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Amending soil with insect exuviae improves herbivore tolerance, pollinator attraction and seed yield of *Brassica nigra* plants

Katherine Y. Barragán-Fonseca [a,c,*], Liana O. Greenberg [a,1], Gerrit Gort [b], Marcel Dicke [a], Joop J.A. van Loon [a]

[a] Laboratory of Entomology, Wageningen University & Research, Wageningen, the Netherlands
[b] Biometris, Wageningen University & Research, P.O. Box 16, 6700 AA Wageningen, the Netherlands
[c] Grupo en Conservación y Manejo de Vida Silvestre, Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá, Colombia, Universidad Nacional de Colombia, Bogotá, Colombia

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ABSTRACT

To promote circularity in agriculture, the residual –streams from the production of insects as feed and food, such as insect exuviae (moulted skins), can be a sustainable novel organic soil amendment for crop production. Organic soil amendments can influence soil composition by providing nutrients and stimulating the growth of beneficial microorganisms. Soil composition significantly impacts plant growth and resistance against herbivores. However, little is known about the effect of soil composition on flower visitors and seed production. Here, black soldier fly (BSF; *Hermetia illucens*) exuviae was added to soil to investigate the effects on plant growth, plant resistance against two insect herbivores, and the consequences for attraction of pollinators and seed yield. *Brassica nigra* seeds were sown in field soil or in field soil mixed with powdered BSF exuviae. Three-week-old plants and the soil of each pot were planted in a common garden. The effects on vegetative and flower traits, interactions with flower visitors and seed yield were quantified. In addition, the performance of the leaf-chewing larvae of *Pieris brassicae* and the piercing-sucking aphid *Brevicoryne brassicae* were assessed. *Brassica nigra* plants grown on soil amended with BSF exuviae showed increased growth, had more flowers and flower visitors, and higher seed production. Herbivory neither affected number of flowers nor seed production, suggesting tolerance and compensatory growth responses to herbivory compared to plants growing in soil without the addition of BSF exuviae. After five weeks fewer aphids were found on plants growing in amended soil. Our findings show that BSF exuviae added to soil can positively affect tolerance to herbivory and seed production of *B. nigra*, an insect-pollinated plant species. Moreover, adding insect exuviae as soil amendment in the field benefits plant-pollinator mutualism and seed yield even during herbivore attack in the field. Use of by-products from insect production as soil amendment can contribute to sustainable agricultural practices along with the conservation of ecosystem services.

1. Introduction

The production of insects as a high-quality source of animal proteins has received increasing attention as a viable and sustainable alternative to the traditional livestock production (Van Huis, 2021). Currently the most widely used insect produced for livestock feed is the black soldier fly (*Hermetia illucens* L.; BSF). The production of BSF generates residual streams such as insect exuviae (moulted skins) and frass (excrements). Exuviae, the outer layer of the exoskeleton shed during a moult, are rich in chitin. Chitin has potential as a plant growth enhancer through stimulation of beneficial rhizobacteria (De Tender et al., 2019; Sharp, 2013). Research on the agricultural applications and mechanisms underlying the effects of chitin as soil amendment focussed on plant protection against biotic and abiotic stresses (Malerba and Cerana, 2020). Chitin and its derivatives may enhance plant growth by increasing the density of beneficial microorganisms in the soil to the detriment of plant pathogens (Sharp, 2013). The addition of chitin into the soil also causes soil microbes to increase their production of chitinolytic enzymes that

* Corresponding author at: Laboratory of Entomology, Wageningen University & Research, Wageningen, the Netherlands.
E-mail address: katherine.barraganfonseca@wur.nl (K.Y. Barragán-Fonseca).
1 Current address: Biosystematics Group, Wageningen University & Research, Wageningen, the Netherlands.

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have a negative impact on nematodes (Sarathchandra et al., 1996). Exuviae may be used for new applications, for example, as a soil amendment in crop production, thereby representing a possible alternative to synthetic fertilisers (Nurfikari and de Boer, 2021; Barragán-Fonseca et al., 2022a, 2022b). The use of soil amendments can impact plant traits that may influence the interactions with the plant-associated community (Nurfikari and de Boer, 2021; Barragán-Fonseca et al., 2022a, 2022b).

Plants are engaged in interactions with insects (Van Dam and Heil, 2011) and microbes above and belowground. Aboveground interactions include mutualistic interactions with pollinators and antagonistic interactions with insect herbivores; belowground interactions involve, among others, microorganisms such as fungi and bacteria that can be detrimental or beneficial to the plant (Van Dam and Heil, 2011; Friman et al., 2020). Moreover, organisms associated with either the shoot or the root system may influence each other through plant-mediated interactions (Biere and Goverse, 2016; Heinen et al., 2018).

Herbivore feeding induces changes in the morphological or chemical phenotype of plants thereby affecting plant reproduction (Chrétien et al., 2018; Jacobsen and Raguso, 2018; Rusman et al., 2018). For example, herbivory induces the biosynthesis of secondary plant metabolites that defend plants against herbivores. Moreover, herbivory changes flower morphology, flower volatile emissions, and pollinator rewards, which influence pollinator behaviour (Chauta et al., 2017; Rusman et al., 2019). Furthermore, the evolution of plant traits can be affected by the interaction of the activities between pollinators and herbivores (Ramos and Schiestl, 2019). Insect herbivory has direct and indirect effects on many members of the plant-associated community and may influence the composition of the pollinator community (Rusman et al., 2018). Overall, a trade-off between plant defense and plant reproduction has been proposed (Herms and Mattson, 1992).

Plant reproduction can be affected not only by insect herbivory, but also by soil-borne microorganisms which is one possible mechanism by which chitin amendments affect plant growth. Soil amendment with insect residual streams may stimulate the activity of beneficial microbes (Barragán-Fonseca et al., 2022a, 2022b). For instance, amending soil with mealworm exuviae resulted in higher densities of Bacilli, a group of bacteria commonly used for crop protection to control insects as well as plant diseases (Bai, 2015). Beneficial bacteria, such as plant-growth-promoting rhizobacteria (PGPR), can promote plant performance by increasing plant defence against aboveground attackers through the induction of systemic resistance (ISR), which proved to be effective against a wide range of pathogens and insect pests (Gadhave et al., 2016; Pineda et al., 2017). For example, exposure of *Brassica oleracea* roots to *Bacillus* spp. reduces the infestation of the plant by cabbage aphids *Brevicoryne brassicae* L., a phloem feeder specialising on brassicaceous species (Gadhave et al., 2016). PGPR can also increase plant tolerance against attackers by improving nutrient and water uptake, enabling faster regrowth of plant biomass after herbivory (Pineda et al., 2010). PGPR can furthermore stimulate flower abundance, and consequently influence plant reproduction. For example, rhizobacteria from the genus *Bacillus* enhanced the abundance of *Tagetes erecta* flowers (Flores et al., 2007). In previous studies, flower abundance was the best predictor of the abundance of insect pollinators (Salisbury et al., 2015).

Many studies have addressed the effects of different soil amendments on plant growth and resistance against insect herbivores (Sharp, 2013; Pineda et al., 2017; Barragán-Fonseca et al., 2022a, 2022b). However, less is known about the effects of soil amendments on pollinator behaviour, on plant-mediated interactions between antagonists and mutualists and on plant yield (Barber and Soper Gorden, 2014; van Gils et al., 2016), or on the trade-off between plant defence against herbivores and pollinator-mediated reproduction. The aim of this study was to evaluate whether BSF exuviae can enhance plant growth and/or plant resistance against herbivory, and to assess the effects of soil amendment with black soldier fly exuviae on the behaviour of flower visitors. Specifically, the effects of exuviae were studied on (1) plant growth, (2) seed production (3) performance of insect herbivores and (4) herbivore-pollinator interactions.

2. Materials and methods

2.1. Plant growth conditions

Black mustard (*Brassica nigra* (L.) Koch., Brassicaceae), an annual plant native to Europe, was used as model plant. The seeds were acquired from the Centre for Genetic Resources (CGN, Wageningen, the Netherlands), with accession number CGN06619, and were propagated by open pollination. Plants were cultivated in 1 L pots placed individually in saucers in a greenhouse (22 ± 1 °C, 50 % RH, L16:D8). The greenhouse natural daylight was supplemented with 400 Watt metal halide lamps (200 μmol m^{-2} s^{-1}) when photosynthetically active radiation (PAR) dropped below 400 μmol m^{-2} s^{-1}. Plants were watered twice per week in the saucers until the top soil was moist. When the plants were four weeks old, they were transplanted to an experimental field site in Wageningen, the Netherlands (Supplementary Fig. S1).

2.2. Insect rearing

The herbivores used to infest the plants were naïve neonate larvae of the large cabbage white butterfly, *Pieris brassicae* L. (Lepidoptera: Pieridae), and wingless adult cabbage aphids, *Brevicoryne brassicae* L. (Hemiptera: Aphididae). These insect species are native to Europe and are specialist herbivores of plants in the Brassicaceae family. Insects were originally collected in Wageningen and were reared at the insect rearing facility of the Laboratory of Entomology, Wageningen University on Brussels sprouts plants (*Brassica oleracea* variety *gemmifera* cultivar Cyrus) in greenhouse conditions (22 ± 1 °C, 50–70 % RH, L16:D8).

2.3. Experimental design

Brassica nigra plants were randomly divided into four groups of fifty, each assigned to receive one of the four treatments in a two-way factorial design with factors soil amendment (with two levels: control (C) and soil amendment with BSF exuviae (A)) and herbivory (with two levels: control (H) and soil amendment with BSF exuviae (AH)).

2.3.1. Soil amendment

To test whether flowering is affected by a chitin-based soil amendment, a common garden field experiment was set up in which *B. nigra* plants sown and grown in amended soil were compared to plants sown and grown in soil without amendment as a control. Soil was amended by mixing two grams of powdered BSF exuviae per kg of soil. The soil amendment was derived from the exuviae of black soldier fly (*Hermetia illucens* L.: Diptera, Stratiomyidae). BSF exuviae were acquired from a commercial mass rearing of these insects (Protix Biosystems BV, Dongen, the Netherlands), and used in powdered form following a heat treatment of 60 °C for 24 h. Plants grown in field soil amended with BSF exuviae are labelled with (A). Soil was collected from the experimental fields of Wageningen University and sieved to remove pebbles. To mix the soil and 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 plants (C), except that no powdered exuviae was added (Supplementary Fig. S1). Seeds were then sown in 1 L pots of amended (100 plants) and unamended soil (100 plants).

Brassica nigra seeds were sown in a greenhouse on six staggered dates, with 3–4 days in between, so that flowering in the field would occur gradually over an extended period and a sufficient number of pollinator observations could be conducted.

2.3.2. Herbivory

The effect of a combined infestation with *P. brassicae* caterpillars and *B. brassicae* aphids on plants growing in amended or unamended soil was investigated. One day after transplanting to the field, 25 days after sowing, five *P. brassicae* neonate caterpillars and 10 wingless *B. brassicae* adult aphids of similar size were placed on half of the plants on amended soil (50 plants; AH) and on half of the control plants (50 plants; H) (Supplementary Fig. S1). Caterpillars and aphids were placed with a fine-haired brush on one of the two youngest leaves that had fully unfolded. The insects were checked after 24 h, and if there were fewer than three aphids or three caterpillars remaining, these plants were reinfested to carry five caterpillars and 10 aphids. The herbivores remained on the plant feeding freely for the rest of the season. Plants that were experimentally infested with herbivores are referred to as infested plants (treatments H and AH) in the following.

2.3.3. Common garden setup

After three weeks of growth in the greenhouse compartment, the plants were moved to an outdoor mesh tent to acclimatise to the natural weather conditions while being protected from herbivores. Four-week-old plants were planted in a common garden field design in the recently tilled experimental fields of Wageningen University. Soil composition was 81 % sand, 14 % silt and 2 % clay, the soil organic matter content was 3.2 % with a nitrogen delivery capacity of 80 kg/ha, as determined by Eurofins Agro (Wageningen, the Netherlands) in 2018. Plants were placed into 1 L holes along with the soil contents of the pot in which they were growing; the pot itself was removed. The plants were placed in a 32 m x 20 m field surrounded by a flowering edge of *B. nigra*. The field was split into two blocks. Each of the two blocks was split further into six main plots. In block I each main plot consisted of 5 rows, each containing 4 randomised planting positions so that every row had all four treatments, whereas in Block II a main plot consisted of 4 rows or 3 rows of 4 randomised planting positions. Sixty-eight three-week-old seedlings were planted on three different planting dates. Planting dates were randomised over the main plots within blocks, and treatments over planting positions within rows of the main plots (Supplementary Fig. S2).

2.3.4. Plant growth

To assess whether soil amendment and herbivory influenced plant growth we measured three plant traits: plant height, plant width and leaf length. All traits were measured at two time points: 3 and 7 weeks after sowing, except for number of flowers which was measured once, 7–9 days after the first flower opened. Plant size was measured and used as a proxy for biomass to assess the growth of the plants in a non-destructive way. Plant size and biomass are strongly correlated in many plant species (Veley et al., 2017). We measured initial stem height from the soil surface to the highest point of the plant, at the time of planting in the field. Vegetative height from the soil surface to the tip of the highest leaf was measured. The width of the plant at its widest point (leaf tip to leaf tip) and maximum leaf length were recorded, measured as the length of the longest leaf from the base of the lamina to the leaf tip.

2.3.5. Flowering status

Every two days, the flowering stage of the plant was recorded, noting the time at which the first flower buds were visible, the start and end of anthesis, and the time at which siliques were fully ripe and ready for harvest. We calculated the number of days between sowing and first flower bud, first open flower, and end of flowering as well as how long open flowers were present. We counted the number of flowers per plant and measured the height of the inflorescence from the base of the plant immediately prior to the pollinator observation.

2.3.6. Seed production

To investigate whether soil amendment and herbivore treatment affected seed production, siliques were harvested when they became fully dry and brown but had not yet dehisced. Immature siliques and flowers were left on the plants and daily checked for ripeness. Siliques and seeds were stored in paper bags in a dry storage room (20 °C) after which the seeds were separated from siliques and cleaned. An electronic seed counting machine (Contador, Pfeuffer GmbH, Germany) was used to count the total number of seeds.

2.3.7. Herbivore abundance

The effect of soil amendment on herbivore survival and development was investigated as a proxy of plant resistance to herbivores. The number of caterpillars and aphids was recorded after one week, nine days, two weeks, three weeks, and one month since the onset of infestation. Caterpillars and aphids fed freely on the plants and, therefore, could disperse to neighbouring plants.

2.3.8. Pollinator activity

To investigate whether soil amendment altered pollinator attraction to the plants or their behaviour while visiting flowers, pollinator visitation was recorded to flowers of *B. nigra* in the four treatment groups, following the methods described by Rusman et al. (2019). Pollinator visitation was recorded within 7–9 days after the first flower had fully opened on the plant. Each plant was monitored for a 15-min period. Pollinator activity was recorded using a handheld computer (Psion Workabout Pro™ 3, London, UK) programmed with The Observer XT software (version 10, Noldus Information Technology, Wageningen, the Netherlands). When a pollinator made contact with a flower, the identity of the pollinator was recorded as one of the following: honey bee (*Apis mellifera*), bumble bee (*Bombus* spp.), syrphid fly (Syrphidae), solitary bee (all Apidae excluding *A. mellifera* and *Bombus* spp.), other flies (other Diptera than Syrphidae). Bumble bees and solitary bees were infrequent visitors and were only included in the analysis of all pollinators combined. If other pollinators visited a plant during the observation of a particular pollinator, only the identity of those other pollinators was recorded. If the same pollinator individual returned to the observed plant, after having visited a different plant, it was scored as a new visit. Observations were carried out when weather conditions were suitable for pollinator activity (15–30 °C – wind speed < 6 m.s^{-1}).

2.4. Statistical analysis

To investigate whether BSF exuviae influenced both herbivore abundance and attraction of flower visitors, several analyses were performed. For plant growth analysis Mixed Linear Models (MLM) were used using fixed effects for treatments and blocks, and random effects for main plots within blocks, for rows within main plots and residual error. Treatment effects were split into main effects for soil amendment and herbivory, and their interaction, using ANOVA with the Kenward-Roger method for calculating degrees of freedom. Pairwise comparisons among treatments were done using P-value adjustment according to the Tukey method. For number of flowers and number of seeds, Generalized linear mixed models (GLMMs) were used with a log link function and a negative binomial distribution to handle the overdispersion of the counts. Focussing on the plants infested by herbivores, the number of aphids over time were analysed using a GLMM with negative binomial distribution (with time-dependent dispersion parameter) and log link function, with extra fixed effects of time and time by treatment interaction and extra random effects for plants. For the number of caterpillars retrieved from the five individuals placed on a plant at four weeks the same GLMM was used, but assuming a beta-binomial distribution with logit link function. Comparisons of treatments per timepoint were extracted from the overall analyses. As the scored count of pollinators visiting the plant is necessarily larger than zero, a zero-truncated distribution was used for the negative binomial distribution. Counts of visiting individual pollinator species were analysed with a GLMM with negative binomial distribution and log link. To test whether the total number of visits is related to flower characteristics the truncated negative binomial distribution was used. Statistical analyses were done using the emmeans (Russell, 2018), glmmTMB (Brooks et al., 2017), lme4 (Bates et al., 2015) and car (Fox and Weisberg, 2019) packages in R version 3.6.1 (R Core Team, 2019). For all tests 0.05 was used as level of significance.

3. Results

3.1. Effects of BSF exuviae on vegetative plant growth in the absence and presence of herbivory

Growth of *B. nigra* plants was affected by soil amendment with BSF exuviae at all time points, except for plant height after seven weeks (Fig. 1). Three-week-old plants grown on soil amended with BSF exuviae were significantly taller than plants grown on field soil alone ($F = 11.63$; df = 1, 147.6; $p < 0.001$). At both timepoints, BSF exuviae significantly affected plant width (3 weeks: $F = 48.94$; df = 1, 121.7; $p < 0.001$; 7 weeks: $F = 20.64$; df = 1, 138.1; $p < 0.001$) and maximum leaf length (3 weeks: $F = 42.82$; df = 1, 121.69; $p < 0.001$; 7 weeks: $F = 20.67$; df = 1, 138.8; $p < 0.001$). After three weeks, plants grown on amended soil had significantly larger plant width and leaf length than control plants irrespective of herbivory. A main effect of herbivory was observed for plant width ($F = 9.41$; df = 1, 149.7; $p = 0.003$) and maximum leaf length ($F = 9.61$; df = 1, 149.2; $p = 0.002$) after seven weeks. There was no significant interaction between soil amendment and herbivory for the growth measurements.

3.2. Effects of BSF exuviae on plant seed production

Soil amendment significantly affected plant reproduction. Plants grown in soil amended with BSF exuviae produced more seeds than plants without soil amendment (Chi-square $X^2 = 10.78$; df = 1; $p = 0.001$; Fig. 2). This effect was independent of herbivory (Amendment * Herbivory: $X^2 = 0.44$; df = 1; $p = 0.51$), and herbivory did not significantly affect plant reproduction ($X^2 = 1.97$; df = 1; $p = 0.16$).

3.3. Effects of BSF exuviae on herbivore performance

No significant differences between numbers of aphids on plants grown on amended soil and control plants were found in the first four time points. After five weeks a significantly larger aphid number was found on control plants ($t = 2.08$; df = 479; $p = 0.038$; Fig. 3a). For none of the five timepoints a significant difference in the numbers of caterpillars on *B. nigra* plants between the two treatments was found (Fig. 3b).

3.4. Effects of BSF exuviae on flowering and pollinator attraction

Plants grown in soil amended with BSF exuviae produced significantly more flowers than plants without soil amendment ($X^2 = 10.37$; df = 1; $p = 0.001$, main effect of soil amendment; Fig. 4a). Flowers of plants grown on amended soil were visited significantly more frequently by insect pollinators compared to flowers of control plants ($X^2 = 12.67$; df = 1; $p < 0.001$; Fig. 4b). Effect of soil amendment on number of flowers and number of insects visiting the plant was independent of herbivory. A generalized linear model, explaining the number of visiting pollinators by the number of flowers and height of the inflorescence, showed a strong positive relationship ($X^2 = 30.53$; df = 1; $p < 0.0001$) between the number of flowers and the number of insect pollinators (Fig. 3c). The most common pollinators visiting the plants were syrphid flies (62.9 %) followed by honey bees (20.6 %) and other flies (13.8 %). All other visitors were relatively rare with solitary bees contributing 1.7 % and bumble bees 1.1 % (Supplementary Fig. S3). Syrphid flies visited flowers of plants grown on amended soil significantly more often than flowers of plants grown on control soil ($X^2 = 7.82$; df = 1; $p = 0.005$).

4. Discussion

Our study is the first to provide insights into the influence of amending soil with insect-derived material on plant growth, pollinator attraction and seed production. This research shows that amending soil with BSF exuviae results in enhanced plant size and seed yield of *B. nigra* plants. Plants grown in soil amended with BSF exuviae were larger, had more flowers, were visited more frequently by insect pollinators and produced more seeds than those without this soil amendment. When infested, plants grown in amended soil were better able to compensate for tissue or assimilate loss and resisted the attack better, not only maintaining their larger size, but also their enhanced seed production. The latter is explained by enhanced flowering and higher attractiveness to pollinators. Together, these results show that soil amendment with powdered BSF exuviae increases tolerance to attack by *P. brassicae* and *B. brassicae.*

4.1. Soil amended with BSF exuviae can positively affect plant tolerance to herbivory

Plants grown in amended soil had an increased plant width, greater maximum leaf length both with and without herbivores, but only increased height in the absence of herbivores (Fig. 1). Herbivory by *P. brassicae* early in *B. nigra*'s development has been shown to reduce the height of this species (Blatt et al., 2008), even when other growth

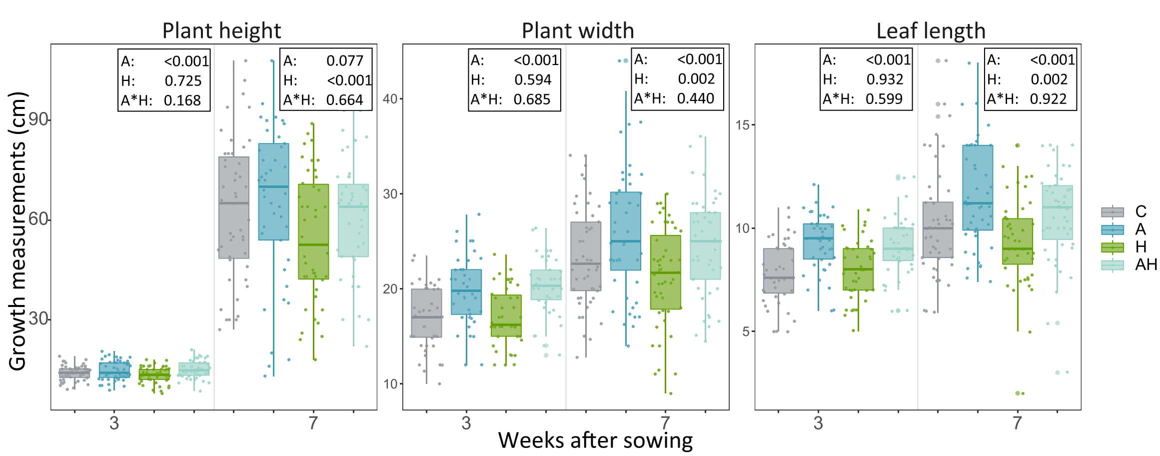

Fig. 1. Growth measurements of *Brassica nigra* plants with or without BSF exuviae and/or herbivory, grown in the field and assessed at different time points. Treatments: C: control plants grown in field soil; A: plants grown in field soil amended with BSF exuviae; H: plants sown in field soil and infested with herbivores; AH: plants grown in field soil amended with BSF exuviae and infested with herbivores. All analyses were performed using a linear mixed model.

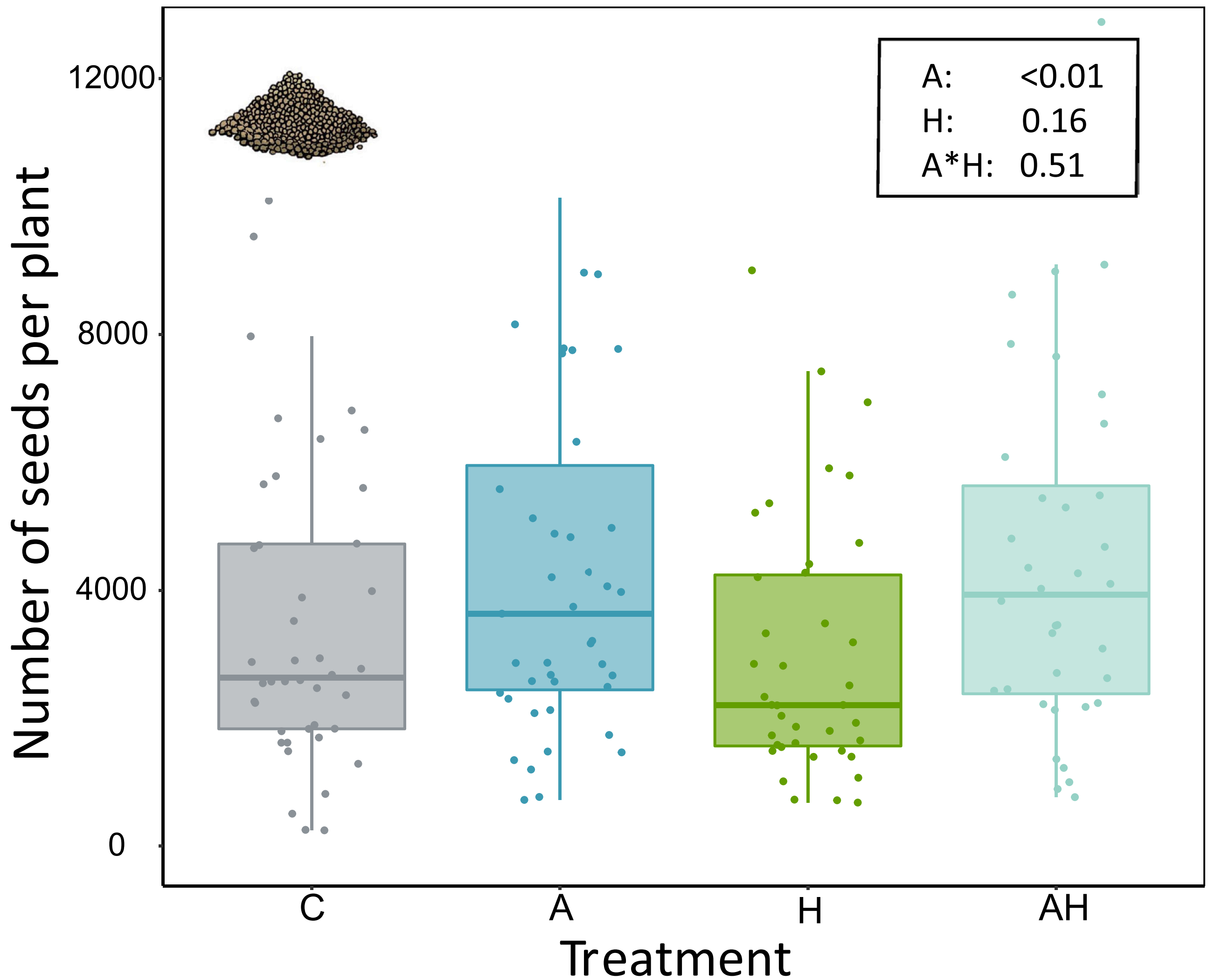

Fig. 2. Effects of BSF exuviae on seed set of *Brassica nigra*. Treatments: C: control plants grown in field soil; A: plants grown in field soil amended with BSF exuviae; H: plants sown in field soil and infested with herbivores; AH: plants grown in field soil amended with BSF exuviae and infested with herbivores.

variables showed compensation. These results indicate a positive effect of the soil amendment on *B. nigra*'s ability to tolerate herbivory, perhaps due to compensation for lost biomass (Blatt et al., 2008). Organic soil amendments, such as manure, have been shown to increase tolerance to herbivory (Allee and Davis, 1996; Altieri and Nicholls, 2003).

In the field, survival of caterpillars was not significantly affected by the amendment. However, the number of aphids was reduced after four weeks (Fig. 2a). Soil amendments can impact herbivore resistance aboveground (Pineda et al., 2017). Changes in nutrient availability (Glynn et al., 2003; Katjiua and Ward, 2006). and microbial composition of the soil (Sarathchandra et al., 1996; Kielak et al., 2013; Sharp, 2013) can enhance resistance against herbivory. Because the soil amendment adds chitin, stimulating microbial growth including that of PGPR, chitinolytic bacteria, and microbial chitinase production, Induced Systemic Resistance (ISR) and PAMP-triggered immunity may be mechanisms for improved herbivore resistance in plants growing in amended soil (Sharp, 2013; Rowen et al., 2019; Barragán-Fonseca et al., 2022a, 2022b). As their name indicates, PGPR promote plant growth (Barragán-Fonseca et al., 2022a, 2022b). This group of bacteria may also prepare plants for enhanced defence against aboveground attackers by the systemic response to root colonisation by these microorganisms (Hol et al., 2010; Pineda et al., 2010; Pieterse et al., 2014) A broad variety of root-associated beneficial microorganisms, including *Bacillus*, *Pseudomonas*, *Trichoderma* and mycorrhiza species have been shown to enable more effective responses to biotic and abiotic stresses via physiological changes in the plant (Pieterse et al., 2014). Furthermore, soil amended with the rhizobacterium *Pseudomonas simiae* or with several *Bacillus* species, have shown to modify the blend of herbivore-induced plant volatiles (HIPV) in brassicaceous species, consequently increasing the recruitment of parasitoids of aphids and caterpillars (Pangesti et al., 2015; Gadhave et al., 2016) Aphid numbers declined after five weeks on plants growing in amended soil. This may have been due to ISR or due to enhanced attraction of natural enemies of aphids (Barragán-Fonseca et al., 2022a, 2022b), but the later was not investigated. How the addition of BSF exuviae affects the soil microbial community associated to the rhizosphere of *B. nigra* plants remains to be determined.

The number of caterpillars declined rapidly which may have been due to predation. However, the number of caterpillars was not affected by the amendment which could be explained by *P. brassicae* being a specialist herbivore. The effectiveness of ISR and resistance priming against insects differs according to feeding guild and host specialisation of the insect, but also on bacterial strains and plant genotype (Pineda et al., 2017; Shikano et al., 2017). Investigating the relative effects of exuviae treatment on specialists versus generalists would be an interesting additional component of a future study.

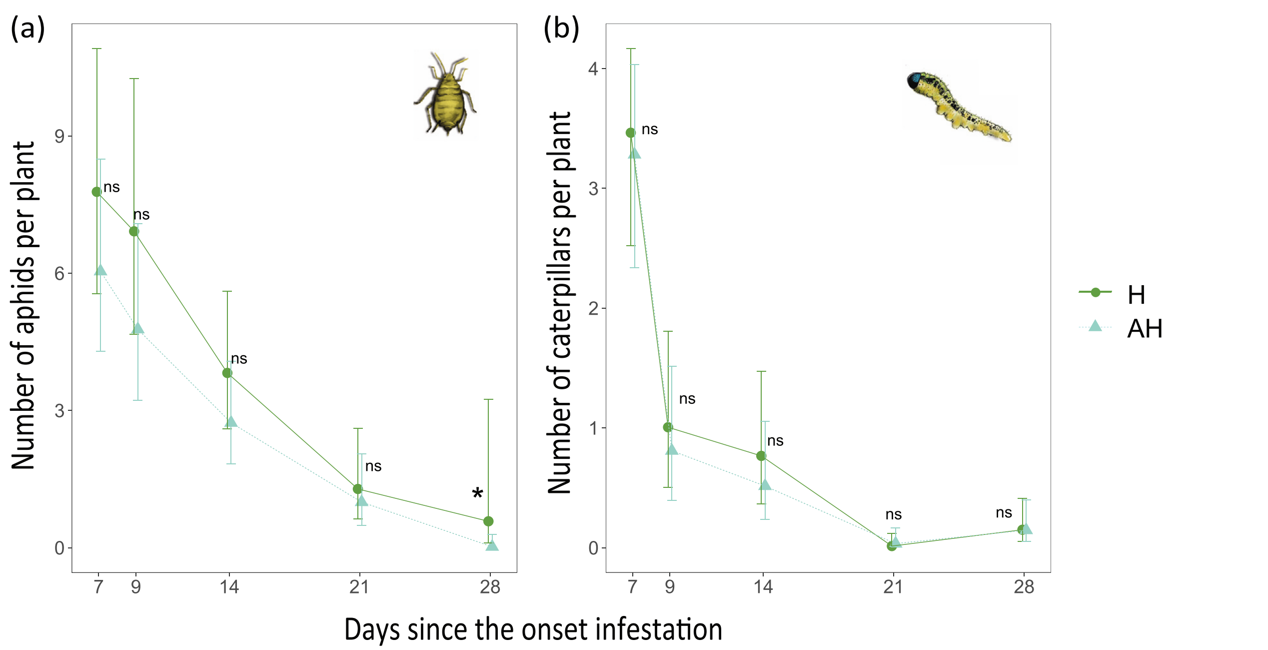

Fig. 3. Number of *Brevicoryne brassicae* (a) and *Pieris brassicae* (b) on *Brassica nigra* plants grown on control soil or on soil amended with BSF exuviae at different time points in the field. Values along the y-axis indicate estimated median number of insect herbivores per plant with 95 % confidence limits; the x-axis indicates the number of days after initial infestation when herbivores were counted. Asterisk (*) indicates a significant difference ($p < 0.05$) between treatments. Plants were sown in field soil and infested with herbivores (H) or in field soil amended with insect exuviae and infested with herbivores (AH).

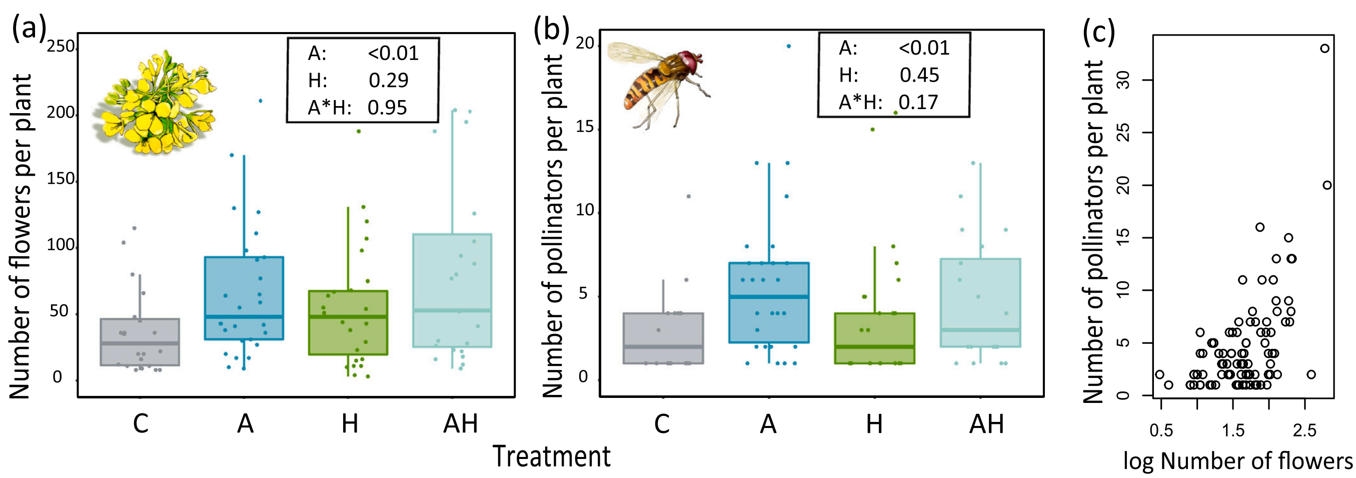

Fig. 4. Effects of BSF exuviae on flowering *Brassica nigra* plants: (a) number of flowers and (b) number of pollinators visiting per 15 min. (c) Relation between the number of visits by pollinators and the number of flowers per plant. Treatments: C: control plants grown in field soil; A: plants grown in field soil amended with BSF exuviae; H: plants sown in field soil and infested with herbivores; AH: plants grown in field soil amended with BSF exuviae and infested with herbivores.

4.2. *Plants grown in amended soil had more flowers and attracted more pollinators*

The number of flowers was higher in plants growing in amended soil than in plants growing in control soil. These results suggest that plants growing in amended soil were able to invest more resources in reproductive tissues. BSF exuviae have a chitin content of 10.9–11.1 % (Nurfikari and de Boer, 2021). An increase in flower biomass can be the result of an active degradation of chitin by chitinase-producing bacteria (De Tender et al., 2019). The use of chitin has been shown to alter rhizosphere microbial composition, stimulating chitinolytic populations able to degrade chitin and release nutrients for both plants and the soil microbiome (Debode et al., 2016). PGPR in the soil act as biofertilizers by making nutrients such as phosphorous and nitrogen available for plants (Vishwakarma et al., 2020). In addition, three-week-old plants grown in amended soil also had significantly increased inflorescence height. Insect exuviae also contain carbohydrates, proteins and lipids that may stimulate the growth of PGPR (Barragán-Fonseca et al., 2022a, 2022b). Changes in the soil microbiome may improve the availability of resources for increasing plant biomass and can increase the absorption of water and nutrients by influencing root architecture (Trivedi et al., 2020).

Pollinators made more visits to plants growing in amended soil with and without herbivores, and during these visits, made contact with more flowers per visit than they did on control plants. The duration of visits and time spent per flower were not significantly affected by any of the treatments. Furthermore, when accounting for the increased number of flowers on amended plants, the number of flowers visited in relation to the number of available open flowers was not affected by treatment. This suggests that pollinators were responding to the increase in number of flowers. This is supported by the strong positive correlation between the number of pollinator visits and the number of flowers per plant. Larger floral displays are more attractive to pollinators (Arroyo et al., 2007) and more flowers on larger amended plants would suggest that floral advertisement is more readily perceived by pollinators (Majetic et al., 2017). Visual cues are integral to pollinator attraction, and in *B. nigra*, visual cues are more important than olfactory cues for long distance attraction of *Episyrphus balteatus* L. (Diptera: Syrphidae) (Barragán-Fonseca et al., 2020a, 2020b). In addition, soil amended with exuviae may influence the olfactory cues exploited by pollinators by altering the volatile blend emitted by the plants in comparison to the control (Barragán-Fonseca et al., 2022a, 2022b). Exploring how other floral traits such as shape, colour or emitted volatiles are altered by amending soil with insect exuviae will allow predictions of pollinator foraging preference since this can vary from one taxonomic group to another depending on the reward they are interested in (Barragán-Fonseca et al., 2020a, 2020b).

Herbivory treatment did not cause a significant increase in the number of flowers, nor in the number of visits of insect pollinators. These results support an increase in herbivore tolerance because herbivore-infested plants growing in amended soil, despite losing height, compensated by investing in an increased density of flowers, and thus remained as attractive to pollinators as amended plants without herbivory. Contrary to expectations based on previous results on effects of herbivory on plant-pollinator interactions (Rusman et al., 2018), no significant shifts in the pollinator community were observed. Rather, the proportion of visitation contributed by each pollinator group was similar between treatments. Interestingly, syrphid flies visited plants amended with BSF exuviae significantly more often than plants without insect exuviae. While some pollinators rely on visual cues to approach the plant from a distance, they also rely on odour to discriminate between flowers once near the plant in combination with visual cues (Barragán-Fonseca et al., 2020a, 2020b). Therefore, syrphid flies may perceive the effects of amended soil on certain flower traits. Exploring the effects of soil amendments on different members of the pollinator community will be an important next step. Our data suggest that exuviae are suitable to be used as a biofertilizer because no negative impact on pollinator attraction has been found.

4.3. Seed production can be enhanced by amending soil with BSF exuviae

The number of seeds produced by amended plants was higher than that of control plants and this effect was independent of herbivory. In previous studies, infestation by *B. brassicae* and *P. brassicae* in the vegetative stage has been found to reduce seed set of *B. nigra* (Rusman et al., 2019) and seed set was not affected when the plant was sufficiently able to compensate for herbivory (Blatt et al., 2008; Rusman et al., 2018), provided that plants are grown in high nutrient conditions (Meyer, 2000).

Our data show that seed production can be increased by amending soil with insect exuviae. This likely results from a greater number of insect pollinators visiting the plants grown in amended soil. These effects on pollinator behaviour increase cross-pollination and thereby benefit plant fitness (Klinkhamer et al., 1994). In order to reduce synthetic fertiliser application, plant fitness can be reliably enhanced by stimulating plant-associated microbiomes that benefit plants through growth promotion, nutrient uptake, stress tolerance and resistance to pathogens (Trivedi et al., 2020; Barragán-Fonseca et al., 2022a, 2022b). Most of the studies on belowground beneficial organisms have investigated the interaction of PGPR with vegetative plants. However, flowering plants have been neglected in this research thus far, in spite of the fact that quantifying plant reproduction is required to assess how successful induced defences are. Our data relate to a single field season. To what extent weather and other seasonal characteristics influence variation in the effects of soil amendment with insect residual streams on flowering plants remains to be investigated. Nevertheless, the reported evidence is important because to our knowledge there are no studies available about the effect of beneficial microbes or soil amendment with residual streams of insect production on pollinator performance and plant fitness.

5. Conclusions

Our study shows that BSF exuviae added to soil as an organic amendment enhance herbivore tolerance and seed yield of *B. nigra*, a plant species that relies on insect pollinators for reproduction. The use of insect exuviae as soil amendment benefits plant-pollinator mutualism and plant fitness even during herbivore attack in the field. Likewise, the use of insect exuviae as soil amendment may stimulate beneficial rhizobacteria that potentially play an important role in pollination-related plant traits, consequently positively affecting plant reproduction. This highlights the importance of exploring organic soil amendments as alternative to conventional synthetic fertilisers that have shown to negatively impact plant health and plant mutualists (Human-n-Guilleminot et al., 2019). Residual streams from insect production that contain chitin may be used as soil amendment, stimulating beneficial rhizobacteria that cause changes in plant traits that are exploited by insects interacting with the plant. Here, for the first time, experimental evidence is presented on how the phenotype of flowering plants is influenced by the use of insect exuviae as soil amendments, and how the subsequent changes in plant phenotype affect interactions with insect herbivores and pollinators. Future studies should investigate the underlying mechanisms such as the effect of soil amendment with exuviae on the soil microbiome associated with plant roots and on the interaction of the plants with natural enemies of insect herbivores, e.g. by affecting gene expression and the production of herbivore-induced plant volatiles in response to herbivory. Farming practices need to consider methods that protect and optimise the ecosystem services provided by insect pollinators (Chen et al., 2022). The use of residual streams from insect production can contribute to developing sustainable and circular crop production systems along with the conservation of ecosystem services, and deserves more attention in future research.

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CRediT authorship contribution statement

K.Y.B-F., G.G., M.D. and **J.J.A.v.L.** designed the study; **K.Y.B-F.** and **L.O.G.** collected the data; **G.G.** and **K.Y.B-F.** conducted the statistical analyses; **K.Y.B-F., M.D.** and **J.J.A.v.L.** interpreted the results and wrote the manuscript and all authors commented on and approved the final version.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: K. Y. BARRAGAN-FONSECA reports financial support was provided by National Department of Science, Technology and Innovation of Colombia. K.Y. BARRAGAN-FONSECA reports a relationship with National Department of Science, Technology and Innovation of Colombia that includes: funding grants.

Data availability

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2022.108219.

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