pathogens can also impact pollinators. For instance, some bacteria and fungi associated with flowers alter floral traits (e.g. VOC emission; Rering et al., 2018, flower morphology; Adler et al., 2018, and nectar production; Vannette & Fukami, 2018), resulting in changes in pollinator attraction and visitation. Thus far, however, the underlying mechanisms and ultimate effects on plant fitness of pathogens attacking vegetative tissues on pollinators via changes in floral traits, particularly floral VOCs, remain largely unstudied (but see Groen et al., 2016), despite representing a large portion (probably most) of plant pathogen indirect interactions with pollinators. Further, studies comparing simultaneously effects of herbivory and plant pathogen infection on pollination and plant fitness have not been conducted, but are needed since attacker-specific plant responses appear to be fairly co (Rusman et al., 2018; Rusman, Karssemeijer, et al., 2019; Rusman, Poelman, et al., 2019).

To address this gap, we investigated the effects of leaf damage by insect herbivores and infection by plant pathogens on floral traits, pollinator attraction, and reproductive success. We used *Brassica rapa* (Brassicaceae), a self-incompatible plant species, which requires pollinators to produce viable seeds, to assess the role of floral VOCs in mediating plant enemy-pollinator indirect interactions. To this end, we performed several field experiments in which we placed plants in large mesh enclosures (12×10×2.5 m) and exposed them to one of the following treatments: (1) leaf feeding by larvae of the generalist moth *Mamestra brassicae* (Lepidoptera: Noctuidae), (2) leaf feeding by the specialist aphid *Brevicoryne brassicae* (Hemiptera: Aphididae), (3) leaf infection by the generalist fungus *Sclerotinia sclerotiorum* (Helotiales: Sclerotiniaceae), (4) leaf infection by the specialist bacterium *Xanthomonas campestris* pv. *campestris* (Xanthomonadales: Xanthomonadaceae), and (5) control (untreated plants). Following an incubation period, we released bumblebees (*Bombus terrestris*) inside the enclosures and measured flower output and VOCs, and subsequent fruit- and seed-set, seed weight and seed germination rate. To further assess the role of floral VOCs on pollinator attraction, we performed a greenhouse experiment in which we determined responses to artificial flowers emitting VOC blends mimicking those produced by herbivore damage plants in the field experiment. To further strengthen conclusions on any such indirect effects via floral VOCs and their implications for plant reproduction, we conducted two complementary assessments. First, we tested for (a) direct effects of the leaf damage treatments on reproductive success (i.e. damage reducing plant allocation to reproduction), an alternative mechanism to indirect effects. We did this by hand-pollinating inflorescences for control versus plants

subjected to each plant enemy. This simulated high pollen loads in response to which plants would allocate more resources to fruit filling and maturation and direct reproductive costs (if present) of leaf damage are more likely to arise. If direct effects are weak or absent this would mean indirect effects (via VOCs or some other floral trait) are the main mechanism. In addition, we also tested whether plants were pollen-limited, as indirect effects via reduced pollinator visitation will occur or be strongest under pollen limitation. The latter was evaluated under both enclosure and open field conditions to compare the strength of pollen limitation between environments and assess whether enclosure results are representative of ecological dynamics in the field. Overall, the present study provides a robust mechanistic assessment of floral VOC-mediated plant-pollinator interactions as modified by different types of plant antagonists (herbivores and pathogens), specificity in such interactions, and their ultimate consequences for plant fitness.

2 | MATERIALS AND METHODS

2.1 | Study system

The turnip green (*Brassica rapa* L. subsp. *rapa*) is an annual crop in the Brassicaceae family commonly grown in temperate climates worldwide for its edible leaves. In the temperate regions, this species starts flowering in late winter (February–March), and matures and disperses its seeds in late spring (May–June) (Cartea et al., 2021). This species is self-incompatible and mostly relies on pollinators for fertilisation and reproduction (Schiestl et al., 2014).

This plant species is attacked by a diverse community of specialist and generalist insect herbivores, mainly leaf chewers (e.g. larval stages of Lepidoptera) and sap-feeders (e.g. aphids and white flies) (Finch & Thompson, 1992). Caterpillars of the generalist *Mamestra brassicae* (cabbage moth) and the specialist aphid *Brevicoryne brassicae* (cabbage aphid) can be highly destructive when feeding on this plant species (Finch & Thompson, 1992). Early-instar larvae of the cabbage moth feed at night on the underside of the external leaves, where they make perforations and cause extensive amounts of damage (Cartea et al., 2010). All life stages of the cabbage aphid feed on the phloem sap on the underside of leaves or on the growing tips of shoots, leading to a reduction in plant size and yield (Finch & Thompson, 1992).

Pathogenic fungi and bacteria are also important enemies on this plant. These include the generalist fungus *Sclerotinia sclerotiorum* and the specialist bacterium *Xanthomonas campestris* pv. *campestris*, which are both necrotrophic pathogens. *Sclerotinia sclerotiorum* causes the so-called white mould disease in leaves and can spread through the whole plant (Johnson & Atallah, 2014). *Xanthomonas campestris* pv. *campestris* produces V-shaped lesions that extend toward the base of the leaf resulting in wilting and necrosis (Vicente & Holub, 2013). Both diseases have devastating effects in agriculture. We did not need any permit to work with these plant and animal species and in the study site.

2.2 | Experiment 1: Effects of insect herbivory and pathogen infection on floral VOCs and plant reproductive success

2.2.1 | Experimental design

In September 2019, we germinated seeds of five *B. rapa* accessions obtained from the germplasm collection of Biological Mission of Galicia (CSIC, Spain). Seeds were sowed in 2-L pots containing potting soil with peat, and plants were grown in a glasshouse under controlled light (12h per day) and temperature (10°C night, 25°C day). Plants were watered twice a week. In October 2019, 4 weeks after sowing, we transplanted plants into four adjacent nylon cages (12×10×2.5m) placed in the field (42.41°N, 8.64°W, Pontevedra, Spain). Within each cage, we randomly allocated 15 plants per plant accession (i.e. 75 plants in total per cage) in a ten (row) by eight (column) grid. Distance between plants was 1 m.

In January 2020, shortly before the onset of flowering, we measured plant height (mean ± SE: 84.61 ± 1.90 cm) and randomly assigned three plants of each accession per cage to one of five leaf damage treatments: (1) herbivory by *M. brassicae* larvae, (2) herbivory by adult *B. brassicae* aphids, (3) pathogen-infection by *S. sclerotiorum*, (4) pathogen-infection by *X. campestris* and (5) control (untreated plants). In total, we used 300 plants, distributed over 4 cages (i.e. blocks) × 5 plant accessions × 5 leaf damage treatments × 3 replicates. For the leaf damage treatments with herbivores, we added 1 s-instar *M. brassicae* larvae or 15 *B. brassicae* adults to each of two average-sized leaves per plant using a fine paintbrush and covered these leaves with a nylon bag to prevent herbivore dispersal. We obtained *M. brassicae* eggs from a colony reared on Brussels sprouts (var Gemmifera, cv Cyrus) for several generations at the Wageningen University (the Netherlands). We then reared *M. brassicae* larvae on wheat germ-based artificial diet. We collected aphids from *B. rapa* plants in surrounding areas to our field site and reared them on potted *B. rapa* plants in a greenhouse. For the leaf damage treatments with pathogens, we applied three punctures to the upper side of two average-sized leaves using an awl of 1 mm in diameter, added agar plugs (0.4 cm in diameter) containing *S. sclerotiorum* mycelia. *Xanthomonas campestris* was injected at three different points of each leaf puncturing the main veins using mouse-tooth forceps wrapped in cotton wool soaked with the bacterial suspension (5×10^8 CFU mL⁻¹) (Madloo et al., 2019). Inoculated leaves were also covered with a nylon mesh bag. We collected both pathogens on *B. napus* plants found in the vicinity to our field site. To provide fresh colonies for the experiments, we cultivated surface-sterilised pathogens on potato dextrose agar (PDA) plates incubated at 24°C in the dark for 4 days. We obtained fresh colonies through routine growth of mycelium-agar plugs from the margin of the fungal colony on PDA incubated at 24°C for 72 h. For the control and leaf damage treatments with herbivores, we also punctured the leaves as above to control for slight mechanical damage caused by puncturing but did not add

the pathogen-containing agar. For control plants, we also covered two medium-sized leaves with a nylon bag but without herbivores or pathogens to control for bagging effects. We daily monitored leaf damage and re-introduced herbivores when dead individuals were found throughout the five-week incubation period. Most leaves that were treated with *M. brassicae* larvae and both pathogens had more than 30% damage.

In February 2020, when plants started flowering, we released bumblebees (*B. terrestris*, Natupol, Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands) into the cages. This species is an efficient pollinator of *B. rapa* and is frequently found visiting *Brassica* flowers on crop plantations in the study area (X. Moreira, personal observation). We placed one nest box containing 20 bumblebees in a corner of each of the four cages. This pollinator-plant density ratio (approx. 0.20) is similar to that found in crop plantations of the study area (X. Moreira, personal observation). To avoid attacker dispersal among plants, 1 week after introducing the bumblebees we clipped off all leaves on which the herbivores or pathogen were introduced to completely remove herbivores and pathogens. Effects of leaf removal on plant reproduction were assumed to be negligible given that only two leaves were subjected to damage and plants had on average 15 leaves. Bumblebees foraged on *B. rapa* flowers during the day and at night returned to their hive. The nest boxes were kept inside the cages until the end of the flowering phase (March 2020).

2.2.2 | VOC collection

Immediately after removing the leaves with herbivores and pathogens, we collected floral VOCs from 60 plants in one of the cages (5 treatments $\times$ 5 plant accessions $\times$ 2–3 replicates) following Moreira et al. (2021). Briefly, of each plant, we bagged one flowering stalk bearing 20–30 open flowers with a 2 L Nalophan bag. We trapped floral VOCs on a charcoal filter (SKC sorbent tube filled with Anasorb CSC coconut-shell charcoal) for 60 min at a rate of 0.25 L min⁻¹ using a Sidekick 224-52MTX pump (0.25 L min⁻¹ airflow of technical air N₂O₂). We eluted traps with 150 μ L dichloromethane (CAS#75-09-2, Merck, Dietikon, Switzerland) to which we had previously added an internal standard (nonyl acetate [CAS#143-13-5], 200 ng in 10 μ L dichloromethane). We subsequently injected 1.5 μ L of each sample onto an Agilent 7890B gas chromatograph coupled with a 5977B mass selective detector fitted with a 30 m $\times$ 0.25 mm $\times$ 0.25 μ m film thickness HP-5MS fused silica column (Agilent, Santa Clara, California, United States). We operated the GC in split-less mode with helium as the carrier gas (flow rate 1 mL min⁻¹). The GC oven temperature program was: 3.5 min hold at 40°C, 5°C min⁻¹ ramp to 250°C, and 1 min hold at 250°C. We identified VOCs using the library NIST Standard Reference Database 1A v17 and by comparison with commercial standards when available. We measured total emission of individual VOCs as a proportion of the internal standard (Moreira, Nell, et al., 2018).

2.2.3 | Plant reproductive success

From February to March 2020, we counted the number of flowering stalks on each plant (total of three surveys). Because the treatment effect on flowering stalk production was consistent across surveys (non-significant treatment by survey interaction, $F_{4,791} = 1.52$, $p = 0.195$), we used the total number of flowering stalks per plant across surveys. During the last survey (March 2020), we also counted the number of flowers from a subset of plants ($n = 63$). In this subset, we found a strong correlation between the number of flowering stalks and the number of flowers ($R^2 = 0.71$, $p < 0.001$; flowers = $27.2 \times$ flowering stalks – 266.1). We, therefore, used this regression equation to predict flower number per plant from the total number of flowering stalks. In May 2020, at the end of the growing season (20 weeks after applying leaf damage treatments) when the plants started senescing, we removed nest boxes of the bumblebees and estimated the number of seed siliques per stalk on all plants. We collected all mature siliques from a subsample of four randomly chosen stalks per plant and counted the number of siliques per stalk. We estimated the number of siliques per plant by multiplying the total number of stalks per plant by the mean number of siliques per stalk obtained from the subsample. Based on this, we then calculated fruit-set as the estimated number of siliques per plant divided by the number of flowers per plant (predicted values from the above regression between flowering stalk vs. flower number). Fruit-set is a good proxy for reproductive success and pollinator service in self-incompatible species such as *B. rapa* (Sutherland & Delph, 1984). We shelled the collected siliques and counted the number of seeds in 20 randomly chosen siliques per plant to estimate the mean number of seeds per silique. We weighed these seeds to the nearest 0.0001 g to estimate mean seed weight. To evaluate treatment effects on seed germination, we sowed groups of 25 randomly chosen seeds per plant on wet cotton fibre in Petri dishes (6 cm in diameter by 1.2 cm high) inside growth chambers at 25°C in the dark. We counted the proportion of germinated seeds per Petri dish after 1 week.

2.2.4 | Statistical analyses

Volatile organic compounds

We analysed the effects of leaf damage treatment and plant accession (all fixed factors) on the emission of individual floral VOCs and on total VOC emission using linear models with PROC GLM in SAS 9.4 (Littell et al., 2006). We did not evaluate genetic variation in the inducibility of floral VOCs (i.e. leaf damage treatment $\times$ plant accession) due to insufficient replication. Preliminary analyses including plant height as a covariate indicated that this variable did not contribute significantly to explain variation in VOC emission and was therefore removed from the final models. We also ran a permutational multivariate analysis of variance (PERMANOVA) to test for an effect of leaf damage treatment on floral VOC composition

(using abundances of each compound). This analysis was based on 10,000 permutations and was performed with the 'vegan' package in R ver. 4.0.2 software (Oksanen et al., 2016). To visualise these results, we conducted a principal coordinates analysis based on Bray–Curtis pairwise dissimilarities, and graphed the centroids of each leaf damage treatment effect (Moreira et al., 2021). We also identified floral VOCs that correlated strongly ($R^2 > 0.60$) with the first two ordination axes (using 'envfit' in vegan; Oksanen et al., 2016), and displayed these relationships using biplot arrows with length scaled to R^2 values.

Plant reproductive traits

We analysed the effects of leaf damage treatment, plant accession, and their interaction (all fixed factors) on flower number, fruit-set, mean number of seeds per silique, mean seed weight and seed germination (i.e. proportion of germinated seeds) using linear mixed effect models with PROC MIXED in SAS 9.4 (Littell et al., 2006). We included the cages as a random factor and plant height as a covariate to account for differences in plant size affecting reproductive output and success. We log-transformed proportions of germinated seeds to achieve normality of residuals.

2.3 | Experiment 2: Test of plant enemy effects on pollinator attraction and direct effects on plant reproductive success

2.3.1 | Experimental design

In September 2022, we germinated seeds of the same five *B. rapa* accessions used in experiment 1. In October 2022, 4 weeks after sowing, we transplanted plants to the field site used in the previous experiment. We kept plants in a nylon mesh enclosure (same as those used in Experiment 1) and randomly allocated 12 plants per plant accession (i.e. 60 plants) in a ten (row) by six (column) grid. In January 2023, shortly before the onset of flowering, we measured plant height (mean $\pm$ SE: 67.29 $\pm$ 3.40 cm) of all surviving plants with flowers ($n=35$ out of 60) and randomly assigned plants in roughly similar numbers to no damage or to one of the four leaf damage treatments (same as in Experiment 1). Accessions were similarly represented across treatments. Three weeks after applying leaf damage treatments, we clipped off leaves used for the experiments to completely remove herbivores and pathogens and estimated the percentage of leaf area consumed by *M. brassicae* larvae and infected by both pathogens using the professional mobile application BioLeaf–Foliar Analysis™ (Brandoli Machado et al., 2016) and ImageJ software (Version 1.51n), respectively. Percentage of leaf area consumed by *M. brassicae* larvae was 26.22 $\pm$ 5.69 whereas the percentage of leaf area infected by *S. sclerotiorum* and *X. campestris* was 46.28 $\pm$ 9.22 and 45.01 $\pm$ 12.57, respectively. Although we did not systematically measure herbivore and pathogen damage during Experiment 1, observations suggest that damage levels were similar in the Experiments 1 and 2.

To test for direct effects of leaf damage on reproductive success, when plants started flowering (February 2023), we selected one flowering stalk per plant for which flowers had not opened yet and covered it with a nylon mesh bag to avoid pollination by bumblebees. Once all flowers opened, we counted them, manually hand-pollinated all flowers per inflorescence using a fine paintbrush with pollen of a *B. rapa* accession not used in the experiments and covered again the flowers with the nylon mesh bag. With this, we aimed to mimic a scenario of high pollen load and therefore increased allocation to fruit formation and filling under which costs of leaf damage on reproduction would be more likely to arise. Immediately after hand pollination, we followed the same methodology as for the field enclosure experiment and placed one nest box containing eight bumblebees in a corner of the cage (resulting in a 0.20 pollinator: plant ratio observed under field conditions) and released bumblebees into the cage. Then, in late April 2023, when plants started senescing, we counted the number of seed siliques on these stalks. We calculated fruit-set as the number of siliques divided by the number of flowers for these hand-pollinated stalks. We then collected these siliques, shelled them, and counted the number of seeds to estimate the mean number of seeds per silique. Finally, we weighed all these seeds to estimate mean seed weight and performed the germination trials in Petri dishes to estimate the proportion of germinated seeds following the approach in Experiment 1. It is important to note that seed number and germination rate were much higher in Experiment 1 compared to Experiment 2 (see Section 3). One potential explanation is that plants in Experiment 2 were a bit smaller and started to senesce 1 month before than plants in Experiment 1 (April 2023 vs. May 2020). Despite this difference, treatments and magnitude of effects were similar, suggesting that the nature of the effects remained consistent across different levels of reproductive output.

To test for leaf damage effects on pollinator attraction, in the 2 weeks after introducing the bumblebees, we carried out three-minute observations of each plant (total of six surveys) to estimate the number of pollinator visits on each plant. We used the total number of visits across surveys per plant for statistical analyses. We performed all observations from 11:00 to 12:00 AM during six consecutive days. From February to March 2023, we also counted the number of unbagged flowering stalks on each plant (total of two surveys) to account for variation in display size in testing for leaf damage effects on pollinator visitation.

2.3.2 | Statistical analyses

To test for direct effects of damage, using data from bagged flowering stalks, we analysed the effects of leaf damage treatment on total number of pollinator visits, fruit-set, mean number of seeds per silique, mean seed weight and seed germination (i.e. proportion of germinated seeds) using linear mixed effect models with PROC MIXED in SAS 9.4. For all these models, we included the effect of plant accession as a random factor and plant height as a covariate. Moreover,

to test for leaf damage effects on pollinator visits, we tested for the leaf damage treatment as well as plant accession as a random effect and also included the total number of flowering stalks (across the two surveys) as a covariate. We log-transformed number of pollinator visits and fruit-set to achieve normality of residuals.

2.4 | Experiment 3: Test of candidate floral VOCs mediating pollinator attraction

2.4.1 | Experimental design

To assess the role of specific floral VOCs in pollinator attraction, in November 2021, we performed two dual-choice greenhouse bioassays. In both cases, we constructed artificial inflorescences by cutting paper circles from yellow construction paper (10 cm diameter) (Schiestl et al., 2014). We attached a filter paper disk (3 cm diameter) to the centers of each yellow paper disk. Based on the findings obtained from Experiment 1 (see Section 3.1), we prepared two synthetic blends of VOCs: 'Control' and 'B. brassicae', using four chemical compounds (nonanal: Sigma Aldrich, CAS number: 124-19-6; 2-butyl-1-octanol: Sigma Aldrich, CAS number: 3913-02-8; tetradecane: Sigma Aldrich, CAS number: 629-59-4; pentadecane: Sigma Aldrich, CAS number: 629-62-9), of which the emission rates significantly changed in response to *B. brassicae* feeding (see VOC Results). To achieve the mean amounts emitted by *B. rapa* inflorescences in control and damaged plants, we diluted 0.88 mL of nonanal, 0.16 mL of 2-butyl-1-octanol, 0.52 mL of tetradecane and 0.54 mL of pentadecane in 20 mL of dichloromethane (Sigma Aldrich, CAS number: 75-09-2) for the control blend and 0.44 mL of nonanal, 0.12 mL of 2-butyl-1-octanol, 0.48 mL of tetradecane and 0.48 mL of pentadecane in 20 mL of dichloromethane for the *B. brassicae* blend. For the first dual-choice bioassay, we soaked the center filter paper disks of artificial inflorescences with either 200 μ L of control blend or 200 μ L of *B. brassicae* blend and attached one inflorescence of each treatment at either end of 20 plastic cylinders, that is replicates (height, 60 cm; diameter, 10 cm). Subsequently, we released one bumble bee into each plastic cylinder through a hole (4 cm diameter) at the centre of the cylinder and recorded each bumble bees' first choice between control and *B. brassicae* blend ('0' for not landing vs. '1' for landing on an inflorescence type of each replicate). We conducted the choice experiment once (i.e. one trial) for each replicate and in each case used a different bumble bee. In the second dual-choice bioassay, we fixed the artificial inflorescences at the top of white plastic sticks (height, 45 cm; diameter, 2 cm) and placed them in 1 L pots containing potting soil with peat (Gramoflor GmbH & Co. KG Produktion, Vechta, Germany). We again soaked the filter paper disks with 200 μ L of control blend or 200 μ L of *B. brassicae* blend and placed pairs of artificial inflorescences (one of each treatment) in 20 plastic flight cages (37.5 $\times$ 37.5 $\times$ 96.5 cm). Within each cage, pairs of artificial inflorescences were placed 30 cm apart

horizontally. We released a single bumblebee into each flight cage and recorded the bumblebees' first choice as above.

2.4.2 | Statistical analyses

For each dual-choice bioassay type, we analysed the effect of VOC blend type (two levels: control vs. *B. brassicae* blend, fixed factor) on the odds of bumble bees' first choice using a generalised linear mixed model with a binomial distribution and logit-link function (PROC GLIMMIX in SAS 9.4). Odds ratio values are the ratio between successful and unsuccessful events (i.e. bees landing vs. not landing on an inflorescence of a given type, respectively) for each treatment level, that is a likelihood of a bee being attracted to the control or *B. brassicae* blend treatments. In both cases, we included the effect of the choice testing device or set-up (i.e. cylinder or flight cage) as a random factor to control for non-independence of each pair of inflorescences per replicate.

2.5 | Experiment 4: Test of pollen limitation

2.5.1 | Experimental design

In September 2022, we germinated seeds of the same five *B. rapa* accessions used previously. In October 2022, 4 weeks after sowing, we transplanted plants to the same field site used in the previous experiments. We placed a group of plants inside a nylon mesh enclosure as in Experiment 1 and another group in an adjacent open field site. In both cases, we randomly allocated 12 plants per plant accession (i.e. 60 plants) in a ten (row) by six (column) grid. Distance between plants was 1 m in both cases.

In February 2023, at the onset of flowering, we had 25 plants with flowers under open field conditions and 33 in the enclosure (plant height mean $\pm$ SE: 70.96 $\pm$ 3.77 cm at the open field site; 60.15 $\pm$ 4.42 cm in the enclosure). The accessions were similarly represented in both groups. We marked two flowering stalks per plant for which flowers had not opened yet and covered it with a nylon mesh bag to avoid pollination by bumblebees. Once all flowers opened, and following standard procedures to test for pollinator-mediated pollen-limitation (Ashman et al., 2004), for one stalk we counted all flowers and hand-pollinated them using a fine paintbrush with pollen of a *B. rapa* accession not used in the field experiments (same accession as the test of leaf damage direct effects in Experiment 2) and covered again the flowers with the nylon bag. For the other stalk, we also counted all flowers but kept it without the nylon bag to measure effects of open pollination on plant reproduction. The same day after conducting hand pollinations and following the same procedure as in Experiment 1, for plants in the enclosure we placed one nest box with eight bumblebees in a corner and released them, resulting again in a pollinator: plant ratio of approx. 0.20. Over the following 2 weeks, on each plant we conducted

three-minute observations (six times per plant over six consecutive days) of pollinator visits on unbagged stalks (surveys conducted from 11:00AM to 12:00AM). These visitation data complemented measurements of fruit-set used to test for pollen limitation (see next section). We used the total number of pollinator visits across all surveys for statistical analyses. Then, in late April 2023, when plants started senescing, we counted the number of seed siliques on the marked flowering stalks (open vs. hand pollinated). For each stalk, we calculated fruit-set as the number of siliques divided by the number of flowers. From February to March 2023, we also counted the number of unbagged flowering stalks on each plant (total of two surveys). Plants under open field conditions received little to no damage by insects or pathogens during the course of the experiment (X. Moreira, personal observation).

2.5.2 | Statistical analyses

We assessed pollen limitation (i.e. difference in fruit-set for hand-pollinated vs. open-pollinated inflorescences) and whether it varied in enclosures vs. open field conditions by testing for the effect of stalk treatment (open vs. hand-pollinated), environment type (enclosure vs. open field), and their interaction on fruit-set using a linear mixed model with PROC MIXED in SAS 9.4. We additionally ran the same model on total number of pollinator visits per plant to assess differences in pollinator activity between environments potentially related to effects on fruit-set. For both models, we included the total number of flowering stalks per plant as a covariate to account for an effect of overall flowering intensity in testing for the pollination treatment on the focal stalks. In addition, both models included the effects of plant accession and individual plant (to control for non-independence of paired stalks per plant) as random factors, as well as plant height as a covariate to account for residual variation in plant size affecting reproductive output and success. We log-transformed number of pollinator visits to achieve normality of residuals.

TABLE 1 Effects of leaf damage treatment (five levels: control, herbivory by *Mamestra brassicae*, herbivory by *Brevicoryne brassicae*, infection by *Sclerotinia sclerotiorum*, infection by *Xanthomonas campestris*), plant accession and their interaction on flower number (predicted from the total number of flowering stalks), fruit-set (estimated as the number of siliques per plant divided by the predicted number of flowers per plant), mean number of seeds per silique, mean seed weight, seed germination (estimated as the proportion of germinated seeds throughout a one-week period) in *Brassica rapa* plants.

	Damage treatment (T)			Plant accession (PA)			T × PA			Height		
	df	F	p	df	F	p	df	F	p	df	F	p
Flower number	4, 264	2.12	0.078	4, 264	1.42	0.229	16, 264	1.12	0.338	1, 264	262.22	<0.001
Fruit-set	4, 226	0.45	0.773	4, 226	2.41	0.049	16, 226	0.80	0.688	1, 226	130.46	<0.001
Seed number	4, 237	0.57	0.684	4, 237	7.54	<0.001	16, 237	1.00	0.457	1, 237	1.60	0.207
Seed weight	4, 237	3.35	0.011	4, 237	11.48	<0.001	16, 237	1.59	0.073	1, 237	0.77	0.381
Seed germination	4, 235	3.97	0.004	4, 235	1.11	0.353	16, 235	1.13	0.328	1, 235	8.67	0.004

Note: For all models, we used plant height as a covariate. F-values, degrees of freedom (numerator, denominator) and associated significance levels (p) are shown. Significant p values ($p < 0.05$) are in bold.

3 | RESULTS

3.1 | Experiment 1: Effects of insect herbivory and pathogen infection on floral VOCs and plant reproductive success

Flower number, fruit-set and mean seed number per silique were not significantly affected by leaf damage treatment (Table 1; Figure 1a,b). However, plants subjected to feeding by *B. brassicae* produced seeds that were lower in weight and germinated less well than seeds from the other treatments and the control (Table 1; Figure 1c,d).

We identified a total of 15 floral VOCs emitted by *B. rapa* plants (Table 2). The leaf damage treatment did not significantly influence the total emission (Table 3; Figure 2a) or composition (PERMANOVA: Table 3; Figure 2b) of floral VOCs. Nonetheless, analyses of individual compounds showed a significant effect of leaf damage in several cases, namely: nonanal (PERMANOVA: $R^2 = 0.73$, $p < 0.05$), tetradecane ($R^2 = 0.70$, $p < 0.05$), and pentadecane ($R^2 = 0.67$, $p < 0.05$) (Figure 2b). Post-hoc pair-wise comparisons of treatment level means indicated that plants subjected to feeding by *B. brassicae* emitted a significant lower amount of nonanal and 2-butyl-1-octanol compared with control plants (Table 2). Likewise, in plants subjected to *S. sclerotiorum* infection the emission of nonanal was significantly reduced compared with controls (Table 2).

3.2 | Experiment 2: Test of plant enemy effects on pollinator attraction and direct effects on plant reproductive success

We found a significant effect of the leaf damage treatment on the number of pollinator visits to unbagged flower stalks (Table 4). Consistent with results from experiment 1, plants subjected to herbivory by *B. brassicae* exhibited a lower number of pollinator visits relative to control plants as well as to plants subjected to damage by all other plant enemies (Figure 3a).

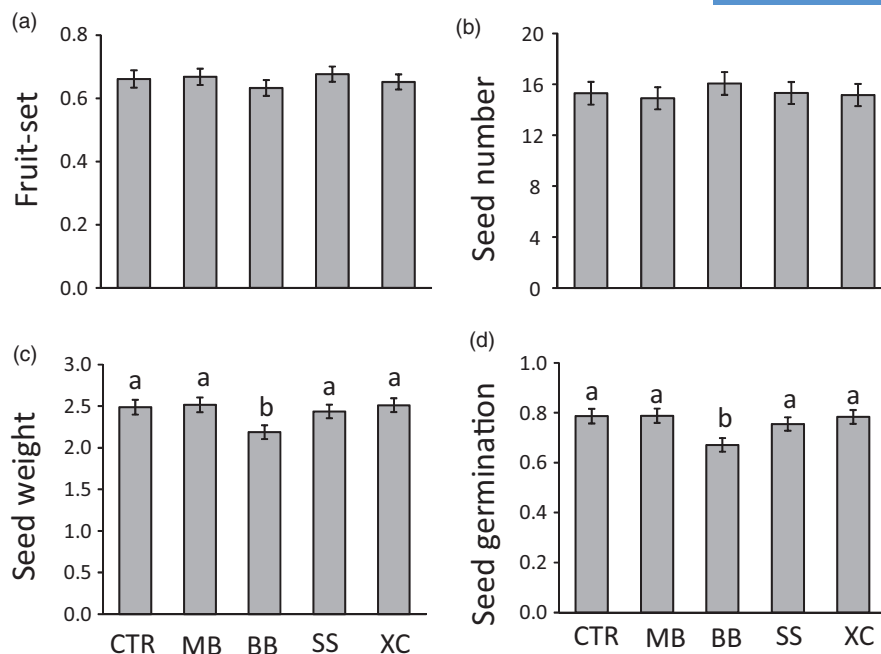


FIGURE 1 Effects of leaf damage treatment (five levels: control [CTR], herbivory by *Mamestra brassicae* [MB], herbivory by *Brevicoryne brassicae* [BB], infection by *Sclerotinia sclerotiorum* [SS] and infection by *Xanthomonas campestris* [XC]) on (a) fruit-set (estimated as the number of siliques per plant divided by the predicted number of flowers per plant), (b) mean number of seeds per silique, (c) mean seed weight, (d) seed germination (estimated as estimated the proportion of germinated seeds in a one-week period) in *Brassica rapa* plants pollinated by bumblebee (*Bombus terrestris*). Bars are least square means $\pm$ SEM ($N = 60$). Different letters indicate significant differences between leaf damage treatments. Statistics are shown in Table 1.

TABLE 2 Effects of leaf damage treatment (five levels: control [CTR], herbivory by *Mamestra brassicae* [MB], herbivory by *Brevicoryne brassicae* [BB], infection by *Sclerotinia sclerotiorum* [SS] and infection by *Xanthomonas campestris* [XC]) on the emission of individual volatile compounds (VOCs) released by flowers of *Brassica rapa* plants (nanograms per hour).

Compound	Control	MB	BB	SS	XC
Benzaldehyde	17.12 $\pm$ 4.65	10.59 $\pm$ 0.73	9.70 $\pm$ 1.31	13.34 $\pm$ 2.08	16.89 $\pm$ 3.46
5-hepten-2-one, 6-methyl-	28.23 $\pm$ 3.57	22.28 $\pm$ 2.45	19.73 $\pm$ 2.03	22.83 $\pm$ 5.10	24.51 $\pm$ 3.15
trans- α -bergamotene	8.93 $\pm$ 0.50	8.70 $\pm$ 0.85	8.10 $\pm$ 0.48	8.79 $\pm$ 0.83	9.26 $\pm$ 0.77
2-hexen-1-ol, acetate, (Z)-	13.39 $\pm$ 4.21	15.35 $\pm$ 1.57	10.71 $\pm$ 2.59	10.92 $\pm$ 2.29	9.63 $\pm$ 0.84
Limonene	3.21 $\pm$ 0.35	3.17 $\pm$ 0.18	2.70 $\pm$ 0.21	3.16 $\pm$ 0.33	3.14 $\pm$ 0.23
Linalool	4.15 $\pm$ 0.48	4.07 $\pm$ 0.32	3.52 $\pm$ 0.34	3.76 $\pm$ 0.51	3.81 $\pm$ 0.45
Nonanal	41.58 $\pm$ 8.18a	30.02 $\pm$ 5.10ab	21.65 $\pm$ 6.08b	17.17 $\pm$ 4.36b	27.64 $\pm$ 4.24ab
(E)- β -farnesene	2.68 $\pm$ 0.40	2.29 $\pm$ 0.41	2.30 $\pm$ 0.49	4.20 $\pm$ 1.28	5.50 $\pm$ 2.27
2-butyl-1-octanol	7.93 $\pm$ 0.32a	7.52 $\pm$ 0.44a	5.93 $\pm$ 0.41b	6.66 $\pm$ 0.54ab	7.74 $\pm$ 0.55a
Tridecane	9.92 $\pm$ 0.71	9.69 $\pm$ 0.58	7.78 $\pm$ 0.46	8.61 $\pm$ 0.78	9.88 $\pm$ 0.86
Decanal	3.55 $\pm$ 0.25	3.58 $\pm$ 0.12	3.02 $\pm$ 0.26	3.57 $\pm$ 0.30	3.65 $\pm$ 0.19
Farnesane	9.26 $\pm$ 1.06	8.31 $\pm$ 0.55	7.07 $\pm$ 0.37	7.86 $\pm$ 0.76	8.05 $\pm$ 0.71
Tetradecane	34.90 $\pm$ 2.91	34.76 $\pm$ 2.38	29.60 $\pm$ 0.98	32.05 $\pm$ 2.81	32.48 $\pm$ 2.34
Pentadecane	26.66 $\pm$ 2.57	27.33 $\pm$ 2.45	23.89 $\pm$ 1.22	26.16 $\pm$ 3.14	24.67 $\pm$ 2.42
α -farnesene	8.78 $\pm$ 2.07	7.17 $\pm$ 0.70	6.95 $\pm$ 0.57	6.77 $\pm$ 1.05	7.09 $\pm$ 0.75

Note: Least-square means $\pm$ SE ($N = 12$). Different letters indicate significant differences within leaf damage treatments at $p < 0.05$ based on Tukey post hoc tests. Floral VOCs that significantly changed after herbivore attack or pathogen infection are in bold.

TABLE 3 Effects of leaf damage treatment (five levels: control, herbivory by *Mamestra brassicae*, herbivory by *Brevicoryne brassicae*, infection by *Sclerotinia sclerotiorum* and infection by *Xanthomonas campestris*) and plant accession on total emission rates and composition of floral volatile organic compounds (VOCs) from *Brassica rapa* plants.

	df	Damage treatment		Plant accession	
		F/pseudo-F	p	F/pseudo-F	p
VOC emission	4, 51	2.28	0.074	0.44	0.782
VOC composition	4, 51	1.52	0.106	1.25	0.241

Note: For VOC composition, we used a permutational multivariate analysis of variance (PERMANOVA) model. F-values (pseudo-F in the case of PERMANOVA), degrees of freedom (numerator, denominator) and associated significance levels (p) are shown.

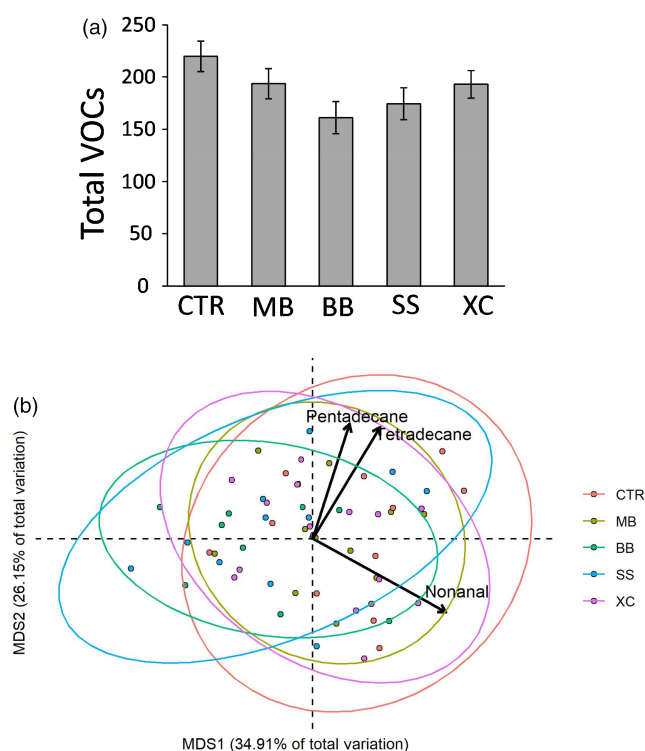


FIGURE 2 (a) Effects of leaf damage treatment (five levels: control [CTR], herbivory by *Mamestra brassicae* [MB], herbivory by *Brevicoryne brassicae* [BB], infection by *Sclerotinia sclerotiorum* [SS] and infection by *Xanthomonas campestris* [XC]) on the emission rates of volatile organic compounds (VOCs) released by flowers of *Brassica rapa* plants. Bars are least-square means $\pm$ SEM ($N = 12$). Statistics are shown in Table 2. (b) Unconstrained ordinations showing the effects of leaf damage treatment on composition of VOCs released by flowers of *B. rapa* plants. Biplot arrows show associated linear trends with volatiles, scaled to reflect relative magnitude of effects based on R^2 values ($R^2 > 0.60$, $p < 0.001$). The leaf damage treatment ordination displays control and herbivore- or pathogen-induced centroids and 95% ellipses. The first two axes of this ordination accounted for ca. 61% of the treatment effect in volatile composition (26% and 35% respectively). See Table 3 for Permutational Analysis of Variance (PERMANOVA) test on VOC composition related to this ordination.

On the other hand, the test of direct effects (i.e. not mediated by reduced pollinator attraction) of leaf damage on plant reproduction indicated no significant effects on fruit-set, mean seed number

per silique, seed weight or seed germination rate for bagged, hand-pollinated flowers (Table 4; Figure 3b–e).

3.3 | Experiment 3: Test of candidate floral VOCs mediating pollinator attraction

Results from both dual-choice bioassays with artificial VOC emitters indicated that the '*B. brassicae*' blend was chosen a significantly lower number of times (i.e. was less attractive) than the 'Control' blend for both the plastic cylinder experiment (z -value = 3.51, $p < 0.001$, $df = 1$, Figure 4a) and the flight cage experiment (z -value = 2.46, $p = 0.014$, $df = 1$, Figure 4b).

3.4 | Experiment 4: Test of pollen limitation

We found a significant effect of environment type (open field vs enclosures) on pollinator visits ($F_{1,50} = 19.81$, $p < 0.001$), whereby plants in the enclosure had 59% more visits on average than plants in the open field (Figure 5a). Plants in the open field were visited mainly by bumblebees ($46.6 \pm 6.47\%$) and honeybees ($41.8 \pm 6.72\%$). In addition, we found a significant effect of flowering stalk pollination treatment on fruit-set ($F_{1,44} = 37.16$, $p < 0.001$). Fruit-set was 42% greater in hand-pollinated stalks (0.69 ± 0.02) than in open-pollinated stalks (0.48 ± 0.02). Despite the higher pollinator visitation rates in the enclosures, we did not find a significant effect of environment type ($F_{1,44} = 0.59$, $p = 0.446$; cage: 0.59 ± 0.03 ; field: 0.57 ± 0.02) or an interaction between flowering stalk pollination treatment and environment type on fruit-set ($F_{1,44} = 0.27$, $p = 0.608$; Figure 5b).

4 | DISCUSSION

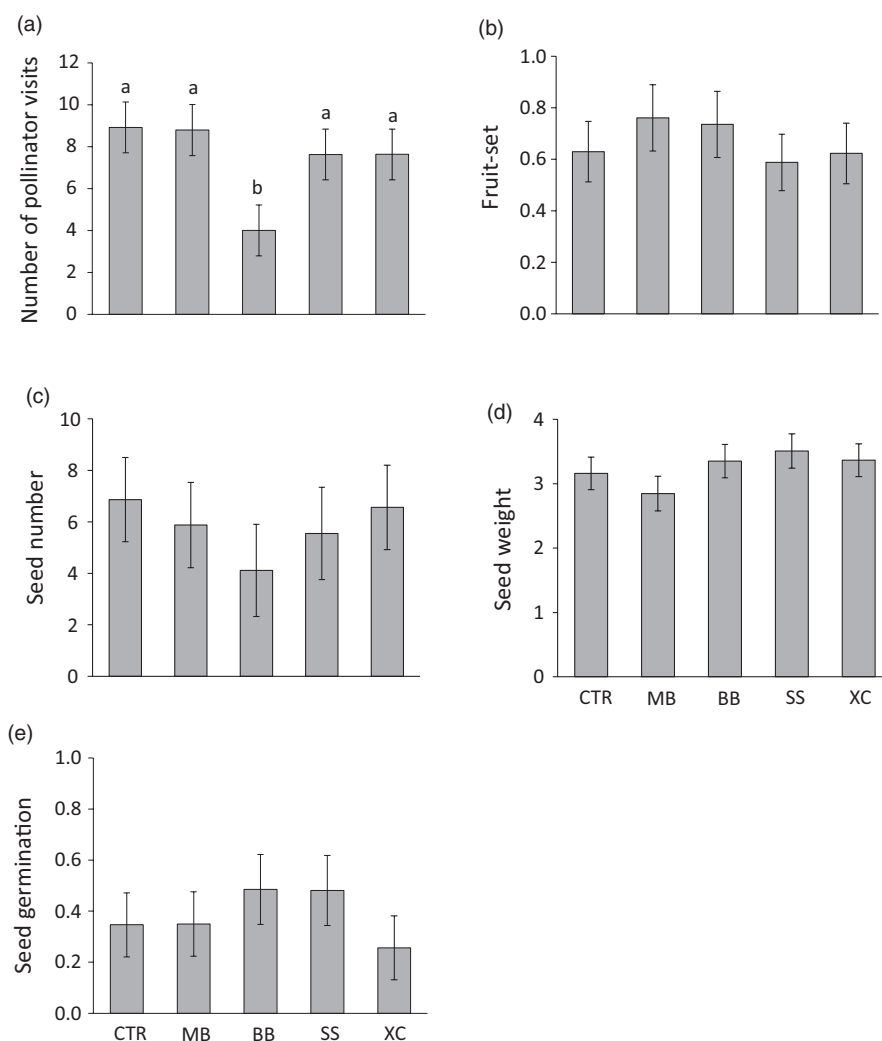
The field experiments showed that plants exposed to herbivory by the specialist aphid *B. brassicae* emitted lower amounts of two floral VOCs (nonanal and 2-butyl-1-octanol) and were less visited by bumblebees. Likewise, findings from the dual-choice greenhouse bioassays showed that low emission rates of these floral VOCs (mimicking induced changes in response to aphid feeding) resulted in decreased bumblebee attraction, thus providing a mechanistic support for the link between aphid herbivory, lowered emissions

TABLE 4 Effects of leaf damage treatment (five levels: control, herbivory by *Mamestra brassicae*, herbivory by *Brevicoryne brassicae*, infection by *Sclerotinia sclerotiorum*, infection by *Xanthomonas campestris*) on the number of bumblebee (*Bombus terrestris*) visits (across six surveys), fruit-set (estimated as the number of siliques divided by the number of flowers per plant), mean number of seeds per silique, mean seed weight, seed germination (estimated as the proportion of germinated seeds throughout a one-week period) in hand-pollinated *Brassica rapa* plants.

	Damage treatment			Plant height			Flowering stalks		
	df	F	p	df	F	p	df	F	p
Pollinator visits	4, 24	2.93	0.042	1, 24	4.95	0.036	1, 24	0.47	0.501
Fruit-set	4, 19	0.39	0.811	1, 19	2.96	0.102	—	—	—
Seed number	4, 18	0.39	0.816	1, 18	0.83	0.375	—	—	—
Seed weight	4, 18	1.58	0.223	1, 18	6.64	0.019	—	—	—
Seed germination	4, 18	0.56	0.694	1, 18	0.00	0.999	—	—	—

Note: For all models, we used plant height as a covariate. In the case of pollinator visits, we also included the total number of flowering stalks (across two surveys) as a covariate. F-values, degrees of freedom (numerator, denominator), and associated significance levels (p) are shown. Significant p values ($p < 0.05$) are in bold.

FIGURE 3 Effects of leaf damage treatment (five levels: control [CTR], herbivory by *Mamestra brassicae* [MB], herbivory by *Brevicoryne brassicae* [BB], infection by *Sclerotinia sclerotiorum* [SS] and infection by *Xanthomonas campestris* [XC]) on (a) the total number of bumblebee (*Bombus terrestris*) visits (across six surveys), (b) fruit-set (estimated as the number of siliques divided by the number of flowers per plant), (c) mean number of seeds per silique, (d) mean seed weight, (e) seed germination (estimated as estimated the proportion of germinated seeds in a one-week period) in hand-pollinated *Brassica rapa* plants. Bars are least square means $\pm$ SEM ($N = 7$). For pollinator visits, bars are back-transformed least-square means $\pm$ SE from a linear mixed model ($N = 7$). Statistics are shown in Table 4.



of these compounds, and reduced pollinator visitation. Nonanal is an aldehyde that has been reported to act as an insect attractant of mosquitoes (Syed & Leal, 2009), bark beetles (de Groot & Poland, 2003), and pollinators (Klatt et al., 2013), as well as a repellent

to parasitoids (Desurmont et al., 2020). In the case of pollinators, a recent study using electroantennogram recordings and behavioural observations reported that nonanal released by flowers of three plant species (*Sapium sebiferum*, *Ligustrum compactum* and *Castanea*

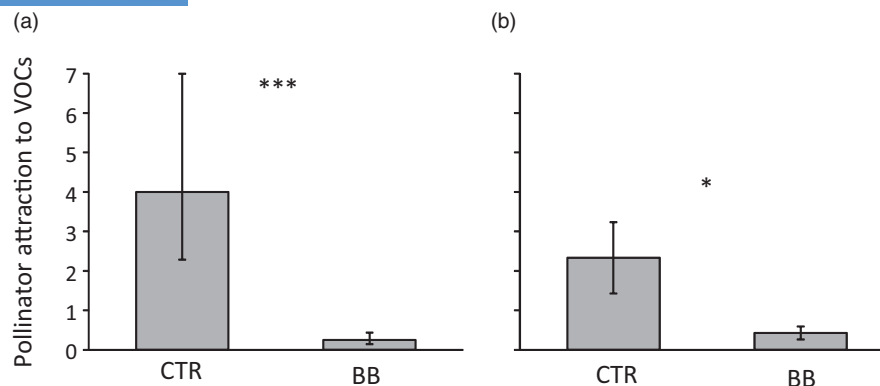


FIGURE 4 Pollinator (*Bombus terrestris*) attraction (measured as odds values) to artificial flowers of two emission types: those containing synthetic blends of volatile organic compounds (VOCs) which mimic the emission rates in response to *Brevicoryne brassicae* (BB) or control (CTR) emissions based on dual-choice bioassays using (a) plastic cylinders or (b) flight cages. Bars are the mean odds ratio value ($\pm$ SE) for each VOC exposure treatment obtained from a generalised linear mixed model ($N=20$ assay replicates, see Experiment 3 Methods). Odds values were calculated as the ratio between successful and unsuccessful events (i.e. bees landing vs. not landing on an inflorescence of a given type, respectively) for each inflorescence of each treatment level, that is a likelihood of a bee being attracted to the CTR or BB treatments. Asterisks above the bars indicate significant differences between VOC exposure treatments at $p < 0.05$ (*) and $p < 0.001$ (***).

henryi) increased honeybee attraction (Liu et al., 2022). Similarly, another recent study using electroantennographic recordings showed that inexperienced females of the wild bee *Osmia bicornis* had higher antennal responses to nonanal identified from floral volatile extracts of strawberry plants than to controls of air and paraffin oil (Klatt et al., 2013). Likewise, 2-butyl-1-octanol is an aliphatic alcohol released by plants at low amounts and acts as a pheromone attractant of some pollinating insects (Wang & Tan, 2019). In turn, and consistent with our findings, several studies have reported that leaf herbivory changes the emissions of these and other floral compounds which then reduces the attraction of pollinators (e.g. Hoffmeister et al., 2016; Kessler et al., 2011; Schiestl et al., 2014). For example, related work by Schiestl et al. (2014) using *B. rapa* found that herbivory by two leaf chewers reduced the emission of a number of floral VOCs (e.g. 6-methyl, 5-hepten-2-one, phenylacetaldehyde, acetophenone, phenylethyl alcohol, phenylethyl acetate, and decanal), and that this correlated with reduced plant attractiveness to pollinators. Whereas most of those floral compounds are odorous volatiles which have been shown to elicit bee responses, the roles of nonanal and 2-butyl-1-octanol are less clear and warrant further investigation. Follow-up work assessing the individual effects of these compounds on pollinators and their potential interactions with other compounds in VOC mixtures are needed.

Induced changes in floral VOCs due to aphid feeding have been little studied. In one of the few available studies, Pareja et al. (2012) found that feeding by two aphids (the specialist *Lipaphis erysimi* and the generalist *Myzus persicae*) reduced the emission of floral volatiles in the white mustard *Sinapis alba* relative to undamaged plants and those attacked by the specialist leaf chewer *Plutella xylostella*. However, contrary to our findings, aphid herbivory did not affect pollinator visits (Pareja et al., 2012), highlighting variability and likely also specificity in outcomes from VOC induced changes. In interpreting aphid-induced suppression

of floral VOC emission in our study, it is worth noting that volatile benzenoids were an important component of *B. rapa* floral scent. These chemicals are derived from the breakdown of phenylalanine by phenylalanine ammonia-lyase (PAL) (Muhlemann et al., 2014), an enzyme shown to be involved in plant defence against aphids (van Eck et al., 2010). It is, therefore, possible that aphids use a biochemical mechanism (affecting PAL activity) that alters the biosynthesis of floral VOCs similarly to biosynthetic changes in leaf VOCs in response to phloem feeding (Zhang et al., 2009). Further work is needed to test this possibility by looking at plant biochemical effects of aphid feeding on *B. rapa* and how these relate to induced changes in floral VOCs emissions. More broadly, studies assessing the biochemical mechanisms underlying induced floral (and leaf) VOC responses to different species or guilds of plant enemies controlling for traits such as feeding mode or diet breadth would be highly valuable. Inclusion of pollinator responses in these studies would increase our understanding of whether and how plant enemy-interactions indirectly affect pollination services and the mechanisms behind specificity.

Results from the field experiments also showed that herbivory by *B. brassicae* feeding affects plant fitness, reducing seed weight and germination rate. In contrast, fruit-set was not affected by aphid feeding. A possible explanation for these results is that pollinator efficiency (e.g. via changes in visit duration or movement patterns among flowers within or between plants) impacts fruit filling but not fruit setting (Chautá-Mellizo et al., 2012; Ghazoul, 2006). Pollen limitation, as evidenced by greater fruit-set for hand- versus open-pollinated stalks, was of similar magnitude in enclosure relative to open field conditions despite there being more than two-fold higher visits in the enclosure. This suggests a threshold beyond which no further gains in fruit-set are achieved despite increases in visitation rates, and that plant reproductive success depends on aspects of pollinator behaviour that determine their efficiency independently

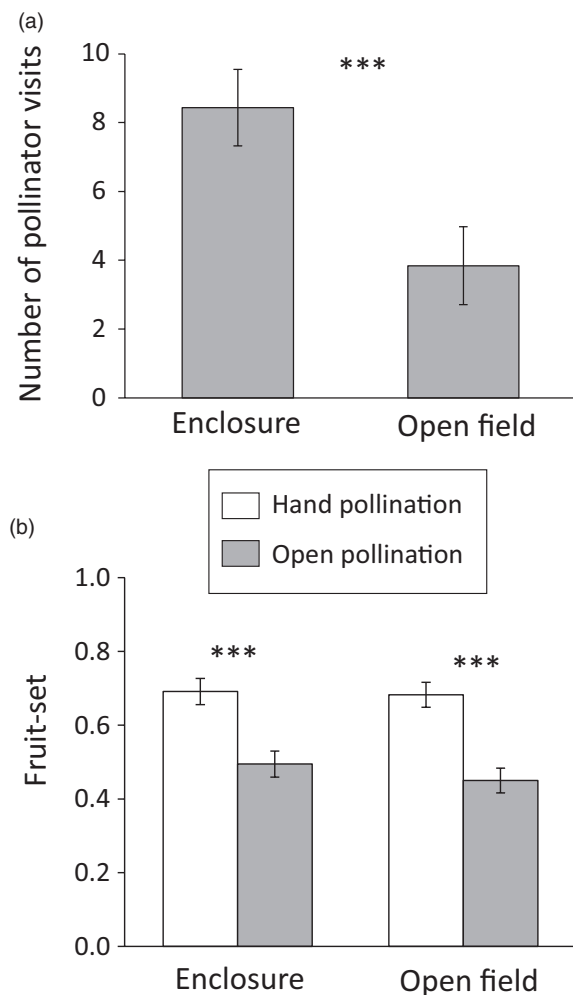


FIGURE 5 (a) Number of pollinator visits (across six surveys) to *Brassica rapa* plants under two experimental conditions (enclosure vs. open field conditions). In the case of open field conditions, pollinator visits were by bumblebees, honeybees and wasps. In the case of the enclosure, pollinator visits were exclusively by bumblebees (*Bombus terrestris*). Bars are back-transformed least-square means $\pm$ SE from a linear mixed model ($N=25$ under open field conditions and $N=33$ in the enclosure). Asterisks above the bars indicate significant differences between experimental conditions (enclosure vs. open field conditions) at $p < 0.001$ (***). (b) Fruit-set (calculated as the number of seed siliques divided by the number of flowers) in open (with bumblebees) versus hand pollinated flowering stalks of *B. rapa* under enclosure vs. open field conditions. Bars are least square means $\pm$ SEM ($N=22$ under open field conditions and $N=33$ in the enclosure). Asterisks above the bars indicate significant differences between open versus hand pollination treatments within both experimental conditions (enclosure vs. open field conditions) at $p < 0.001$ (***).

of visit rates. Detailed observations of pollinator behavioural responses are needed to test this possibility.

It is important to point out that we cannot discard that floral traits other than VOC emissions were affected by aphid herbivory and contributed to observed indirect effects. We do note, however, that there was no effect of leaf damage treatment on flower number

(see Table 1), indicating that display size, an important predictor of pollinator attraction (Harder & Johnson, 2009), did not drive effects on pollinators. Complementing this, we controlled for floral display size by including flowering stalk number in the field experiment testing for leaf damage effects on pollinator visitation. In their study with *B. rapa*, Schiestl et al. (2014) additionally reported no effect of leaf chewer herbivory on traits such as floral colour and flower diameter, traits not measured in our study. We call for future work adopting integrative approaches measuring multiple floral traits, including VOCs (e.g. herbivory-induced shifts in correlated floral traits), to better explain these floral trait-mediated effects.

Collectively, results from this study provide strong evidence for indirect floral VOC-mediated effects of aphid feeding on pollinator attraction, which ultimately affect *B. rapa* reproductive success. Additional aspects to consider in future work are biochemical and molecular-level changes associated to plant defence signalling pathways underlying aphid effects on floral VOC emissions, as well as assessments of effects of simultaneous or sequential attacks by multiple plant antagonists to shed insight into plant-mediated interactions occurring in natural communities. While some plant enemies may not exert on their own an influence on floral VOCs and plant-pollinator interactions (as observed here), they could act in combination with other attackers and result in potentially important non-additive effects on floral traits and pollinators.

AUTHOR CONTRIBUTIONS

Xoaquín Moreira formulated the idea of the manuscript. Xoaquín Moreira, Elena Cartea and Luis Abdala Roberts designed the experiment. Xoaquín Moreira, Elena Cartea, Pilar Soengas and Beatriz Lago Núñez performed the experiment. Sergio Rasmann and Gregory Röder performed the chemical analyses. Xoaquín Moreira, Elena Cartea, Rieta Gols and Sergio Rasmann contributed reagents/materials/analysis tools. Xoaquín Moreira and Carla Vázquez González analysed the data. Xoaquín Moreira wrote the first draft of the manuscript. Luis Abdala Roberts, Sergio Rasmann and Rieta Gols contributed critically to the writing.

ACKNOWLEDGEMENTS

We thank Andrea Galmán, Ignacio Vicente-Díez, Lucía Martín-Cacheda, Felisa Covelo, Juan Carlos Fernández and Rosaura Abilleira for helping with field and greenhouse tasks. This research was financially supported by two grants from the Spanish Ministry of Science, Innovation and Universities (RTI2018-096591-BI00 to EC and RTI2018-099322-B-I00 to XM) and a grant from the Regional Government of Galicia (IN607A 2021/03) to XM, PS, CVG and EC. We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

CONFLICT OF INTEREST STATEMENT

We declare we have no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/1365-2745.14242>.

DATA AVAILABILITY STATEMENT

The data used in this study is archived at the Dryad Digital Repository: <https://doi.org/10.5061/dryad.rbnzs7hjh> (Moreira, et al. 2023).

ORCID

Xoaquín Moreira  <https://orcid.org/0000-0003-0166-838X>

Luis Abdala-Roberts  <https://orcid.org/0000-0003-1394-3043>

Rieta Gols  <https://orcid.org/0000-0002-6839-8225>

Sergio Rasmann  <https://orcid.org/0000-0002-3120-6226>

Gregory Röder  <https://orcid.org/0000-0002-7110-294X>

Carla Vázquez-González  <https://orcid.org/0000-0001-6810-164X>

REFERENCES

- Adler, L. S., Michaud, K. M., Ellner, S. P., McArt, S. H., Stevenson, P. C., & Irwin, R. E. (2018). Disease where you dine: Plant species and floral traits associated with pathogen transmission in bumble bees. *Ecology*, 99, 2535–2545.
- Ashman, T.-L., Knight, T. M., Steets, J. A., Amarasekare, P., Burd, M., Campbell, D. R., Dudash, M. R., Johnston, M. O., Mazer, S. J., Mitchell, R. J., Morgan, M. T., & Wilson, W. G. (2004). Pollen limitation of plant reproduction: Ecological and evolutionary causes and consequences. *Ecology*, 85, 2408–2421.
- Bagchi, R., Gallery, R. E., Gripenberg, S., Gurr, S. J., Narayan, L., Addis, C. E., Freckleton, R. P., & Lewis, O. T. (2014). Pathogens and insect herbivores drive rainforest plant diversity and composition. *Nature*, 506, 85–88.
- Biere, A., & Govers, A. (2016). Plant-mediated systemic interactions between pathogens, parasitic nematodes, and herbivores above- and belowground. *Annual Review of Phytopathology*, 54, 499–527.
- Brandoli Machado, B., Orue, J. P. M., Arruda, M. S., Santos, C. V., Sarath, D. S., Goncalves, W. N., Silva, G. G., Pistori, H., Roel, A. R., & Rodrigues-Jr, J. F. (2016). BioLeaf: A professional mobile application to measure foliar damage caused by insect herbivory. *Computers and Electronics in Agriculture*, 129, 44–55.
- Bronstein, J. L., Huxman, T. E., & Davidowitz, G. (2007). Plant-mediated effects linking herbivory and pollination. In T. Ohgushi, T. P. Craig, & P. W. Price (Eds.), *Ecological communities: Plant mediation in indirect interaction webs* (pp. 75–103). Cambridge University Press.
- Burke, L. A., & Runyon, J. B. (2016). Drought and leaf herbivory influence floral volatiles and pollinator attraction. *Global Change Biology*, 22, 1644–1654.
- Cartea, M. E., Di Bella, M. C., Velasco, P., Soengas, P., Toscano, S., & Branca, F. (2021). Evaluation of Italian and Spanish accessions of *Brassica rapa* L.: Effect of flowering earliness on fresh yield and biological value. *Agronomy*, 11, 29.
- Cartea, M. E., Francisco, M., Lema, M., Soengas, P., & Velasco, P. (2010). Resistance of cabbage (*Brassica oleracea* capitata group) crops to *Mamestra brassicae*. *Journal of Economic Entomology*, 103, 1866–1874.
- Chautá-Mellizo, A., Campbell, S. A., Argenis-Bonilla, M., Thaler, J. S., & Poveda, K. (2012). Effects of natural and artificial pollination on fruit and offspring quality. *Basic and Applied Ecology*, 13, 524–532.
- Cipollini, D., & Heil, M. (2010). Costs and benefits of induced resistance to herbivores and pathogens in plants. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, 5, 1–25.
- de Groot, P., & Poland, T. M. (2003). Attraction of *Hylastes opacus* (Coleoptera: Scolytidae) to nonanal. *The Canadian Entomologist*, 135, 309–311.
- Desurmont, G. A., von Arx, M., Turlings, T. C. J., & Schiestl, F. P. (2020). Floral odors can interfere with the foraging behavior of parasitoids searching for hosts. *Frontiers in Ecology and Evolution*, 8, 148.
- Desurmont, G. A., Xu, H., & Turlings, T. C. J. (2016). Powdery mildew suppresses herbivore-induced plant volatiles and interferes with parasitoid attraction in *Brassica rapa*. *Plant, Cell & Environment*, 39, 1920–1927.
- Fernández-Conradi, P., Jactel, H., Robin, C., Tack, A. J. M., & Castagnayrol, B. (2018). Fungi reduce preference and performance of insect herbivores on infected plants. *Ecology*, 99, 300–311.
- Finch, S., & Thompson, A. B. (1992). Pests of cruciferous crops. In B. G. McKinlay (Ed.), *Vegetable crop pests* (pp. 87–138). McMillan Press.
- Ghazoul, J. (2006). Floral diversity and the facilitation of pollination. *Journal of Ecology*, 94, 295–304.
- Groen, S. C., Jiang, S., Murphy, A. M., Cuniffe, N. J., Westwood, J. H., Davey, M. P., Bruce, T. J. A., Caulfield, J. C., Furzer, O. J., Reed, A., Robinson, S. I., Miller, E., Davis, C. N., Pickett, J. A., Whitney, H. M., Glover, B. J., Carr, J. P., & Whitfield, A. (2016). Virus infection of plants alters pollinator preference: A payback mechanism for susceptible hosts? *PLoS Pathogens*, 12, e1005790.
- Haas, S. M., & Lortie, C. J. (2020). A systematic review of the direct and indirect effects of herbivory on plant reproduction mediated by pollination. *PeerJ*, 8, e9049.
- Harder, L. D., & Johnson, S. D. (2009). Darwin's beautiful contrivances: Evolutionary and functional evidence for floral adaptation. *New Phytologist*, 183, 530–545.
- 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, 1241–1249.
- Jacobsen, D. J., & Raguso, R. A. (2018). Lingering effects of herbivory and plant defenses on pollinators. *Current Biology*, 28, R1164–R1169.
- Janzen, D. H. (1970). Herbivores and the number of tree species in tropical forests. *The American Naturalist*, 104, 501–528.
- Johnson, D. A., & Atallah, Z. (2014). Disease cycle, development and management of *Sclerotinia* stem rot of potato. *American Journal of Plant Sciences*, 5, 3717–3726.
- Jones, P. L., & Agrawal, A. A. (2017). Learning in insect pollinators and herbivores. *Annual Review of Entomology*, 62, 53–71.
- Kessler, A., Halitschke, R., & Poveda, K. (2011). Herbivory-mediated pollinator limitation: Negative impacts of induced volatiles on plant–pollinator interactions. *Ecology*, 92, 1769–1780.
- Klatt, B. K., Burmeister, C., Westphal, C., Tscharnkte, T., & von Fragstein, M. (2013). Flower volatiles, crop varieties and bee responses. *PLoS ONE*, 8, e72724.
- Lehtilä, K., & Strauss, S. Y. (1997). Leaf damage by herbivores affects attractiveness to pollinators in wild radish, *Raphanus raphanistrum*. *Oecologia*, 111, 396–403.
- Littell, R. C., Milliken, G. A., Stroup, W. W., Wolfinger, R., & Schabenberger, O. (2006). *SAS System for mixed models* (2nd ed.). SAS Institute Inc.
- Liu, Y. B., Zeng, Z. J., Barron, A. B., Ma, Y., He, Y. Z., Liu, J. F., Li, Z., Yan, W. Y., & He, X. J. (2022). The involvement of a floral scent in plant–honeybee interaction. *Naturwissenschaften*, 109, 30.
- Lohmann, D. J., Zangerl, A. R., & Berenbaum, M. R. (1996). Impact of floral herbivory by parsnip webworm (*Oecophoridae: Depressaria pastinacella* Duponchel) on pollination and fitness of wild parsnip (*Apiaceae: Pastinaca sativa* L.). *American Midland Naturalist*, 136, 407–412.
- Lucas-Barbosa, D. (2016). Integrating studies on plant–pollinator and plant–herbivore interactions. *Trends in Plant Science*, 21, 125–133.
- Lucas-Barbosa, D., Sun, P., Hakman, A., van Beek, T. A., van 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, 431–441.
- Lucas-Barbosa, D., van Loon, J. J. A., & Dicke, M. (2011). The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. *Phytochemistry*, 72, 1647–1654.
- Madloo, P., Lema, M., Francisco, M., & Soengas, P. (2019). Role of major glucosinolates in the defense of kale against *Sclerotinia sclerotiorum* and *Xanthomonas campestris* pv. *campestris*. *Phytopathology*, 109, 1246–1256.
- Maron, J. L. (1998). Insect herbivory above- and belowground: Individual and joint effects on plant fitness. *Ecology*, 79, 1281–1293.
- Moreira, X., Abdala-Roberts, L., Gols, R., Lago-Núñez, B., Rasmann, S., Röder, G., Soengas, P., Vázquez-González, C., & Cartea, M. E. (2023). Data from: Insect herbivory but not plant pathogen infection drive floral volatile-mediated indirect effects on pollinators and plant fitness in *Brassica rapa*. *Dryad Digital Repository*, <https://doi.org/10.5061/dryad.rbnzs7hj>
- Moreira, X., Abdala-Roberts, L., & Castagneyrol, B. (2018). Interactions between plant defence signaling pathways: Evidence from bioassays with insect herbivores and plant pathogens. *Journal of Ecology*, 106, 2353–2364.
- Moreira, X., Castagneyrol, B., Abdala-Roberts, L., & Traveset, A. (2019). A meta-analysis of herbivore effects on plant attractiveness to pollinators. *Ecology*, 100, e02707.
- Moreira, X., Granjel, R. R., de la Fuente, M., Fernández-Conradi, P., Pasch, V., Soengas, P., Turlings, T. C. J., Vázquez-González, C., Abdala-Roberts, L., & Rasmann, S. (2021). Apparent inhibition of induced plant volatiles by a fungal pathogen prevents airborne communication between potato plants. *Plant, Cell and Environment*, 44, 1192–1201.
- Moreira, X., Nell, C. S., Katsanis, A., Rasmann, S., & Mooney, K. A. (2018). Herbivore specificity and the chemical basis of plant-plant communication in *Baccharis salicifolia* (Asteraceae). *New Phytologist*, 220, 703–713.
- Mothershead, K., & Marquis, R. J. (2000). Fitness impacts of herbivory through indirect effects on plant-pollinator interactions in *Oenothera macrocarpa*. *Ecology*, 81, 30–40.
- Muhlemann, J. K., Klempien, A., & Dudareva, N. (2014). Floral volatiles: From biosynthesis to function. *Plant, Cell and Environment*, 37, 1936–1949.
- Ohgushi, T. (2005). Indirect interaction webs: Herbivore-induced effects through trait change in plants. *Annual Review of Ecology, Evolution, and Systematics*, 36, 81–105.
- Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens, M. H. H., & Wagner, H. (2016). *vegan: Community ecology package*. R package version 2.4-1.
- Pareja, M., Qvarfordt, E., Webster, B., Mayon, P., Pickett, J., Birkett, M., & Glinwood, R. (2012). Herbivory by a phloem-feeding insect inhibits floral volatile production. *PLoS ONE*, 7, e31971.
- Ponzio, C., Gols, R., Pieterse, C. M. J., & Dicke, M. (2013). Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens. *Functional Ecology*, 27, 587–598.
- 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.
- Ramos, S. E., & Schiestl, F. P. (2019). Rapid plant evolution driven by the interaction of pollination and herbivory. *Science*, 364, 193–196.
- Rering, C. C., Beck, J. J., Hall, G. W., McCartney, M. M., & Vannette, R. L. (2018). Nectar-inhabiting microorganisms influence nectar volatile composition and attractiveness to a generalist pollinator. *New Phytologist*, 220, 750–759.
- Rusman, Q., Karssemeijer, P. N., Lucas-Barbosa, D., & Poelman, E. H. (2019). Settling on leaves or flowers: Herbivore feeding site determines the outcome of indirect interactions between herbivores and pollinators. *Oecologia*, 191, 887–896.
- Rusman, Q., Lucas-Barbosa, D., Hassan, K., & Poelman, E. H. (2020). Plant ontogeny determines strength and associated plant fitness consequences of plant-mediated interactions between herbivores and flower visitors. *Journal of Ecology*, 108, 1046–1060.
- Rusman, Q., Lucas-Barbosa, D., & Poelman, E. H. (2018). Dealing with mutualists and antagonists: Specificity of plant-mediated interactions between herbivores and flower visitors, and consequences for plant fitness. *Functional Ecology*, 32, 1022–1035.
- 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 and Environment*, 42, 1882–1896.
- Schiestl, F. P. (2015). Ecology and evolution of floral volatile-mediated information transfer in plants. *New Phytologist*, 206, 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, 257–266.
- Song, X., & Corlett, R. T. (2022). Do natural enemies mediate conspecific distance- and density-dependence of trees? A meta-analysis of exclusion experiments. *Oikos*, 2022, e08509.
- Strauss, S. Y., Conner, J. K., & Rush, S. L. (1996). Herbivory affects floral characters and plant attractiveness to pollinators: Implications for male and female plant fitness. *The American Naturalist*, 147, 1098–1107.
- Sutherland, S., & Delph, L. F. (1984). On the importance of male fitness in plants: Patterns of fruit-set. *Ecology*, 65, 1093–1104.
- Syed, Z., & Leal, W. S. (2009). Acute olfactory response of *Culex* mosquitoes to a human- and bird-derived attractant. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 18803–18808.
- van Eck, L., Schultz, T., Leach, J. E., Scofield, S. R., Peairs, F. B., Botha, A. M., & Lapitan, N. L. V. (2010). Virus-induced gene silencing of WRKY53 and an inducible phenylalanine ammonia-lyase in wheat reduces aphid resistance. *Plant Biotechnology Journal*, 8, 1023–1032.
- Vannette, R. L., & Fukami, T. (2018). Contrasting effects of yeasts and bacteria on floral nectar traits. *Annals of Botany*, 121, 1343–1349.
- Vicente, J. G., & Holub, E. B. (2013). *Xanthomonas campestris* pv. *campestris* (cause of black rot of crucifers) in the genomic era is still a worldwide threat to brassica crops. *Molecular Plant Pathology*, 14, 2–18.
- Wang, Z., & Tan, K. (2019). Honey bee alarm pheromone mediates communication in plant-pollinator-predator interactions. *Insects*, 10, 366.
- Zhang, P.-J., Zheng, S.-J., van Loon, J. J. A., Boland, W., David, A., Mumm, R., & Dicke, M. (2009). Whiteflies interfere with indirect plant defense against spider mites in Lima bean. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 21202–21207.

How to cite this article: Moreira, X., Abdala-Roberts, L., Gols, R., Lago-Núñez, B., Rasmann, S., Röder, G., Soengas, P., Vázquez-González, C., & Cartea, M. E. (2024). Insect herbivory but not plant pathogen infection drive floral volatile-mediated indirect effects on pollinators and plant fitness in *Brassica rapa*. *Journal of Ecology*, 112, 402–415. <https://doi.org/10.1111/1365-2745.14242>