Soil amendment with black soldier fly frass and exuviae to control the cabbage root fly Max Wantulla 2023

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Propositions

1. Soil amendment with insect frass or exuviae can only provide reliable crop protection through co-application of microbial agents.
(this thesis)

2. Searching for new microbial pest control agents among isolates from non-target organisms is illogical and inefficient.
(this thesis)

3. Competition in science increases the quantity and reduces the quality of research output.

4. As modern scientists advance knowledge in their field, they become unable to pursue the scientific ideal of polymathy.

5. Deintensification of agriculture serves the purpose of preserving cultural landscapes but hinders nature conservation.

6. Cultivating friendships outside of the academic bubble is essential for understanding society.

Propositions belonging to the thesis, entitled

Soil amendment with black soldier fly frass and exuviae to control the cabbage root fly

Max Wantulla
Wageningen, 26 June 2023

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This research was conducted under the auspices of the Graduate School Experimental Plant Sciences.

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 26 June 2023
at 11 a.m. in the Omnia Auditorium.

Max Wantulla
Soil amendment with black soldier fly frass and exuviae to control the cabbage root fly
154 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2023)
With references, with summaries in English, German and Dutch

ISBN: 978-94-6447-651-4
DOI: https://doi.org/10.18174/590856

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Chapter 1

General introduction

Belowground pests as a challenge for crop production

Pests represent the main limiting factor for crop production and have been estimated to cause global potential losses of 50% to more than 80% (Oerke, 2006). Soil-dwelling pests that feed on plant roots are among the most devastating and challenging to manage, not least due to their cryptic lifestyles and persistence (Johnson et al., 2016). Plants in general are much less tolerant of root herbivory than shoot herbivory and damage to root crops can be particularly severe. The acute damage caused by root-feeding insects can have various consequences, such as facilitated pathogen invasion, reduced water and nutrient uptake or inadequate anchorage of the plant (Herbst et al., 2017; Shuhang et al., 2016; Spencer et al., 2009). By exacerbating other biotic and abiotic plant stresses, these factors often lead to secondary damage.

The control of root-feeding pests is complicated by the fact that they are hidden in the soil or within the root tissue, where they cannot easily be reached with pesticides. Therefore, effective chemical control often relies on systemic active ingredients and usually involves seed, granule or soil drench treatments (Collier et al., 2020; Spencer et al., 2009). As it is especially difficult to notice and react to soil pest infestations on time, prophylactic pesticide applications are common practice (Johnson et al., 2016). The consequent extensive use of insecticides without consideration of economic thresholds has repeatedly resulted in the development of resistance in important root-feeding insect pests (Gray et al., 2009; Myrand et al., 2015). Furthermore, legal restrictions are limiting the availability of effective pesticides, with the ban of neonicotinoids in the European Union being a notable instance. In the light of diminishing numbers of available control options, the need for new strategies for the management of soil pests has often been stressed (Collier et al., 2020; Johnson et al., 2016).

The role of soil microbes in plant protection

Various bacteria and fungi with the capacity to protect plants, including plant mutualists, entomopathogens or antagonists of plant pathogens, can be found in the soil (Cawoy et al., 2011; Jaronski, 2007; Kupferschmied et al., 2013). These microbes are generally able to colonize plant roots or insects and control herbivores or root diseases via the production of toxic or inhibitory secondary metabolites. Root colonizers known as plant growth-promoting rhizobacteria or fungi can moreover induce plant resistance to pathogens and insects (Pieterse et al., 2014; Pineda et al., 2010). In naturally disease-suppressive soils, plant-mediated effects and direct antagonistic activity of the resident microbial community are both thought to contribute to the suppression of plant pathogens (Pieterse et al., 2014;

Schlatter et al., 2017). Soil microbes with a range of biocontrol properties have repeatedly been isolated and represent promising alternatives to conventional pesticides. The variety of mechanisms by which these organisms can provide crop protection offers great potential advantages, e.g., in mitigating the development of pest resistance (Cawoy et al., 2011).

A considerable number of microbial strains are commercialized as so-called biopesticides or soil inoculants, with bacterial agents making up the majority of the available products (Cawoy et al., 2011; Kupferschmied et al., 2013). The activity of these microbes depends heavily on both biotic and abiotic factors in the local environment. While foliar applications are not uncommon, soil is typically considered to provide more favorable conditions, i.e., moderate temperatures, optimal moisture and protection from UV radiation (Jaronski, 2007; Kupferschmied et al., 2013). Nonetheless, introduced microbial agents commonly fail to persist through the desired functional period. As a result of competitive interactions with endemic soil microbes, microbial cell densities often decline rapidly soon after introduction into the soil (Kaminsky et al., 2019; Mazzola & Freilich, 2017). Whereas applications to plant surfaces may be repeated, soil applications are usually only practicable preceding or during planting. In view of these limitations to the use of non-native microbial strains, the management of naturally occurring microbes with biocontrol potential has been proposed as an alternative strategy. By amending soil with organic substrates, elements of the native microbiome that are functional in crop protection can be stimulated (Mazzola & Freilich, 2017).

By-products of insect production as soil amendments

The practice of farming insects for human consumption or animal feed has recently developed into a rapidly growing industry. Many companies are now producing insects, especially as feed for livestock and fish farming or as pet food (van Huis, 2021). In the light of ongoing legislative reforms, such as the recent approval of insects in poultry and pig feed by the European Union, the insect production sector is expected to continue expanding (European Union, 2021a). Various insects are able to transform organic residual streams into high-quality animal protein and can convert their feed into body mass much more efficiently than cattle and pigs (Dicke, 2018). Nonetheless, they also generate residual streams themselves, namely feces and exuviae (molted exoskeletons). The mixture of these two products in combination with unconsumed feed, commonly referred to as ‘frass’, is produced in large quantities during insect farming.

Applications of insect frass for soil fertilization have been widely investigated (Fuhrmann et al., 2022; Houben et al., 2020; Poveda et al., 2019; Watson et al., 2021). In

general, observed fertilization effects are not only related to direct nutrient supply but also to the stimulation of soil microbial activity. Similarly, soil amendment with insect exuviae and frass has great potential for stimulating microbes that can contribute to crop protection. However, studies investigating the capacity of such amendments to control plant pests are still largely lacking. Research on the effects of soil amendment with insect residual streams on specific pest organisms as well as on the resident microbes involved is clearly needed.

Objective and research questions

The main objective of the research presented in this thesis was to develop new methods for the microbial control of an important root-feeding insect pest. For this purpose, soil amendment with insect residual streams was used as a means to recruit naturally occurring soil bacteria with biocontrol potential. The following research questions were addressed:

1. How does soil amendment with residual streams derived from different insect species affect the performance of a root-feeding insect?

2. Do residual streams derived from different insect species differentially affect bacterial communities in the plant rhizosphere?

3. Does amendment with an insect residual stream have the same effects on a root-feeding insect in different types of soil?

4. Do bacteria isolated from a root-feeding insect after exposure to soil amended with an insect residual stream have insecticidal activity?

Study system

Many plants in the family Brassicaceae, also known as Cruciferae, are economically important crops. In terms of global production, cabbages (*Brassica oleracea* L.) are the main vegetable species produced after tomatoes, onions and cucumbers, while rapeseed (*Brassica napus* L.) is the main source of vegetable oil after oil palm fruit and soybean (FAO, 2022). In the research presented here, *B. oleracea* L. var. *gemmifera* cv. Cyrus (Brussels sprouts) was used for greenhouse experiments. For the most part, experiments were conducted using soil from a field on which different brassicaceous plant species had been grown for many years.

The insect herbivore that is the focus of this thesis is the cabbage root fly or cabbage maggot, *Delia radicum* L. (Diptera: Anthomyiidae). It is a major pest of various brassicaceous crops, primarily in Europe and North America (Dosdall et al., 2000; Herbst et al., 2017; Joseph & Iudice, 2020; Shuhang et al., 2016). In these regions, it has been estimated to cause annual economic losses of up to $100 million. Adult *D. radicum* flies feed on flower nectar and females oviposit on the soil surface close to the stem base of the host plant. The eggs are ca. 1 mm in diameter and hatch within 4-6 days (Santolamazza-Carbone et al., 2017). Larvae feed on root tissue and on the lower stem, which may lead to symptoms such as yellowing of the foliage, stunting or wilting. After ca. 3 weeks of feeding, they pupate in the soil surrounding the roots (Joseph & Iudice, 2020). Finally, the second generation of flies emerges within 2-4 weeks after pupation. Currently, there are no reliable options to control *D. radicum* in several countries of the European Union (Collier et al., 2020; Herbst et al., 2017).

Insect frass as well as separated exuviae were used as soil amendments to investigate potential effects on *D. radicum* or soil microbes. The different residual streams were derived from black soldier fly larvae, *Hermetia illucens* L., house crickets, *Acheta domesticus* L., or yellow mealworms, *Tenebrio molitor* L., all of which are important species for the commercial production of insects (van Huis, 2021).

Thesis outline

Chapter 2 of this thesis presents a literature review on the potential of insect residual streams to promote plant growth and health. The properties of insect frass and exuviae are described and related to possible applications as fertilizer or substrate for beneficial soil microbes. Examples of bacteria that may be stimulated by soil amendment with these materials are provided, with an emphasis on the mechanisms through which they may protect crops or enhance crop growth. The prospects for implementation in crop production and implications for the sustainability of agriculture are discussed.

In Chapter 3, the effects of soil amendment with the exuviae or frass of different insect species on the performance of *D. radicum* are reported. Residual streams derived from black soldier fly larvae, house crickets or mealworms were compared with synthetic fertilizer treatments. Larvae were placed on plants growing in amended soil and were either collected before pupation or were allowed to complete their development and emerge as flies. Possible explanations for positive or negative effects on *D. radicum* performance are discussed and the suitability of certain amendments as a means to control *D. radicum* is evaluated.

Chapter 4 describes the effects of exuviae from different insect species on the bacterial community in the *B. oleracea* rhizosphere. Plants were grown in soil amended with the exuviae of black soldier fly larvae, house crickets or mealworms and bacterial abundance as well as the diversity and composition of the rhizosphere community were compared over time. Emphasis is placed on differentially enriched groups of bacteria and their potential to improve plant growth or provide plant protection is discussed.

In Chapter 5, the impact of amendment with black soldier fly frass on the performance of *D. radicum* in different types of soil is reported. A minimum soil amendment ratio for effective *D. radicum* control was determined and effects on *D. radicum* performance were compared between soil from a field on which brassicaceous plant species had been grown, soil from a field on which non-brassicaceous species had been rotated and potting soil. Larvae were placed on plants growing in amended soil and pupae were collected to assess *D. radicum* performance. The role of cropping history and inherent *D. radicum* suppressiveness of soil for efficacy of the amendment are discussed and implications for using black soldier fly frass to control *D. radicum* are considered.

In Chapter 6, the effects of bacteria isolated from *D. radicum* larvae that had been exposed to soil amended with black soldier fly frass on *D. radicum* performance are reported. In laboratory bioassays, larvae were placed on swede slices treated with bacterial suspensions to assess insecticidal activity. An insecticidal isolate was subsequently tested in the greenhouse by placing larvae on plants growing in treated soil and collecting pupae to assess *D. radicum* performance. The potential role of the isolate in natural or frass-induced *D. radicum* suppression and its suitability as a control agent are discussed.

Chapter 7 provides a general discussion taking into account all previous chapters, with a focus on implications for the management of *D. radicum*. The materials and methods used are revisited and suggestions for future research are made.

Acknowledgements

I thank Joop van Loon and Marcel Dicke for providing helpful comments on an earlier version of this chapter.

Chapter 2

Insect frass and exuviae to promote plant growth and health

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Published in: *Trends in Plant Science, 27*(7), 646-654
doi: 10.1016/j.tplants.2022.01.007

Abstract

Beneficial soil microorganisms are known to contribute to biocontrol of plant pests and diseases, induce systemic resistance (ISR) against attackers and enhance crop yield. The use of organic soil amendments has been suggested to stimulate the abundance and/or activity of beneficial indigenous microbes in the soil. Residual streams from insect farming (frass and exuviae) contain chitin and other compounds that may stimulate beneficial soil microbes that have ISR and biocontrol activity. In addition, changes in plant phenotype that are induced by beneficial microorganisms may directly influence plant-pollinator interactions, thus affecting plant reproduction. Here, we explore the potential of insect residual streams derived from the production of insects as food and feed to promote plant growth and health, as well as their potential benefits for sustainable agriculture.

Glossary

Exuviae: molted exoskeletons of insects.

Frass: insect feces.

Herbivore-induced plant volatiles (HIPV): volatile compounds produced by plants in response to arthropod herbivory.

Induced systemic resistance (ISR): enhanced plant resistance against below- and aboveground pathogens and herbivores that is induced by root-colonizing microbes.

Insect residual streams: by-products of farming insects for food and feed.

Microbe-associated molecular pattern (MAMP): molecules conserved in microbes that are recognized by plants.

Plant growth-promoting rhizobacteria (PGPR): plant-symbiotic soil bacteria that colonize roots and enhance plant growth.

Insect-derived products affect species interactions

Terrestrial plant roots are embedded in soil, a biodiverse substrate rich in microorganisms (Berendsen et al., 2012). These microbes affect plant phenotype and, consequently, plant-mediated interactions with herbivores and other members of the plant-associated community (Berendsen et al., 2012; Friman et al., 2021c; Heinen et al., 2018; Pineda et al., 2010). Soils also contain a high diversity of organic and inorganic substances that affect these plant-mediated interactions (Rowen et al., 2019). These substances may influence the community composition of soil microbiota and understanding the underlying mechanisms may allow to steer this process towards specifically promoting beneficial microbiota (Bonanomi et al., 2018). The use of organic soil amendments enhances soil microbial activity and modifies the microbial community composition. It increases soil fertility, consequently improving plant biomass and crop yield (Bonanomi et al., 2020). Exploiting these positive effects of organic materials on plant growth and resistance to herbivory can address rising environmental concerns about the use of artificial fertilizers and synthetic pesticides (Poveda, 2021; van Huis, 2021).

A novel organic soil amendment is emerging from the production of a new source of animal proteins, i.e., the production of insects such as yellow mealworm (*Tenebrio molitor*),

lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), black soldier fly (*Hermetia illucens*) or housefly (*Musca domestica*) for food and feed (van Huis, 2021). This new industry can use organic residual streams as a resource (Fowles & Nansen, 2020; van Huis, 2021) and is rapidly developing to an estimated market volume of 730,000 metric tons in 2030, having a compounded annual growth rate of 27.8% (Anon, 2019). The production of insects for food and feed results in **residual streams** (see Glossary) like insect **exuviae** and **frass**. These residual streams are considered a potential alternative to conventional fertilizers and pesticides (van Huis, 2021). An important component of insect exuviae is chitin, a high-molecular-weight amino-sugar polysaccharide that is also present in fungal cell walls and the exoskeleton of many crustaceans (Roer et al., 2015). Chitin-containing soil amendments have been demonstrated to promote plant growth (Sharp, 2013). Likewise, the addition of insect frass to the soil has been shown to supply nitrogen and other nutrients to plants that increase their biomass and nutritional content (Poveda, 2021). Both chitin and insect frass amendments impact the soil microbiome composition and this may be an important factor in promoting plant growth and health (Heinen et al., 2018). However, information on the potential of insect-derived products to improve plant growth and their effects on the plant-associated community is limited. In this paper, we discuss the potential effects of adding insect-derived products to plants in five sections: effects on (i) beneficial soil microbes, (ii) plant growth, (iii) plant resistance, (iv) microbial antagonism against plant pathogens and insects and (v) plant reproduction (Figure 1).

Beneficial soil microbes

Various plant beneficial soil microbes are commonly applied in agriculture and are often considered to be promising alternatives to agrochemicals (Kaminsky et al., 2019). Most notably, different soil bacteria have been found to possess a range of beneficial properties (Cawoy et al., 2011; Kupferschmied et al., 2013). For example, so-called **plant growth-promoting rhizobacteria (PGPR)** can enhance resistance to pests and diseases (Pieterse et al., 2014; Pineda et al., 2010). Several strains have been reported to trigger **induced systemic resistance (ISR)** against pathogens and herbivores in their host plants or stimulate the attraction of the natural enemies of herbivores (Pangesti et al., 2015; Pineda et al., 2010). Furthermore, many beneficial bacteria control herbivores and plant pathogens via direct interactions. They can be pathogenic to insects or prevent the growth of other microbes and thus contribute to plant protection independently of the plant itself (Cawoy et al., 2011; Kupferschmied et al., 2013).

While the potential of beneficial microbes for sustainable agriculture is great, the outcomes of microbial applications in field-based crop production are often inconsistent

(Kaminsky et al., 2019). Beneficial microbes are commonly inoculated into soil, the success of which depends on their establishment both in the soil and on plant roots. However, the colonization by microbial inoculants can be constrained by competitive interactions with indigenous microbes (Mazzola & Freilich, 2017). The lack of capacity of the microbial inoculants to establish in the target environment may also result in a rapid decline in inoculant density (Kaminsky et al., 2019).

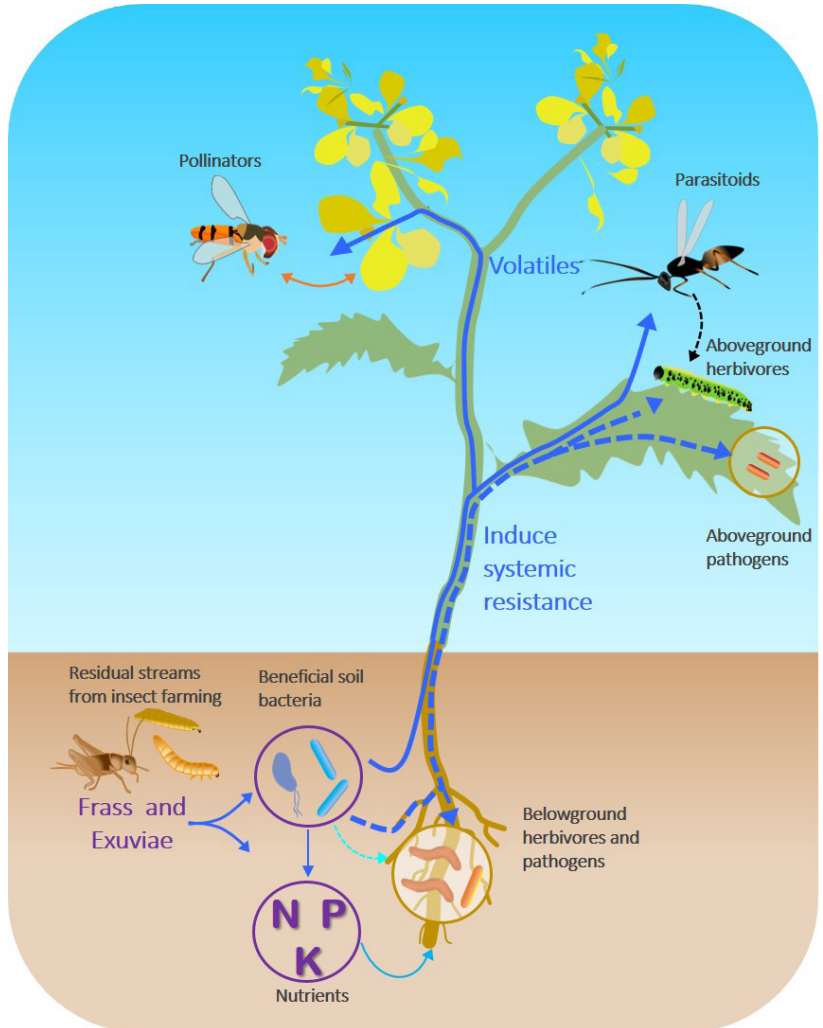

Figure 1. Schematic representation of potential pathways along which insect-derived products may affect plant growth and health. Arrows indicate the effects of organisms from the plant-associated community on the plant and each other. Solid arrows indicate positive effects; dashed arrows indicate negative effects. ISR: Induced systemic resistance. HIPV: Herbivore-induced plant volatiles.

To stimulate soil bacteria that possess biological control or ISR potential, the use of soil amendments that promote the activity and growth of beneficial endemic species has been suggested (de Boer, 2017; Mazzola & Freilich, 2017). Amendment-mediated stimulation of indigenous microorganisms has a clear advantage over the employment of microbial inoculants because the enriched soil-borne microbes are well-adapted to local soil conditions (Mazzola & Freilich, 2017). During decomposition in soils, mealworm exuviae were shown to stimulate a high diversity of chitinolytic bacteria, with a notable increase in the abundance of Bacilli (Bai, 2015). Different members of this class of bacteria, such as *Bacillus thuringiensis*, *Bacillus cereus* or *Lysinibacillus sphaericus*, are commercially provided for biological pest control (Francesca et al., 2015). Besides their ability to form spores, which facilitates production and storage, the success of these Bacilli as crop protection agents is also due to the fact that they can possess virtually all the beneficial properties mentioned above. As prime examples, root-colonizing *B. cereus* and *Bacillus subtilis* both promote plant growth, mediate ISR and have antagonistic activity against a broad range of plant pathogens and pests (Gadhave et al., 2016; Gadhave & Gange, 2016). In view of the increased abundance of Bacilli associated with applying insect exuviae as soil amendment, the utilization of insect-derived products to promote plant growth and health seems to bear good prospects.

Plant growth

PGPR can enhance plant growth and productivity. Forming a symbiotic relationship with their host, they benefit from energy-rich root exudates. In turn, they may synthesize plant growth hormones like cytokinins, auxins and gibberellins or provide increased access to nutrients such as phosphorus or iron. For instance, they may solubilize minerals from sedimentary rocks or fix atmospheric nitrogen in soils where these nutrients would otherwise not be available to plants (Pineda et al., 2010). Although PGPR are especially represented among *Bacillus* and *Pseudomonas* species, plant growth promotion is induced by different members of very diverse bacterial taxa (Hol, et al., 2013). Examples of other genera known to include growth-promoting strains are *Azospirillum*, *Burkholderia*, *Enterobacter*, *Flavomonas*, *Kluyvera*, *Paenibacillus*, *Rhizobium*, *Serratia* and *Streptomyces*. Furthermore, associations with PGPR have been described for various plant species. For example, *B. subtilis* is known to promote the growth of cabbage, cotton, maize, pea, peanut, soybean, sweet pepper and tomato among other crops (Cawoy et al., 2011; Friman et al., 2021c).

While it has been noted that enhanced plant growth can also improve food supply for pests, it provides a means to compensate for possible yield losses at the same time (Pineda et al., 2010). By promoting plant growth, PGPR are thought to facilitate

the allocation of resources to plant defense and, thus, additional protection to the host (Pangesti et al., 2013). The high abundance of Bacilli promoted by insect exuviae suggests a stimulation of beneficial *Bacillus* or *Paenibacillus* spp. by these materials (Bai, 2015). Similarly, others have attributed enhanced plant growth after soil amendment with insect frass not only to nutrient supply, but to stimulated soil microbial activity as well (Houben et al., 2020; Poveda et al., 2019).

Most of the emerging research on the fertilizer effects of insect residual streams focuses on frass rather than exuviae (Houben et al., 2020; Poveda, 2021). However, insect exuviae also contain a considerable amount of nitrogen, mainly in the form of chitin and proteins. Because plants lack the ability to utilize chitin directly, they rely on a cascade of microbial enzymatic activities to break down chitin, which releases compounds beneficial for plant growth such as plant-available nitrogen or short-chain chitin oligomers (de Tender et al., 2019; Shamshina et al., 2020). While the prospect of crustacean-derived chitin to enhance plant nutrient availability has been well-documented (Shamshina et al., 2020; Sharp, 2013), insect exuviae have not yet been investigated for this purpose. In addition to chitin, other compounds in insect exuviae such as proteins and lipids may be mediating increased plant performance (Roer et al., 2015). The bacterial class Bacilli appeared to be strongly involved in the decomposition of mealworm exuviae in soil but not in the decomposition of purified shrimp chitin, whereas both contain high levels of chitin (Bai, 2015).

Frass is defined as insect excrement, but in the context of the insect farming industry, it refers to a mix of predominantly insect feces, remnants of shed exoskeletons and undigested feed (Fowles & Nansen, 2020). Frass is rich in readily extractable nutrients (Lovett & Ruesink, 1995; Watson et al., 2021). Frass deposition can result in a short-term pulse of plant-accessible nutrients due to stimulation of local activity of microbial decomposers (Lovett & Ruesink, 1995), which can also accelerate the decomposition of recalcitrant organic matter (Zimmer & Topp, 2002). Fragments of chitin-containing exuviae, which are present in frass as a minor component, may also provide additional benefits of frass application on plant growth and health (Watson et al., 2021). In addition to improved plant productivity (Houben et al., 2020), frass application may also result in induced plant resistance to abiotic stresses (Poveda et al., 2019). These beneficial effects of frass are mainly ascribed to plant-accessible nutrients, although frass-associated microbes are also likely to play a role (Poveda, 2021). The microbes commonly present in frass are bacterial groups belonging to Gammaproteobacteria and Bacilli as well as fungal groups belonging to Ascomycota (Boiocchi et al., 2017; Poveda et al., 2019). Similar microbial communities were also found in insect digestive tracts (Mogouong et al., 2020; Muratore et al., 2020). Frass-associated microbial isolates were shown to exhibit various PGPR traits, such as the

capability to solubilize phosphate and produce siderophores (Poveda et al., 2019). These microbes may play a significant role in changing the natural soil community and improving plant growth, because the removal of microbes by sterilization of frass resulted in lower plant yield compared to the use of non-sterilized frass (Poveda et al., 2019).

Plant resistance

The addition of insect derivatives to soil may improve plant health by increasing plant growth and tolerance against herbivores, but also by stimulating plant defense. The sparse research on how insect derivatives affect plant resistance shows that effects differ between plant species, insect species that produced the frass, and plant organ to which the frass was supplied (Ray et al., 2016). For example, caterpillar frass suppresses caterpillar-induced defenses in maize plants, while it increases the defense against pathogens and aphids (Ray et al., 2020). However, the direct opposite is observed in rice plants exposed to caterpillar frass, where caterpillar-induced defenses were increased and pathogen defenses decreased (Ray et al., 2016).

Chitin is recognized by plants as a **microbe-associated molecular pattern (MAMP)**, eliciting diverse defense responses in plants including, but not limited to: systemic expression of defense-related genes (Parada et al., 2018; Ramonell et al., 2005), programmed cell death (Newman et al., 2013), and release of reactive oxygen species (Kishimoto et al., 2010; Parada et al., 2018). Its efficiency in stimulating plant defenses against pathogens after application as a soil amendment or as a foliar spray has been shown in numerous systems (Ramírez et al., 2010; Shamshina et al., 2020). Therefore, the addition of chitin-rich insect residual streams to agricultural soil is expected to benefit plant resistance.

In addition to inducing plant defenses directly via chitin, insect residual streams have the potential to stimulate PGPR. Besides increasing tolerance to herbivory by promoting plant growth, PGPR may also sensitize plants for enhanced defense against a broad range of below- and aboveground attackers. This systemic response to root-colonization by beneficial microorganisms is called ISR (Hol et al., 2013; Pieterse et al., 2014; Pineda et al., 2010). To activate ISR, plant roots must recognize beneficial microbes through MAMPs, such as cell surface molecules and compounds that are excreted by these microbes (Segarra et al., 2009; van Wees et al., 2008). Subsequent ISR signalling throughout the root and shoot system is dependent on the plant hormones jasmonic acid (JA) and ethylene (ET) (Pieterse et al., 2014; van Wees et al., 2008). Typically, no direct change in defense-related gene-expression is found, but rather a faster and stronger response upon pathogen or insect attack (Hol et al., 2013; Pieterse et al., 2014; Pineda et al., 2010). The phenomenon enabling more effective

responses to biotic and abiotic stresses via physiological changes in the plant is known as priming (Pineda et al., 2010). A wide variety of root-associated symbionts, including *Pseudomonas*, *Bacillus*, *Trichoderma*, and mycorrhiza species have been shown to prime the plant immune system, without directly activating costly defenses (Pieterse et al., 2014). For example, soil inoculation with different PGPR, including *Bacillus* species, has been shown to mediate ISR of plants against various insects, such as root-feeding beetle larvae or shoot-feeding aphids and whiteflies (Friman et al., 2021c; Gadhave & Gange, 2016). Furthermore, PGPR may affect the recruitment of natural enemies of herbivorous insects, by modifying the blend of **herbivore-induced plant volatiles (HIPV)**. Soil amendment with the rhizobacterium *Pseudomonas simiae* or with several *Bacillus* species, for example, resulted in an altered plant volatile blend and increased recruitment of parasitoids of two aphid species and a caterpillar by *Arabidopsis thaliana* and *Brassica oleracea*, respectively (Gadhave et al., 2016; Pangesti et al., 2015). Selectively stimulating PGPR by adding insect residual streams to the soil may thus induce systemic resistance in the plant, reducing herbivore performance and increasing recruitment of natural enemies.

Microbial antagonism against plant pathogens and insects

In addition to plant-mediated mechanisms, insect residual streams can also exert positive effects on plant survival through the stimulation of native soil microbes with natural biological control activity. Several greenhouse and field studies have shown that the application of chitin-containing amendment coincided with a reduction in disease incidence caused by root-infecting fungi, such as *Verticillium dahliae* (Cretoiu et al., 2013), *Fusarium oxysporum* (Randall et al., 2020) and *Rhizoctonia solani* (Andreo-Jimenez et al., 2021). The key mechanism for this suppression of pathogens is attributed to increased abundance and activity of chitinolytic bacteria and fungi, particularly members of Actinobacteria, Gamma-proteobacteria, Bacilli, and Mortierellomycetes (Debode et al., 2016; Wieczorek et al., 2019). The chitinases produced by these microbes, in combination with other cell-wall-degrading enzymes and antibiotics, can weaken and disrupt the developing cell wall of fungal pathogens (Cretoiu et al., 2013; de Boer et al., 2001). In a similar way, chitinases can affect the development of root herbivores and have been shown to reduce larval feeding and biomass when ingested. The underlying mechanism is thought to be the degradation of chitin in the insect midgut peritrophic matrix (Veliz et al., 2017). Chitinolytic activity is only one of many mechanisms underlying microbial antagonism. Several native soil microbial species have the inherent capacity to produce a wide array of bioactive metabolites to neutralize detrimental organisms (Box 1).

Box 1. Microbial secondary metabolites with biocontrol activity

By means of toxic or inhibitory allelochemicals and proteins, beneficial microbes can control various soil pests and pathogens directly. For instance, the compounds produced by different *Bacillus* spp. are known to have insecticidal, antibiotic or nematicidal properties (Cawoy et al., 2011; Francesca et al., 2015). The well-known Cry and Cyt proteins of *B. thuringiensis* are potent insect-specific toxins that are effective against various members of the Coleoptera, Diptera and Lepidoptera. Similarly, certain cyclic lipopeptides produced by *B. subtilis* have insecticidal activity against fruit flies and mosquitoes. However, *B. subtilis* is mainly known for its antimicrobial activity, which is due to the production of various antibiotic peptides. While lantibiotics, for example, have strong antibacterial activity, different cyclic lipopeptides of *B. subtilis* are involved in the suppression of fungal and oomycete plant pathogens such as *R. solani* or *Pythium aphanidermatum* (Cawoy et al., 2011). Besides *Bacillus* species, soil bacteria that are entomopathogenic or inhibit the growth of plant pathogens can be found in many other genera. Examples are plant growth-promoting *Kluyvera* and *Pseudomonas* species, which can exhibit oral toxicity to insects or suppress plant diseases (Kupferschmied et al., 2013; Laurentis et al., 2014).

Unfortunately, natural levels of antagonistic microbial activity are often insufficient to be effective and consistent (Garbeva et al., 2004). However, the selective enrichment of beneficial microbes, for example, by the addition of chitin-rich soil amendments can serve to enhance pest and disease suppression (Cretoiu et al., 2013). Chitin-containing organic amendments can be applied as an inoculant carrier of beneficial microbes to improve their efficacy. In some cases, combined application of chitin-containing material with beneficial microbes resulted in synergistic positive effects in terms of plant growth and disease suppression. When used as a seed treatment, formulations of *B. subtilis* in combination with chitin-containing materials showed a steady increase in *B. subtilis* over time and a better control of *Aspergillus niger* (causing crown rot) and *Fusarium udum* (causing wilt) in groundnut and pigeon pea plants, respectively (Manjula & Podile, 2001). Similarly, *B. thuringiensis* is known to use chitin as a carbon source and the application of chitin to stimulate its growth has been suggested. Furthermore, co-application of *B. thuringiensis* and chitinase has been shown to increase its insecticidal activity, for example against *Choristoneura fumiferana* caterpillars (Sharp, 2013). These studies suggest that chitinous amendments can enhance the establishment and antagonistic activity of introduced biocontrol strains and render the soil environment more suitable for the successful establishment of introduced biocontrol agents.

Plant reproduction

Changes in the soil- and rhizosphere microbiome induced by insect-derived products may impact plant phenotype such as floral phenology (Heinen et al., 2018). Marigold plants grown in soil inoculated with *B. subtilis* produced more and heavier flowers, with a significantly increased color intensity (Flores et al., 2007). Similarly, the addition of chitin and its derivative, chitosan, can affect flowering phenology (Salachna et al., 2015; Sharp, 2013), speeding up flower production by as much as 15 days (Ohta et al., 1999). These effects were related to increases in chitinolytic microorganisms (Sharp, 2013). The increase in nutrient availability as a result of microbial activity stimulated by the insect-derived amendments may allow the plant to increase resource investment in flower production (Burkle & Irwin, 2009). For example, addition of nutrients has been found to increase the number of flowers, flowering duration and nectar quantities in scarlet trumpet plants (Burkle & Irwin, 2009). Also, petunia flowers showed increases in corolla size, display size and consequently, in the number of flower visitors in response to an increase in soil nitrogen (Rebolleda-Gómez et al., 2019). These patterns suggest that plants are likely to alter their phenotype in response to the availability of nutrients influenced by insect-derived products, affecting plant-pollinator interactions and directly influencing plant fitness and yield (Cardoza et al., 2012). While such effects have not been examined for insect-derived materials, their nutrient content and potentially stimulating effect on Bacilli seem promising. Impacts of soil microbes on flowering phenology are also expected to influence plant reproduction. Although the effect of flowering duration on plant reproduction varies between different plant species, pollinator visitation and subsequent seed set increased with flowering duration in plants with unspecialized flowers (Lázaro et al., 2013).

To the best of our knowledge the effects of insect residual streams on flower traits, interactions with pollinators and, consequently, plant reproduction have not been reported in the literature. First evidence has recently been collected that indeed amending soil with insect residual streams can influence plant-pollinator interactions and increase plant reproduction (Barragán-Fonseca et al., unpublished data).

Concluding Remarks and Future Perspectives

As the insect farming industry is growing rapidly, new companies as well as companies already established in the biocontrol sector have entered the market for insects as animal feed. With the development of regulatory frameworks and the recent authorization of insects as components of pig and poultry feed in the European Union (European Union, 2021a), the use of insects in feed is expected to increase rapidly (IPIFF,

2021). At the same time, large amounts of insect residual streams will become available. The application of these residual streams as soil amendments can further contribute to a sustainable and circular agriculture. In the light of legislation that becomes more and more restrictive for the use of synthetic pesticides, these products can provide alternatives to support the development of sustainable pest management (Torgerson et al., 2021).

The use of insect-derived products represents a tremendous opportunity to enhance crop productivity within circular agriculture (Figure 2). The stimulation of important functional groups like PGPR and antagonists of pathogens influences the functioning of

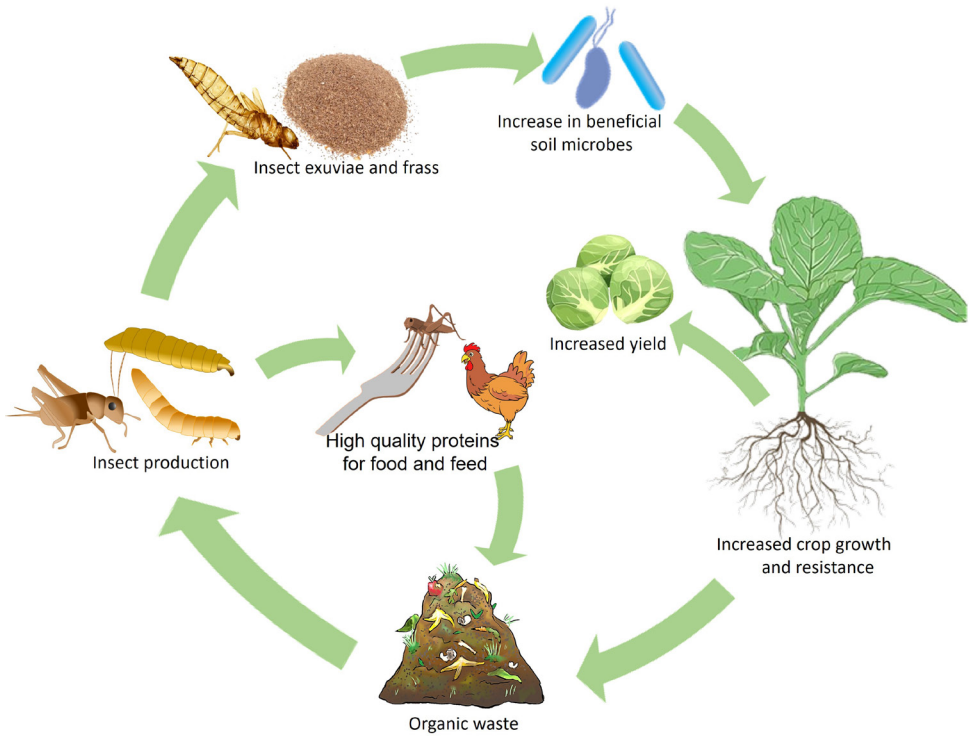

Figure 2. Schematic representation of insect production in a circular food production system. Insects can transform organic waste into high-quality animal protein for food and feed. Here, we discuss the possible use of insect residual streams as soil amendments to stimulate beneficial microbiota and improve soil and crop health.

more complex ecological networks. Beneficial soil bacteria can not only boost plant growth, but also cause changes in plant physiology, attracting mutualist insects, such as pollinators and natural enemies, and suppressing insect pests. To better understand these complex dynamics, studies on the effects of insect-derived products on insect-plant-microbe

interactions should be expanded. In this way, strategies for applying insect residual streams to control pests while maximizing positive effects and avoid negative side-effects on plant traits that are relevant for beneficial insects may be developed. These evaluations should be conducted not only under highly controlled conditions, but also in agro-ecosystems where environmental conditions are variable. Furthermore, the benefits of insect-derived products for agricultural systems compared with conventional management practices need further attention. An improved understanding of key steering factors that are relevant for the successful application of insect residual streams may aid their adoption as a novel approach to develop resilient crop production systems.

Acknowledgements

This work was supported by the Dutch Research Council, NWO (grant number ALWGK.2016.010).

Chapter 3

The potential of soil amendment with insect exuviae and frass to control the cabbage root fly

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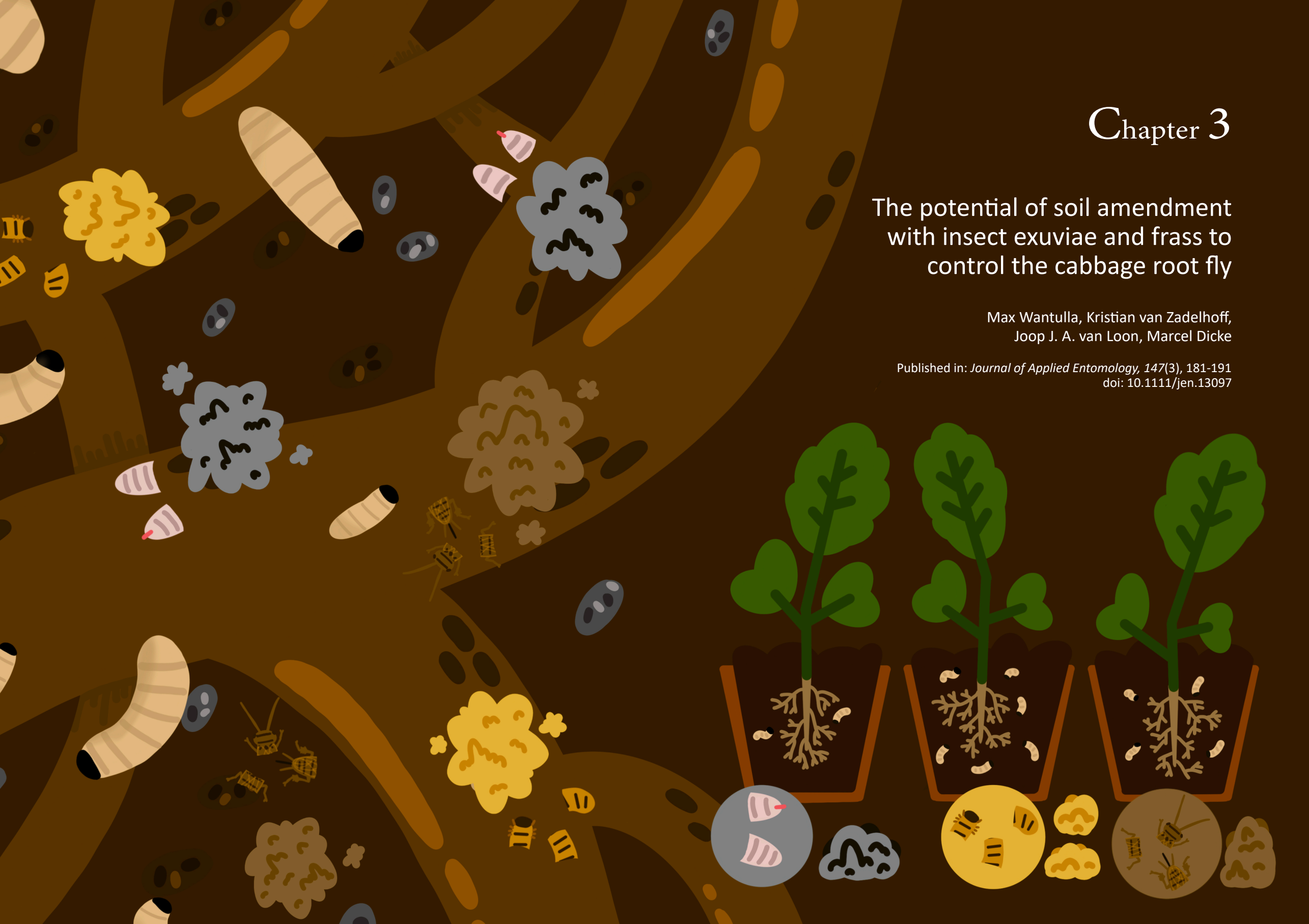
Published in: *Journal of Applied Entomology, 147*(3), 181-191
doi: 10.1111/jen.13097



Abstract

Reliable options to control the cabbage root fly, *Delia radicum* L., are lacking in many countries as restrictions on insecticide use have tightened due to environmental concerns. Although microbial control agents are often considered as a sustainable alternative, their application in agriculture is constrained by inconsistent efficacy owing to low field persistence. To stimulate naturally occurring beneficial microbes, soil amendment with the residual streams of insect production has been suggested as an alternative to synthetic fertilization and a new approach to microbial crop protection. In a set of greenhouse experiments, exuviae and frass of black soldier fly larvae, *Hermetia illucens* L., house crickets, *Acheta domesticus* L., and exuviae of mealworms, *Tenebrio molitor* L., were added to soil from an organically managed field. Exuviae and frass treatments were compared to treatments with synthetic fertilizer. Brussels sprouts, *Brassica oleracea* L., plants were grown in amended soil for 5 weeks before being infested with cabbage root fly larvae. Insect and plant performance were assessed by recording cabbage root fly survival, biomass and eclosion time and seed germination and plant biomass, respectively. Whereas soil amendment with black soldier fly frass or exuviae reduced cabbage root fly survival and biomass, respectively, amendment with house cricket or mealworm residual streams did not negatively affect root fly performance. Furthermore, seed germination was reduced in soil amended with house cricket exuviae, while amendment with either residual stream derived from black soldier fly larvae or house crickets resulted in lower plant shoot biomass compared with the synthetic fertilizer treatment. Amending soil with black soldier fly residual streams could become a novel and low-cost tool to be integrated in cabbage root fly management programs, especially where methods currently available are insufficient. Therefore, the mechanisms underlying the effects of insect-derived soil amendments described here should be the focus of future research.

Introduction

The cabbage root fly or cabbage maggot, *Delia radicum* L. (Diptera: Anthomyiidae), is a major pest of cruciferous crops in northern temperate regions and is considered to be the most damaging pest of turnip and swede (Santolamazza-Carbone et al., 2017). Female flies oviposit in soil close to the base of the host plant, from where the larvae start to feed on root tissue upon hatching. After up to 3 weeks of feeding, pupation occurs in the soil and adult flies eclose between 2 and 4 weeks later (Joseph & Iudice, 2020). Root feeding by *D. radicum* larvae disrupts the transport of water and nutrients and can lead to secondary damage due to the facilitation of pathogen invasion. Symptoms associated with *D. radicum* infestation include yellowing, wilting, stunting and slow growth, which often result in plant mortality (Herbst et al., 2017; Shuhang et al., 2016). As a consequence, crop losses can be as high as 100%, especially among young plants (Razinger et al., 2017). While some damage to roots may be tolerable for leaf or inflorescence vegetables such as cabbage or cauliflower, even slight damage can substantially reduce the quality of root vegetables like radish, turnip or swede (Collier et al., 2020).

Over the past decades, management of the cabbage root fly has largely relied on the use of different synthetic insecticides (Herbst et al., 2017; Shuhang et al., 2016). As the root-feeding larvae are difficult to reach, insecticide application is mostly limited to methods such as seed, granule or drench treatments, particularly using systemic active ingredients (Collier et al., 2020). However, stricter regulations due to environmental concerns as well as the development of insecticide resistance in *D. radicum* have reduced the availability of effective products worldwide (Joseph & Iudice, 2020; Shuhang et al., 2016). As a result of bans and restrictions on the use of relevant insecticides, reliable options for chemical control are lacking in several European countries (Collier et al., 2020; Herbst et al., 2017). With levels of plant resistance to *D. radicum* generally being too low in brassica crops, growers thus increasingly need to rely on physical, cultural and biological control strategies (Santolamazza-Carbone et al., 2017). Unfortunately, methods such as the use of physical barriers, parasitoids or predators of *D. radicum* are often considered to be not effective enough and in some cases can have adverse effects on the crop. While microbial control agents are considered to be more promising alternatives, problems in their practical application and inconsistent effects in the field have prevented their use from becoming widespread (Collier et al., 2020).

In comparison with conventional pesticides, beneficial microorganisms such as entomopathogens or plant mutualists are generally regarded as more sustainable (Kupferschmied et al., 2013). So-called microbial biopesticides commonly exhibit multiple

distinct modes of action, which can be helpful in managing pest resistance. Furthermore, they tend to be environmentally safe, not least due to relatively low persistence (Cawoy et al., 2011). At the same time, however, limited field persistence also represents a major weakness of microbial control agents. As introduced microbes frequently fail to establish in soil, their efficacy has largely remained unreliable under field conditions (Kaminsky et al., 2019). Therefore, instead of applying non-native biocontrol agents, it has been suggested to make use of indigenous soil microbiomes. For this purpose, different organic soil inputs can serve as substrates to selectively enrich endemic microbes with biological control potential (Mazzola & Freilich, 2017).

Many of the properties of substrate inputs would render them particularly suitable for the management of soil pests. Infestations with root-feeding insects are extremely difficult to predict and react to in a timely manner, which often makes it necessary to apply crop protection products prophylactically (Johnson et al., 2016). While this might not always be practical with a control agent, it is convenient for the application of organic substrates, as soil usually has to be amended before or during planting. Although consistent efficacy of crop protection measures through a certain functional period is essential, increased persistence and the possible spread of microorganisms can also pose environmental risks (Kaminsky et al., 2019). Substrate-mediated recruitment of beneficial microbes, however, should limit the presence of colonizers to a definite period. Possible effects of organic inputs on soil organisms should thus wear off as soon as most of the substrate has been decomposed. Another advantage of substrate-based soil treatments is the promotion of potentially useful microbiomes rather than single microbial strains (Mazzola & Freilich, 2017). By stimulating functional consortia of microbes, the risk of resistance development in pests can be reduced greatly.

A relatively new approach to managing indigenous microbial resources is the amendment of soil with by-products of commercial insect production (Barragán-Fonseca et al., 2022). In recent years, mass rearing of insects for food and feed has become an expanding industry that continues to develop rapidly (Poveda, 2021; van Huis, 2021). Growing interest in the production of insects mainly owes to their capacity to convert low quality organic material into animal protein. Nevertheless, insect rearing also generates other outputs, namely a mixture of unconsumed substrate, exuviae (molted exoskeletons) and insect feces, which are thought to be valuable products themselves (Houben et al., 2020). The combination of these three residual products of insects produced for food or feed is commonly referred to as 'frass' (Barragán-Fonseca et al., 2022). Frass has been the primary topic of research as it is produced in large quantities during insect production. In particular, frass of larvae of the black soldier fly, *Hermetia illucens* L., and of yellow

mealworms, *Tenebrio molitor* L., are investigated for potential uses in agriculture (Barragán-Fonseca et al., 2022; Poveda, 2021; Schmitt & de Vries, 2020).

While it has been noted that soil amendment with insect frass could stimulate the activity of beneficial microbes, it is mostly considered for application as a fertilizer (Poveda et al., 2019). In fact, the capacity of frass to supply nutrients to plants and enhance plant growth has been compared to that of synthetic fertilizer and its potential to replace conventional fertilizers has been pointed out (Houben et al., 2020). Whether the use of insect residual streams as fertilizers can also contribute to the management of agricultural pests, however, remains to be investigated. Interestingly, mealworm exuviae, unlike other chitin-containing materials, were found to be colonized by high numbers of Bacilli in both forest and former arable soils (Bai, 2015). Different species and various strains in this diverse group of bacteria are commonly applied for crop protection to control insects as well as plant diseases (Cawoy et al., 2011).

Overall, a great potential to promote soil and crop health has been attributed to insect residual streams, though this has yet to be proven by research (Torgerson et al., 2021). As there might be crucial differences in the composition of exuviae and frass between insect species, it is essential to study the residual streams of various species to determine possible uses beyond fertilization for each of them. Therefore, the objective of the present study was to investigate the effects of different exuviae and frass soil amendments as a replacement for synthetic fertilizers on the root-feeding insect pest *D. radicum*. To this end, the amendments were directly compared with the application of synthetic fertilizer.

Materials and methods

Plants and growth conditions

Brassica oleracea L. var. *gemmifera* cv. Cyrus (Brussels sprouts) plants were kept in a greenhouse compartment at 20 ± 3 °C, 60-80% relative humidity and 16 h light/8 h dark photoperiod. Plants were grown in 1 L plastic pots, which were placed in saucers. For all experiments, pots containing the different treatments were randomly distributed over a single greenhouse bench. Two seeds were sown per pot and gently pressed down. If both seeds germinated, one seedling was randomly removed from each pot after 1 week. Excess seedlings were transplanted to pots of the same treatment in which no seeds had germinated and were used for experiments or were discarded together with ungerminated seeds if not needed. Plants growing in unamended soil were fertilized three times per week with 20 ml of an optimized fertilizer solution (Table S1) in Experiment 1

and 15 ml per pot in Experiments 2 and 3. Fertilizer amounts per 1 L of field soil were based on a nitrogen fertilization advice of 230 kg/ha for cabbage provided by Eurofins Agro (Wageningen, the Netherlands), assuming a topsoil depth of 25 cm and taking into account the duration of each experiment. Plants growing in soil amended with insect residual streams did not receive any synthetic fertilizer. All plants were watered three times per week by filling saucers and emptying them after 2 h. Plants were grown for 5 weeks before being infested with *D. radicum* larvae.

Insect rearing and plant infestation

The *D. radicum* population used was collected in Zeewolde (Flevoland, the Netherlands) in 2013. All life stages were kept in a climate cabinet at 20 ± 1 °C and 16 h light/8 h dark photoperiod. Larvae were kept on *Brassica napus* L. subsp. *rapifera* (swede) roots of 10-week-old plants until pupation. Eclosed adult root flies were kept in gauze cages and were fed with a 1:1:1 mixture of sugar, milk powder and yeast. In addition, a solution of honey in tap water was offered in a Petri dish and tap water was offered in a Petri dish with moist filter paper on top of wet cotton wool. Oviposition was stimulated by providing slices of swede in Petri dishes to the flies in the cages. Eggs were collected and placed on a new swede prior to hatching.

To obtain larvae for plant infestation, eggs were incubated in Petri dishes with moist filter paper. Larvae hatched from the eggs after 4 days and plants were infested by carefully placing five neonate larvae on plastic plant labels that were inserted into the soil surface close to the stem. Labels were checked after 30 min and remaining larvae were replaced. This was repeated until five larvae had moved into the soil in every pot.

Insect residual streams

Five insect residual streams were used in this study: exuviae and frass of larvae of black soldier flies, *H. illucens* (Bestico, Berkel en Rodenrijs, the Netherlands), exuviae and frass of house crickets, *Acheta domesticus* L. (Protix, Bergen op Zoom, the Netherlands), and exuviae of yellow mealworms, *T. molitor* (Nijenkamp Voederdieren, Hellendoorn, the Netherlands). Exuviae and frass were separated by the supplying companies before being delivered. All materials were first inspected for the presence of insects or insect fragments, which were removed, and were then oven-dried at 60 °C for 24 h to allow for soil amendment on a dry matter basis. The dried materials were subsequently ground to a powder with an SM 100 cutting mill (Retsch, Haan, Germany).

Soil

Agricultural soil was collected from the topsoil layer of an organically managed field in Wageningen, the Netherlands in March 2019 for Experiments 1 and 2 and in December 2019 for Experiment 3. The field had been used to grow various brassicaceous plants since 2011 and black mustard (*Brassica nigra* L.) had recently been grown at the location selected for soil collection. Soil composition as assessed for the same field by Eurofins Agro (Wageningen, the Netherlands) in 2018 was 81% sand, 14% silt and 2% clay, while the soil organic matter content was 3.2% with a nitrogen delivery capacity of 80 kg/ha. The soil was homogenized by sieving (particle size < 5 mm) and stored at ambient temperature in a non-heated warehouse for 2 - 8 months before being used.

Experiment 1: effects of black soldier fly and mealworm exuviae on *D. radicum* survival and larval biomass

Soil was mixed with black soldier fly or mealworm exuviae at a ratio of 5 g/kg of dry soil or left unamended for the application of synthetic fertilizer as described above. Exuviae rates were chosen based on previous soil nitrogen content measurements so that the amount of plant-available nitrogen added to soil by amendment approximately corresponded to the amount applied as synthetic fertilizer (Nurfikari, 2022). As plants only grew large enough to sustain feeding *D. radicum* larvae in amended or fertilized soil, an untreated control was not included in the experiment. In a completely randomized design, 11 plants were grown per treatment and infested with *D. radicum* after 5 weeks. Plants were uprooted 2 weeks after infestation and roots were rinsed to remove adhering soil. Roots were checked for remaining larvae and all soil was washed through a Fenwick can (Metaalgaas Twente, Hengelo, the Netherlands) and a 0.5 mm aperture sieve to collect larvae and pupae. Living larvae were counted and weighed on a CP2P-F micro balance (Sartorius, Göttingen, Germany). Plants were oven-dried at 105 °C for 24 h before measuring shoot and root dry biomass.

Experiments 2 and 3: effects of black soldier fly and house cricket residual streams on *D. radicum* survival, fly eclosion time and biomass

For Experiment 2, soil was mixed with black soldier fly exuviae or frass at a ratio of 5 g/kg or 10 g/kg of dry soil, respectively, or left unamended for the application of synthetic fertilizer as described above. In Experiment 3, soil was mixed with house cricket exuviae or frass, both at a ratio of 5 g/kg of soil, or left unamended for the application of synthetic fertilizer. Exuviae and frass rates were chosen based on previous soil nitrogen

content measurements so that the amount of plant-available nitrogen added to soil by amendment approximately corresponded to the amount applied as synthetic fertilizer (Nurfikari, 2022). As plants only grew large enough to sustain feeding *D. radicum* larvae in amended or fertilized soil, an untreated control was not included in the experiments. For each experiment, 21 plants were grown per treatment in a completely randomized design and were infested with *D. radicum* larvae after 5 weeks. Plants were enclosed in mesh sleeves 3 weeks after infestation and eclosing flies were counted and collected daily until no more flies had eclosed for at least 7 days. Flies were stored at -20 °C before being dried at 50 °C for 48 h. Fly dry biomass was then measured on a CP2P-F micro balance (Sartorius, Göttingen, Germany). Plant shoots were harvested 7 weeks after infestation and were oven-dried at 105 °C for 24 h before measuring shoot dry biomass.

Statistical analysis

Statistical tests were performed using R (Version 3.6.3; R Core Team, 2020) and the packages car (Fox & Weisberg, 2019), dunn.test (Dinno, 2017), emmeans (Lenth, 2021), nlme (Pinheiro et al., 2020) and stats (R Core Team, 2020). Seed germination and *D. radicum* survival were analyzed using generalized linear models (GLM) with binomial or quasibinomial distributions, depending on possible overdispersion of the data. Pairwise comparisons were performed using estimated marginal means (EMM). Larval and adult *D. radicum* biomass were analyzed using linear mixed effects models (LMM) with plant as a random factor and EMMs for pairwise comparisons. Average fly eclosion time per plant as well as shoot and root biomass were analyzed using linear models (LM) and pairwise comparisons of EMMs or Kruskal–Wallis and Dunn's tests if the assumption of normality was not met. Models were validated by plotting residuals and, where necessary, homogeneity of variances and normality were confirmed using Levene's test and the Shapiro-Wilk test, respectively.

Results

Experiment 1: effects of black soldier fly and mealworm exuviae on *D. radicum* survival and larval biomass

D. radicum performance

The main effect of soil amendment with insect exuviae on survival of *D. radicum* was significant (GLM: $\chi^2 = 14.827$, $df = 2$, $P < 0.001$). Whereas amendment with black soldier fly exuviae resulted in a significantly lower survival than amendment with mealworm exuviae

(EMM: $P = 0.002$; Figure 1A), survival was not significantly different from the synthetic fertilizer treatment for either of the amendments. Soil amendment had a significant main effect on larval fresh biomass (LMM: $\chi^2 = 28.107$, $df = 2$, $P < 0.001$) and amendment with black soldier fly exuviae significantly reduced larval biomass compared with both the synthetic fertilizer treatment (EMM: $P = 0.021$; Figure 1B) and amendment with mealworm exuviae (EMM: $P < 0.001$; Figure 1B).

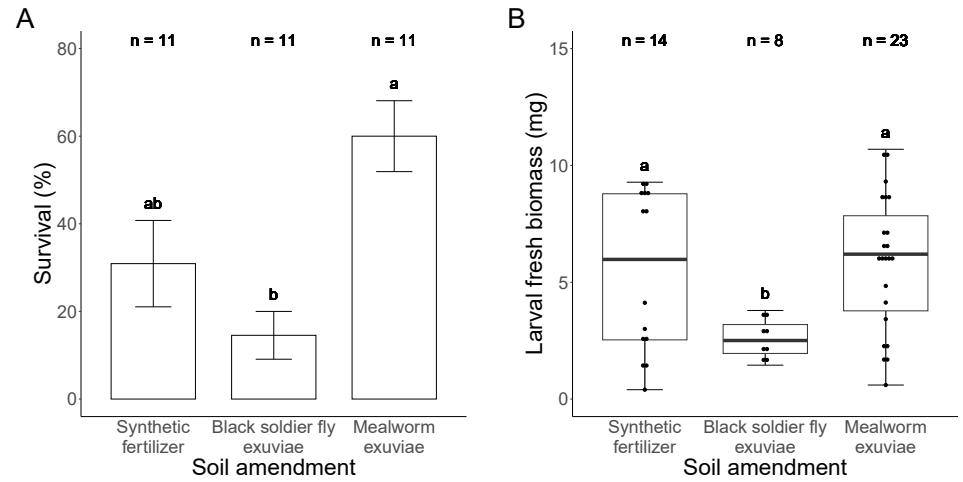


Figure 1. *Delia radicum* survival (A) and larval fresh biomass (B) after 2 weeks of feeding on *Brassica oleracea* plants growing in soil amended with insect exuviae (5 g/kg). Plants grown in unamended soil received synthetic fertilizer. Treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Error bars (A) represent standard errors. Box plot whiskers (B) represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants (A) or larvae (B) are indicated at the top of the panels by n.

Plant performance

Soil amendment with insect exuviae had a significant main effect on both shoot dry biomass (LM: $F = 15.073$, $df = 2$, $P < 0.001$) and root dry biomass (LM: $F = 4.0746$, $df = 2$, $P = 0.027$) of *B. oleracea* plants. Amendment with black soldier fly exuviae resulted in significantly lower shoot biomass than the synthetic fertilizer treatment or amendment with mealworm exuviae (EMM: $P < 0.001$; Figure 2A). Root biomass of plants grown in soil amended with black soldier fly exuviae was significantly higher than that of plants grown in soil amended with mealworm exuviae (EMM: $P = 0.045$; Figure 2B).

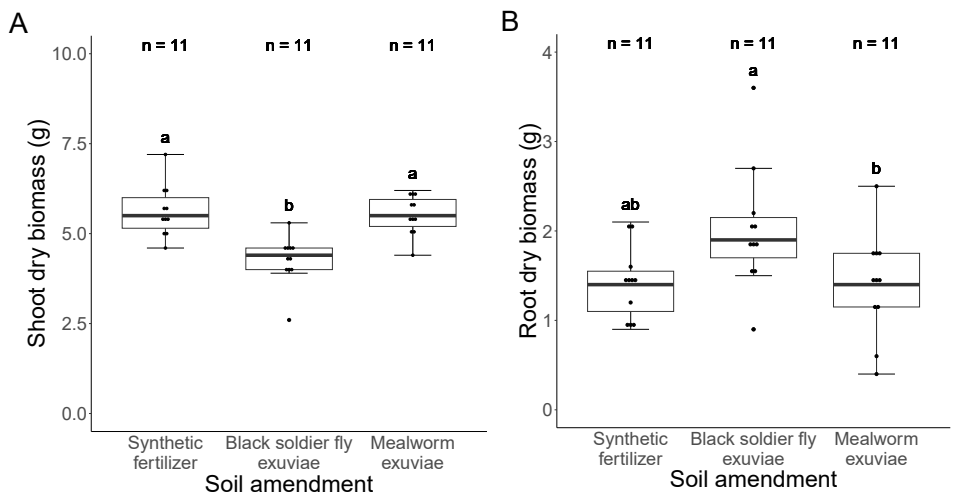


Figure 2. Shoot (A) and root (B) dry biomass of *Brassica oleracea* plants grown in soil amended with insect exuviae (5 g/kg) 7 weeks after planting and 2 weeks after infestation with *Delia radicum* larvae. Plants grown in unamended soil received synthetic fertilizer. Treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Box plot whiskers represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants are indicated at the top of the panels by n.

Experiment 2: effects of black soldier fly residual streams on *D. radicum* survival, fly eclosion time and biomass

D. radicum performance

The main effect of soil amendment with black soldier fly residual streams on *D. radicum* survival was significant (GLM: $\chi^2 = 7.7423$, $df = 2$, $P = 0.021$). Amendment with black soldier fly frass almost halved fly emergence from the soil as compared to the synthetic fertilizer treatment (EMM: $P = 0.02$; Figure 3A). Soil amendment had no significant effect on fly dry biomass (LMM: $\chi^2 = 2.1391$, $df = 2$, $P = 0.343$; Figure 3B). Soil amendment with black soldier fly residual streams did not affect *D. radicum* eclosion time (Kruskal–Wallis test: $H = 3.8389$, $df = 2$, $P = 0.15$; Figure 4).

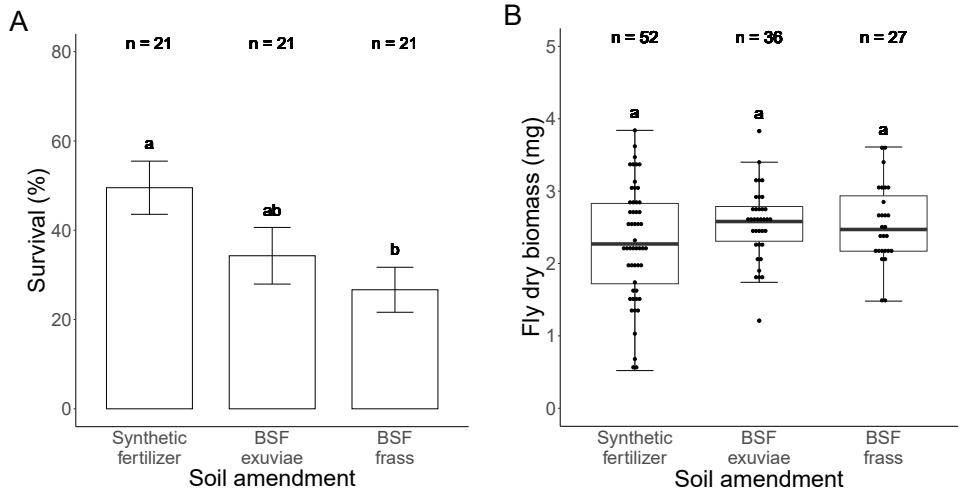


Figure 3. Survival (A) and adult fly dry biomass (B) of *Delia radicum* after larval feeding on *Brassica oleracea* plants grown in soil amended with black soldier fly (BSF) exuviae (5 g/kg) and frass (10 g/kg). Plants grown in unamended soil received synthetic fertilizer. Treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Error bars (A) represent standard errors. Box plot whiskers (B) represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants (A) or flies (B) are indicated at the top of the panels by n.

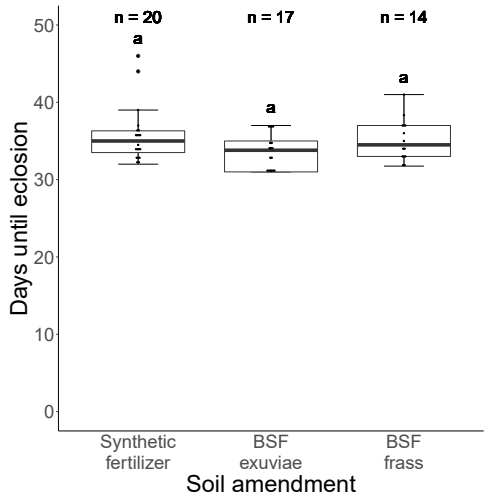


Figure 4. Time until eclosion of *Delia radicum* flies after larval feeding on *Brassica oleracea* plants grown in soil amended with black soldier fly (BSF) exuviae (5g/kg) and frass (10 g/kg). Plants grown in unamended soil received synthetic fertilizer. Time until eclosion did not differ significantly among treatments (Kruskal–Wallis test, $P = 0.15$). Box plot whiskers represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants are indicated at the top of the panels by n.

Plant performance

While soil amendment with black soldier fly residual streams did not affect *B. oleracea* seed germination (GLM: χ^2 = 0.96686, *df* = 2, *P* = 0.617; Figure 5A), amendment significantly affected shoot dry biomass of *B. oleracea* plants (Kruskal–Wallis test: *H* = 7.6925, *df* = 2, *P* = 0.02). Both amendment with black soldier fly exuviae (Dunn's test: *P* = 0.044; Figure 5B) and with black soldier fly frass (Dunn's test: *P* = 0.04; Figure 5B) resulted in lower shoot biomass than the synthetic fertilizer treatment.

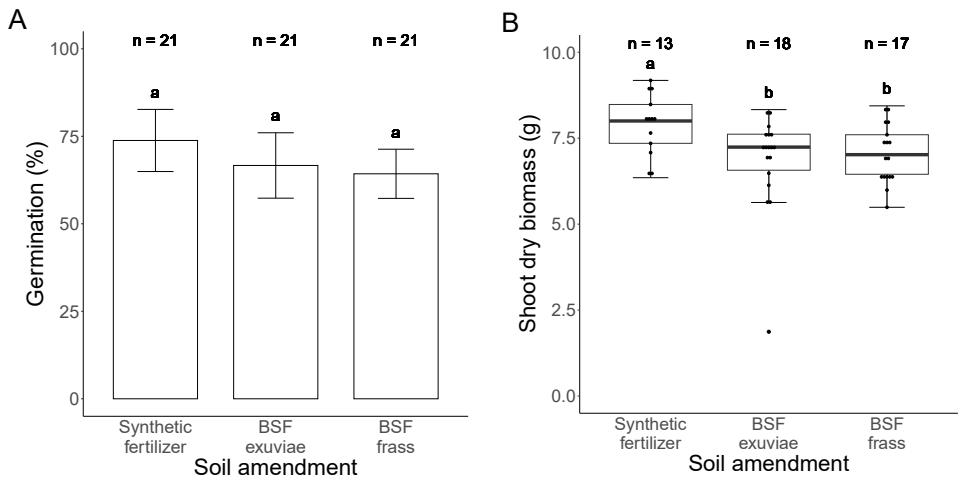


Figure 5. Seed germination (A) and shoot dry biomass (B) of *Brassica oleracea* 12 weeks after planting and 7 weeks after infestation with *Delia radicum* larvae in soil amended with black soldier fly (BSF) exuviae (5g/kg) and frass (10 g/kg). Plants grown in unamended soil received synthetic fertilizer. Treatments denoted with the same letter are not significantly different (Dunn's test, *P* > 0.05). Error bars (A) represent standard errors. Box plot whiskers (B) represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate pots (A) or plants (B) are indicated at the top of the panels by n.

Experiment 3: effects of house cricket residual streams on *D. radicum* survival, fly eclosion time and biomass

D. radicum performance

Soil amendment with house cricket residual streams affected neither *D. radicum* survival (GLM: χ^2 = 4.7617, *df* = 2, *P* = 0.093; Figure 6A) nor fly dry biomass (LMM: χ^2 = 3.7022, *df* = 2, *P* = 0.157; Figure 6B). Soil amendment had a significant main effect on average *D.*

radicum eclosion time (LM: *F* = 5.7051, *df* = 2, *P* = 0.01) and amendment with house cricket frass reduced eclosion time by ca. 3 days (EMM: *P* = 0.011; Figure 7).

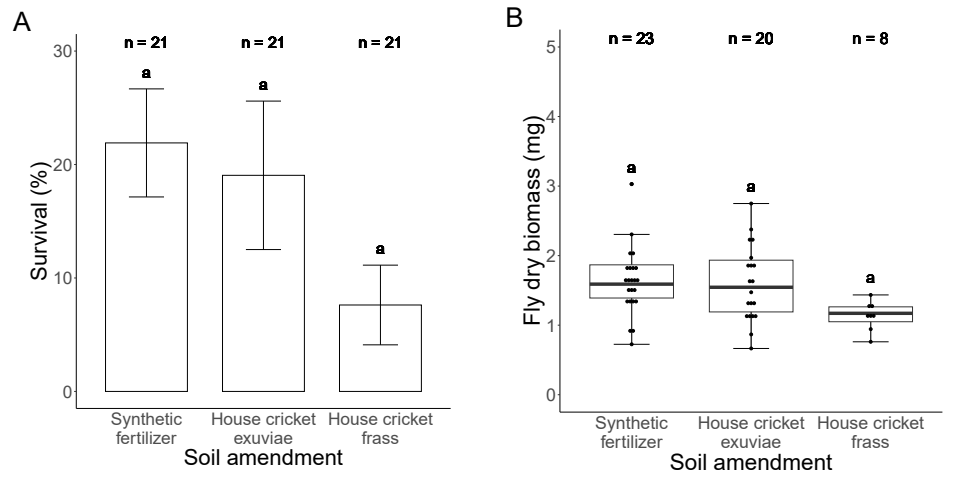


Figure 6. Survival (A) and adult fly dry biomass (B) of *Delia radicum* after larval feeding on *Brassica oleracea* plants grown in soil amended with house cricket residual streams (5 g/kg). Plants grown in unamended soil received synthetic fertilizer. Survival and biomass did not differ significantly among treatments (GLM/LMM, *P* > 0.05). Error bars (A) represent standard errors. Box plot whiskers (B) represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants (A) or flies (B) are indicated at the top of the panels by n.

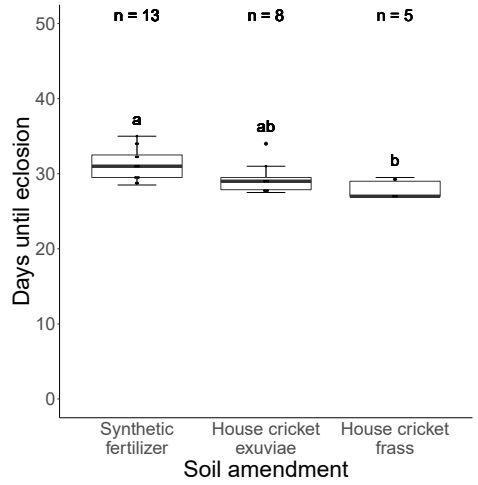


Figure 7. Time until eclosion of *Delia radicum* flies after larval feeding on *Brassica oleracea* plants grown in soil amended with house cricket residual streams (5 g/kg). Plants grown in unamended soil received synthetic fertilizer. Treatments denoted with the same letter are not significantly different (EMM, *P* > 0.05). Error bars represent standard errors. Numbers of replicate plants are indicated at the top of the panels by n.

Plant performance

Soil amendment with house cricket residual streams significantly affected *B. oleracea* seed germination (GLM: χ^2 = 29.142, *df* = 2, *P* < 0.001) and shoot dry biomass (LM: *F* = 17.472, *df* = 2, *P* < 0.001). Amendment with house cricket exuviae reduced seed germination by more than half as compared to the synthetic fertilizer treatment (EMM: *P* < 0.001; Figure 8A). Shoot biomass was significantly lower than in the synthetic fertilizer treatment after soil amendment with cricket exuviae (EMM: *P* = 0.024; Figure 8B) or frass (EMM: *P* < 0.001; Figure 8B) and was significantly lower with frass than with exuviae (EMM: *P* = 0.006; Figure 8B).

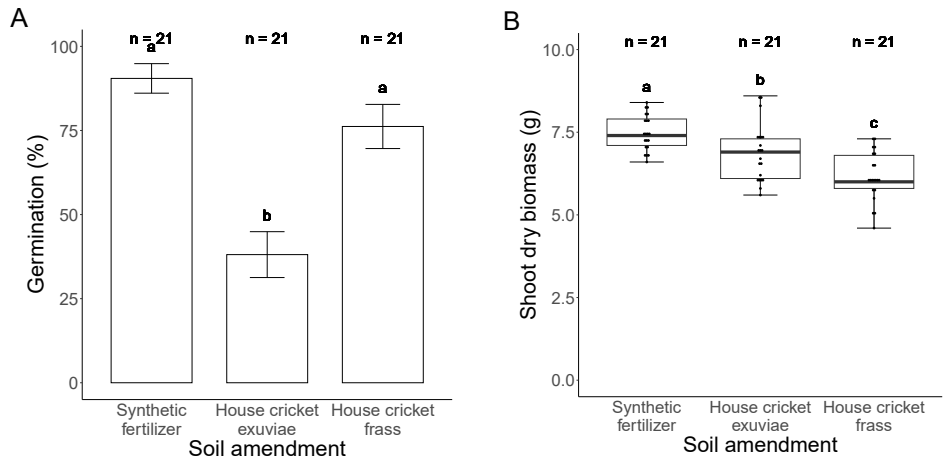


Figure 8. Seed germination (A) and shoot dry biomass (B) of *Brassica oleracea* 12 weeks after planting and 7 weeks after infestation with *Delia radicum* larvae in soil amended with house cricket residual streams (5 g/kg). Plants grown in unamended soil received synthetic fertilizer. Treatments denoted with the same letter are not significantly different (EMM, *P* > 0.05). Error bars (A) represent standard errors. Box plot whiskers (B) represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate pots (A) or plants (B) are indicated at the top of the panels by n.

Discussion

Of the different insect-derived soil amendments tested here, only black soldier fly residual streams had a clear negative effect on the performance of *D. radicum*. While soil amendment with black soldier fly frass nearly halved cabbage root fly emergence as compared to the synthetic fertilizer treatment, amendment with black soldier fly exuviae resulted in a reduction of larval biomass. However, adult fly biomass was affected neither by amendment with frass nor by amendment with exuviae. A possible explanation is that

larvae with a reduced biomass are less likely to complete their development and are not represented in the population of emerging flies. Furthermore, soil amendment with neither black soldier fly residual stream affected adult *D. radicum* eclosion time. It should be noted that black soldier fly frass was applied to soil at a mass ratio twice as high as the one for exuviae, leaving the possibility open that a higher content of exuviae in the soil might result in a significant reduction of *D. radicum* survival. However, as exuviae are produced in considerably smaller amounts in insect farming than frass, it is questionable whether soil amendment with larger quantities per kg would be feasible. Altogether, these results show that soil amendment with different black soldier fly residual streams can reduce *D. radicum* survival or impair larval development.

Interestingly, soil amendment with the same residual streams derived from house crickets or mealworms did not significantly reduce *D. radicum* performance. As the effect of insect residual streams on *D. radicum* might depend on the soil microbes colonizing these streams, varying effects might be explained by differences in these microbial communities. Despite a lack of studies investigating how soil microbial communities respond to insect exuviae and frass, it can be assumed that these materials stimulate various microbes including bacteria and fungi. However, it is likely that soil bacteria play a particularly important role, as they are also generally considered to be the main degraders of chitin and respond to chitin addition more quickly than fungi (Cretoiu et al., 2013; Kielak et al., 2013). Insect exuviae and frass contain various compounds such as chitin, proteins and lipids, the utilization of which as a substrate requires bacteria to have specific properties (Bai, 2015). The exact composition of these compounds may differ between insects so that residual streams derived from different species could also be colonized by specific soil bacteria.

The colonization of black soldier fly, house cricket and mealworm residual streams by distinct bacterial communities seems likely, considering that these species belong to separate insect orders and are thus distantly related. In contrast, the black soldier fly and *D. radicum* are both insects of the order Diptera, which suggests that soil bacteria stimulated by black soldier fly residual streams might potentially colonize *D. radicum*, too. Although many bacterial entomopathogens have a relatively broad host range, some are only active against specific insect taxa. A well-known example for order specificity of insect-associated bacteria are different subspecies of *Bacillus thuringiensis*, whose host ranges include either lepidopteran, dipteran or coleopteran insects (Sanahuja et al., 2011). Overall, it seems possible that bacteria using black soldier fly exuviae or frass as substrates are detrimental to other fly species and that negative effects of these soil amendments on *D. radicum* may indeed be caused by bacterial colonizers.

Bacteria that are promoted by insect-derived soil amendments might also be able to colonize insects or plant roots and could be beneficial in different ways. Insect-colonizing bacteria may be entomopathogenic and may control insects such as *D. radicum* by producing insecticidal chemicals (Cawoy et al., 2011; Kupferschmied et al., 2013). Root-colonizing microbes such as beneficial rhizobacteria can trigger so-called induced systemic resistance (ISR), which involves the priming of plants for enhanced defense. This primed state enables plant cells to react more rapidly and strongly to subsequent attacks by pathogens or insects (Pineda et al., 2013). Bacteria triggering ISR as well as entomopathogenic bacteria are found particularly in genera such as *Bacillus*, *Pseudomonas* and *Serratia* (Kupferschmied et al., 2013; Pineda et al., 2010). While there are several examples of single strains that can control insects via both of the above-mentioned mechanisms, it is also possible that different beneficial bacteria act synergistically to provide plant protection (Cawoy et al., 2011; Kupferschmied et al., 2013). However, ISR is mainly known to be effective against generalist herbivores, whereas negative effects on specialist herbivores such as *D. radicum* are usually not observed (Pineda et al., 2013). In fact, soil inoculation with a rhizobacterium causing ISR was found to improve *D. radicum* performance on plants grown in treated soil (Friman et al., 2021b). This suggests that bacteria colonizing black soldier fly soil amendments are more likely to affect *D. radicum* due to insecticidal activity and pathogenicity.

Since mealworm exuviae had previously been reported to be colonized by Bacilli in different soils (Bai, 2015), they were expected to have negative effects on *D. radicum*. However, soil amendment with mealworm exuviae resulted in *D. radicum* survival being four times higher than when soil was amended with black soldier fly exuviae. Hence, *D. radicum* may in fact benefit from mealworm exuviae or the bacteria colonizing them in soil. Similarly, soil amendment with house cricket frass reduced average time until fly eclosion by 3 days and thus appears to accelerate *D. radicum* development. Indeed, soil microbes that are typically beneficial to plants are often found to have positive effects on insect herbivores, a phenomenon also known as induced systemic susceptibility (Pineda et al., 2013; Pineda et al., 2017). In light of this, it seems possible that some insect-derived soil amendments stimulate bacteria that may benefit an insect, whereas others are colonized by bacteria that will reduce its performance. It should be noted that control survival of *D. radicum* was unusually low in all experiments reported here, which suggests that the field soil used may exhibit a certain inherent suppressiveness to *D. radicum*. While black soldier fly residual streams might mediate the recruitment of soil bacteria responsible for this, mealworm exuviae could inhibit these bacteria or serve as a substrate for other bacteria that antagonize them.

Similar to *D. radicum* performance, the influence of different insect residual streams on the performance of *B. oleracea* plants grown in amended soil varied. Whereas soil amendment with mealworm exuviae did not affect plant dry biomass, amendment with black soldier fly or house cricket residual streams resulted in lower shoot dry biomass than the synthetic fertilizer treatment. House cricket exuviae, moreover, reduced the germination of *B. oleracea* seeds in amended soil by more than half as compared to the synthetic fertilizer treatment. Interestingly, soil amendment with either black soldier fly residual stream led to lower shoot biomass despite reducing *D. radicum* performance. It is possible that insect residual streams may not only potentially promote bacteria with insecticidal activity, but that they can stimulate plant growth-inhibiting microbes as well. Although different organic amendments have successfully been used to suppress soilborne plant diseases, organic substrates are generally thought to mediate the assembly of functionally diverse soil microbiomes (Mazzola & Freilich, 2017). While some elements of the soil microbiome colonizing an insect residual stream could contribute to the control of insects such as *D. radicum*, others may in fact be deleterious to crops. Possible plant growth-inhibiting properties of these materials need to be considered in the future and require further investigation.

Although soil amendment with the different insect residual streams tested here was based on previously reported nitrogen release rates (Nurfikari, 2022), it should be noted that soil nitrogen levels were not measured during the experiments. Thus, despite relatively high application rates, the possibility that nitrogen availability was a plant growth-limiting factor in these treatments cannot be excluded. As plants grown in unamended soil, on the other hand, did receive optimal amounts of synthetic fertilizer, the experimental set up used here is suitable only to a limited extent for explaining effects on plant growth. It should therefore be emphasized that the primary objective of the present study was to investigate possible effects on *D. radicum* rather than effects on plant growth. Based on previous research and considering the relatively low number of larvae added to each plant, it can be assumed that plant size did not limit larval feeding in any of the treatments.

It is striking that root dry biomass of *B. oleracea* plants grown in soil amended with black soldier fly exuviae was higher than when soil was amended with mealworm exuviae and was not different from plants that received synthetic fertilizer, despite shoot dry biomass being lower. This suggests that the supposed benefits of reduced *D. radicum* performance and a reduction in the root damage it caused could outweigh possible nutrient shortage or potentially negative effects of insect-derived soil amendments on plant growth. Finally, it should be pointed out that *B. oleracea* plants were relatively young at the end of the experiments reported here. A clear compensation for plant growth inhibition by stronger *D. radicum* suppression may only become evident after a longer growth period more representative of a *B. oleracea* growing season.

With insect production volumes predicted to grow drastically in the coming years, insect residual streams are soon expected to become available in large amounts (Houben et al., 2020; Schmitt & de Vries, 2020). As soil amendment with the two different black soldier fly residual streams tested here negatively affected *D. radicum* survival and development, these materials could represent a novel tool for managing this insect pest. Although only providing partial control, their implementation in *D. radicum* management programs seems to offer good prospects in view of presumably low costs and a lack of effective control options in many countries (Collier et al., 2020).

Especially organic growers are likely to adopt new control methods and combine them with existing ones within an integrated pest management context as they cannot resort to synthetic insecticides and have particularly few products for *D. radicum* control at their disposal (Herbst et al., 2017). Likewise, conventional growers are expected to depend increasingly on the integration of alternative tools as new legal restrictions continue to limit insecticide availability (Collier et al., 2020; Johnson et al., 2016). When combined with other control methods that separately may not offer sufficient protection against *D. radicum*, black soldier fly soil amendments could thus contribute to an integrated pest management strategy. Most notably, there is prospect for their application in the production of leafy or flowering crops, as a certain degree of *D. radicum* feeding does not damage the marketable parts of these plants and can be tolerated (Collier et al., 2020; Razinger et al., 2017). On the other hand, a successful implementation in root crop production that demands zero tolerance for feeding damage may be less likely.

The experiments reported here suggest that black soldier fly residual streams can effectively reduce *D. radicum* performance for at least 5 weeks after application. Where introduced microbial agents may not persist long enough to provide effective crop protection, insect-derived soil amendments could indeed ensure the long-term establishment of naturally occurring biocontrol agents. Nonetheless, the true mode of action of these amendments and their potential role as a substrate for beneficial soil microbes remain to be investigated. First approaches to elucidate this should be the metagenomic analysis of microbial communities in amended soil or the identification of microbes associated with *D. radicum* larvae that were exposed to it. In this way, positive or negative effects on *D. radicum* could be correlated with the presence or absence of certain groups of microbes that may be known to have attributes relevant for crop protection. Finally, even though field soil was used in the experiments reported here, testing the effects of insect-derived soil amendments on *D. radicum* under field conditions will be essential for future applications.

Acknowledgements

This work was supported by the Dutch Research Council, NWO (grant number ALWGK.2016.010).

Data availability statement

The data that support the findings of this study are openly available in DANS-EASY at https://doi.org/10.17026/dans-xg9-vzpx.

Supporting Information

Table S1

Composition of the optimized fertilizer solution

Macronutrients	(mmol/L)	Micronutrients	(µmol/L)	EC*(S/m)	pH
NH_4^+	1.2	Fe	35.0	2.0	5.5
K	7.2	Mn	8.0		
Ca	4.0	Zn	5.0		
Mg	1.8	B	20.0		
NO_3^-	12.4	Cu	0.5		
SO_4^{2-}	3.3	Mo	0.5		
P	1.0				

*Electrical conductivity.

Chapter 4

Soil amendment with insect exuviae causes species-specific changes in the rhizosphere bacterial community of cabbage plants

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Published in: *Applied Soil Ecology, 188,* 104854
doi: 10.1016/j.apsoil.2023.104854

Abstract

Insect exuviae are a chitin-rich by-product of insect farming that is considered to have great potential for contributing to sustainable agriculture. When used as soil amendment, insect exuviae have been suggested to promote plant growth and health by stimulating naturally occurring beneficial microbes. In a greenhouse experiment, the exuviae of black soldier fly larvae (*Hermetia illucens* L.), house crickets (*Acheta domesticus* L.), and yellow mealworms (*Tenebrio molitor* L.) were added to soil from an organically managed field. Brussels sprouts, *Brassica oleracea* L., plants were grown in amended soil to assess effects on plant growth and investigate bacterial abundance, diversity, and community composition in the rhizosphere. All soil amendments increased plant shoot biomass and stimulated bacterial growth. At the same time, bacterial diversity was diminished and the different amendments resulted in distinct bacterial communities. Most notably, soil amendment with house cricket exuviae increased the relative abundances of the genera *Lysinibacillus* and *Paenibacillus*, whereas the other amendments did not. The exuviae of black soldier fly larvae, however, stimulated the genus *Pseudomonas* and different genera belonging to the *Burkholderiaceae* for a longer period of time than the exuviae of either other insect species. In view of the differential enrichment of potentially plant growth-promoting or plant-protective bacteria, soil amendments with the exuviae of different insect species might have specific uses in agriculture. The present study provides a basis for investigating the combined application of insect exuviae with beneficial bacteria that are commonly used in crop production.

Introduction

Farming insects for food and feed is a fast-developing industry that can produce high-quality animal protein using organic residual streams as input. Some of the most important species currently reared are the black soldier fly, *Hermetia illucens* L., the house cricket, *Acheta domesticus* L., and the yellow mealworm, *Tenebrio molitor* L., among others (van Huis, 2021). As commercial insect production is increasing worldwide, more of the by-products it generates are becoming available for potential applications in agriculture. These insect-derived products include exuviae (molted exoskeletons) and frass, a mixture of insect feces, exuviae and unconsumed feed. The use of insect residual streams for soil amendment has been suggested to stimulate beneficial microorganisms that can promote plant growth, induce plant resistance or have biocontrol activity against plant pathogens and pests (Barragán-Fonseca et al., 2022). In this respect, insect exuviae are particularly interesting as they contain a relatively large proportion of chitin, which is one of the most abundant biopolymers in nature and represents an important substrate for soil microbes.

Soil amendment with chitin has been widely researched for its influence on soil microbial communities and its suppressive effects on plant pathogens (Cretoiu et al., 2013; Ootsuka et al., 2021; Randall et al., 2020). Indeed, the addition of chitin to soil has often been found to increase the relative abundances of various bacterial taxa that are commonly associated with plant protection or plant growth promotion (Andreo-Jimenez et al., 2021; Debode et al., 2016). Although the effects of insect exuviae on soil microbial communities have been only scarcely investigated, one study found that mealworm exuviae, unlike other chitin resources, resulted in a strong increase in the abundance of Bacilli (Bai, 2015). This class of bacteria includes several beneficial species from genera such as *Bacillus*, *Lysinibacillus* or *Paenibacillus*, many of which are used for crop protection or plant growth promotion (Ahsan & Shimizu, 2021; Borriss, 2015; Grady et al., 2016). Interestingly, soil amendment with the exuviae of black soldier fly larvae has been found to reduce the biomass of cabbage maggots, whereas mealworm exuviae did not negatively affect the performance of this insect pest (Wantulla et al., 2022). Such differences are likely related to the composition of the exuviae, as proportions of chitin, different proteins and other components vary between insect species. While mealworm exuviae have a chitin content of 7.9-8.6% and are rich in lipids, exuviae of black soldier fly larvae contain 10.9-11.1% chitin and are thought to have a relatively low lipid content based on their lower hydrophobicity (Nurfikari & de Boer, 2021). Thus, though the exuviae of different species are expected to influence soil microbiomes in a similar way, they may also be colonized and degraded by specific microbes that can affect other soil organisms differentially.

To understand the effects of insect exuviae on soil organisms or plants and specify their potential uses, it is essential to compare how the exuviae of different species affect soil microbial communities. Differential enrichment or depletion of specific microbes by the exuviae of one species can indicate a particular suitability of those exuviae for certain agricultural applications or for a combination with commonly used microbial agents. In this regard, effects on communities in the plant rhizosphere are particularly relevant, as it represents the environment in which interactions with root pathogens and pests but also with most beneficial microbes occur. Positive effects of soil amendment with chitin on plant growth and health have been related to the increased abundance of beneficial microbes in the rhizosphere (Debode et al., 2016). To our knowledge, no studies have yet examined the effects of insect exuviae on rhizosphere microbial communities. While the addition of exuviae to soil may stimulate both bacteria and fungi, bacteria in particular are expected to be important colonizers of these materials. Bacterial communities are known to respond more quickly and strongly to chitin inputs and are generally regarded as key drivers of chitin degradation in soil (Cretoiu et al., 2013; Kielak et al., 2013). The aim of the present study was thus to investigate whether soil amendment with the exuviae of different insect species stimulates bacterial growth and to determine how it influences the diversity and composition of the bacterial community in the rhizosphere of cabbage plants. As exuviae from different species differentially affect the performance of root maggots on cabbage plants, they were expected to induce distinct changes in the rhizosphere bacterial community of this crop (Wantulla et al., 2022).

Materials and methods

Insect exuviae and soil

The exuviae of three different insect species were used in this study: Exuviae of black soldier fly larvae, *Hermetia illucens* (Bestico, Berkel en Rodenrijs, the Netherlands), exuviae of house crickets, *Acheta domesticus* (Protix, Bergen op Zoom, the Netherlands), and exuviae of yellow mealworms, *Tenebrio molitor* (Nijenkamp Voederdieren, Hellendoorn, the Netherlands). All materials were inspected for the presence of insects or insect fragments other than exuviae, which were removed. Although current EU regulations for insect production residues require heating at 70 °C for 1 h (European Union, 2021b), such guidelines did not exist when the experiment reported here was conducted. For the present study, the exuviae were oven-dried at 60 °C for 24 h to allow for soil amendment on a dry matter basis. The dried exuviae were subsequently ground to a powder with an SM 100 cutting mill (Retsch, Haan, Germany).

Agricultural soil was collected from the upper 10 - 20 cm mineral layer of an organically managed field in Wageningen, the Netherlands in October 2018. The field had been used to grow various brassicaceous plants since 2011 and black mustard (*Brassica nigra* L.) had recently been grown at the location from which the soil was collected. Soil composition as assessed for the same field by Eurofins Agro (Wageningen, the Netherlands) in 2018 was 81% sand, 14% silt and 2% clay, while the soil organic matter content was 3.2%. The soil was homogenized by sieving (particle size < 4 mm) and stored at 4 °C for 1 month before being mixed with black soldier fly, house cricket or mealworm exuviae at a ratio of 1 or 10 g/kg of dry soil.

Plants and growth conditions

Brassica oleracea L. var. *gemmifera* cv. Cyrus (Brussels sprouts) plants were kept in a greenhouse compartment at 20 ± 3 °C, 50-70% relative humidity and 16 h light/8 h dark photoperiod. In a completely randomized design, 15 plants per treatment were grown on a single greenhouse bench. Plants were grown in 1 L plastic pots, which were individually placed in saucers. Two seeds were sown per pot and gently pressed down. If both seeds germinated, one seedling was randomly removed from each pot after 1 week. Excess seedlings were transplanted to pots of the same treatment in which no seeds had germinated or were discarded together with ungerminated seeds if not needed. All plants were watered three times per week. Rhizosphere samples were collected from five different plants of each treatment at three different time points as described below. At the end of the experiment, shoots of the 8-week-old plants were harvested separately during rhizosphere sampling and weighed.

Rhizosphere sampling and DNA isolation

Five plants per treatment were uprooted after 2, 4 and 8 weeks of growth to collect rhizosphere samples according to Lundberg et al. (2012). After 8 weeks, three or four, instead of five, plants were sampled for some treatments because in total three plants had died towards the end of the experiment. These three plants had died for unknown reasons. Before the collection of each sample, gloves were cleaned with 70% ethanol. Roots were manually separated from loose soil by kneading and shaking. Entire root systems with a soil layer of ca. 1 mm thickness attached to their surface were then collected in 50 ml tubes containing 25 ml of sterile phosphate buffer (6.33 g $NaH_2PO_4 \cdot H_2O$, 10.96 g $Na_2HPO_4 \cdot 2H_2O$ and 200 µL Silwet L-77 per L). Tubes were vortexed at maximum speed for 15 s, roots were removed and the resulting suspensions were centrifuged for 15 min at 1800 *g*. Supernatants were discarded and pellets were stored at -25 °C until further processing for DNA isolation. DNA was extracted from 50 mg of each rhizosphere sample using the DNeasy PowerSoil Pro Kit (QIAGEN, Venlo, the Netherlands). DNA quantity and quality were checked using

the DeNovix DS-11 Fluorometer and dsDNA Broad Range assay (DeNovix, Wilmington, Delaware, USA). Rhizosphere samples that were too small for the extraction of sufficient DNA were not processed further and excluded from analyses. At each time point, DNA was extracted from 3-5 samples per treatment and all replicates were used for subsequent 16S rRNA gene quantification and sequencing.

Quantification of bacterial 16S rRNA gene numbers

Quantitative PCR of bacterial 16S rRNA genes was carried out in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA). Reaction mixes had a total volume of 20 µL and contained 10 µL SensiFAST SYBR No-ROX mix (Bioline Reagents, London, United Kingdom), 0.4 µL of each primer (25 µM; Eurofins Genomics, Ebersberg, Germany), 4.2 µL H_2O and 5 µL template DNA. Primers used were EUB338/EUB518 (Fierer et al., 2005). Reaction conditions were 3 min at 95 °C, followed by 40 cycles of 95 °C for 10 s, 59 °C for 10 s and 72 °C for 30 s. All DNA sample reactions were run in duplicate. A standard series of 10^3-10^8 *Bacillus circulans* 16S rRNA gene fragments was prepared for each plate by making triplicate 10-fold dilutions and target gene numbers were calculated for each sample using a standard curve.

Amplicon sequencing of the 16S rRNA gene V4 region

Rhizosphere samples from the high exuviae content treatments (10 g/kg) and control samples were submitted to the Centre d'expertise et de services Génome Québec (Montréal, Québec, Canada) for 16S rRNA gene amplicon sequencing on the Illumina MiSeq system. The primers 515F/806R (Caporaso et al., 2011) were used to target the V4 region of the gene. Generated FASTQ files were demultiplexed and non-biological nucleotides were removed by the sequencing provider. Primers used to amplify the V4 region were removed in R (Version 3.6.3; R Core Team, 2020) using the *filterAndTrim* function of the dada2 package and the sequencing data was further processed using the DADA2 pipeline following the standard operating procedure (Callahan et al., 2016). On average, 16028 reads per sample remained after filtering, denoising, merging paired reads and the removal of chimeras. Taxonomy was assigned to amplicon sequence variants (ASVs) up to the genus level using the Silva reference database version 132 (Yilmaz et al., 2014).

Statistical analysis

All statistical analyses were carried out in R. The ASV table produced by the DADA2 pipeline was normalized by cumulative-sum scaling using the *cumNorm* function of the

metagenomeSeq package (Paulson et al., 2013). Shannon indices were calculated as a measure of bacterial alpha diversity using the phyloseq package (McMurdie & Holmes, 2013) and were analyzed with linear models (LM) using the packages stats (R Core Team, 2020) and car (Fox & Weisberg, 2019). Pairwise comparisons were performed using estimated marginal means (EMM) with the package emmeans (Lenth, 2021). For beta diversity, Bray-Curtis dissimilarity matrices were used to perform principal coordinate analyses (PCoA) and permutational multivariate analyses of variance (PERMANOVA) using the packages phyloseq and vegan (Oksanen et al., 2020). Relative abundances of bacterial phyla, families and genera contributing more than 1% to the total number of sequences as well as plant shoot biomass and bacterial 16S rRNA gene numbers were analyzed using LMs and pairwise comparisons of EMMs. LMs were validated by plotting residuals and homogeneity of variances and normality were confirmed with Levene's test and the Shapiro-Wilk test using the packages car and stats, respectively. Log transformations were applied to meet model assumptions where necessary and nonparametric tests were performed using the package dunn.test (Dinno, 2017) if the assumptions of homogeneity or normality were violated. Relative abundances of families and genera that were significantly different between the control and at least one of the soil amendments were used to order samples within heatmaps using PCoA ordination on Bray-Curtis dissimilarities. Heatmaps were created with the phyloseq implementation of the NeatMap approach (Rajaram & Oono, 2010).

Results

Plant growth

Soil amendment with insect exuviae significantly affected shoot fresh biomass of *B. oleracea* plants after 8 weeks of growth (LM: $F = 312.6$, $df = 6$, $P < 0.001$). When soil was amended with exuviae at a ratio of 1 g/kg, shoot biomass was significantly increased by house cricket (EMM: $P < 0.001$; Figure 1) and mealworm exuviae (EMM: $P = 0.008$; Figure 1) compared to the control. Amendment with house cricket exuviae resulted in significantly higher shoot biomass than amendment with black soldier fly (EMM: $P < 0.001$; Figure 1) or mealworm exuviae (EMM: $P = 0.028$; Figure 1). When soil was amended with 10 g/kg, exuviae of all three insect species significantly increased shoot biomass compared to the control (EMM, $P < 0.001$; Figure 1). Amendment with house cricket exuviae resulted in significantly higher shoot biomass than amendment with exuviae of either of the other two insect species (EMM: $P < 0.001$; Figure 1). In all cases, shoot biomass of plants grown in soil amended with 10 g/kg was significantly higher compared to plants grown in soil amended with 1 g/kg (EMM: $P < 0.001$; Figure 1).

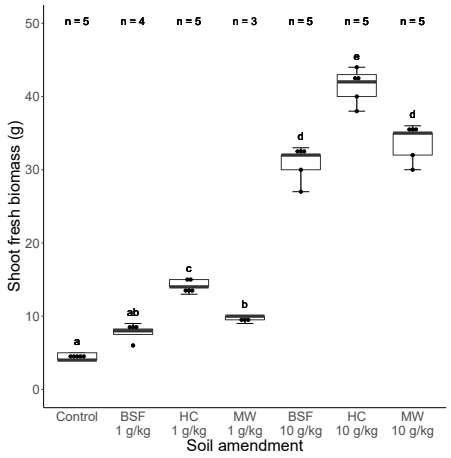


Figure 1. Shoot fresh biomass of 8-week-old Brassica oleracea plants grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 1 or 10 g/kg. Treatments denoted with the same letter are not significantly different (EMM, P > 0.05). Box plot whiskers represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants per treatment (n) are indicated at the top of the panels.

Total bacterial abundance

Soil amendment with insect exuviae had a significant effect on bacterial 16S rRNA gene numbers in the *B. oleracea* rhizosphere after 2 weeks (LM: $F = 18.123$, $df = 6$, $P < 0.001$), 4 weeks (LM: $F = 4.2247$, $df = 6$, $P = 0.004$) and 8 weeks of plant growth (LM: $F = 29.98$, $df = 6$, $P < 0.001$). Compared to the control, 16S rRNA gene numbers were significantly increased by house cricket (EMM: $P = 0.013$; Figure 2A) and mealworm exuviae (EMM: $P = 0.002$; Figure 2A) after 2 weeks and by black soldier fly (EMM: $P = 0.032$; Figure 2B) and mealworm exuviae (EMM: $P = 0.004$; Figure 2B) after 4 weeks when soil was amended with 10 g/kg. After 8 weeks, exuviae of all three insect species significantly increased 16S rRNA gene numbers when soil was amended with 10 g/kg (EMM: $P < 0.001$; Figure 2C).

Bacterial alpha and beta diversity

The Shannon indices of bacterial communities in the *B. oleracea* rhizosphere were significantly affected by soil amendment with insect exuviae after 2 weeks (LM: $F = 11.244$, $df = 3$, $P < 0.001$), 4 weeks (LM: $F = 24.354$, $df = 3$, $P < 0.001$) and 8 weeks of plant growth (LM: $F = 21.178$, $df = 3$, $P < 0.001$). Shannon indices were significantly lower than in the control following amendment with house cricket (EMM: $P = 0.001$; Figure 3A) or mealworm exuviae (EMM: $P = 0.005$; Figure 3A) after 2 weeks, 4 weeks (EMM: $P < 0.001$; Figure 3C) and 8 weeks (EMM: $P < 0.001$; Figure 3E). Soil amendment with black soldier fly exuviae resulted in a significantly lower Shannon index as compared to the control only after 8 weeks (EMM: $P = 0.015$; Figure 3E). The Shannon index was significantly lower following amendment with mealworm exuviae than when soil was amended with black soldier fly exuviae after 4 weeks (EMM: $P = 0.002$; Figure 3C) and was lower following amendment with house cricket exuviae at all three time points (EMM: $P < 0.05$; Figures 3A, C and E).

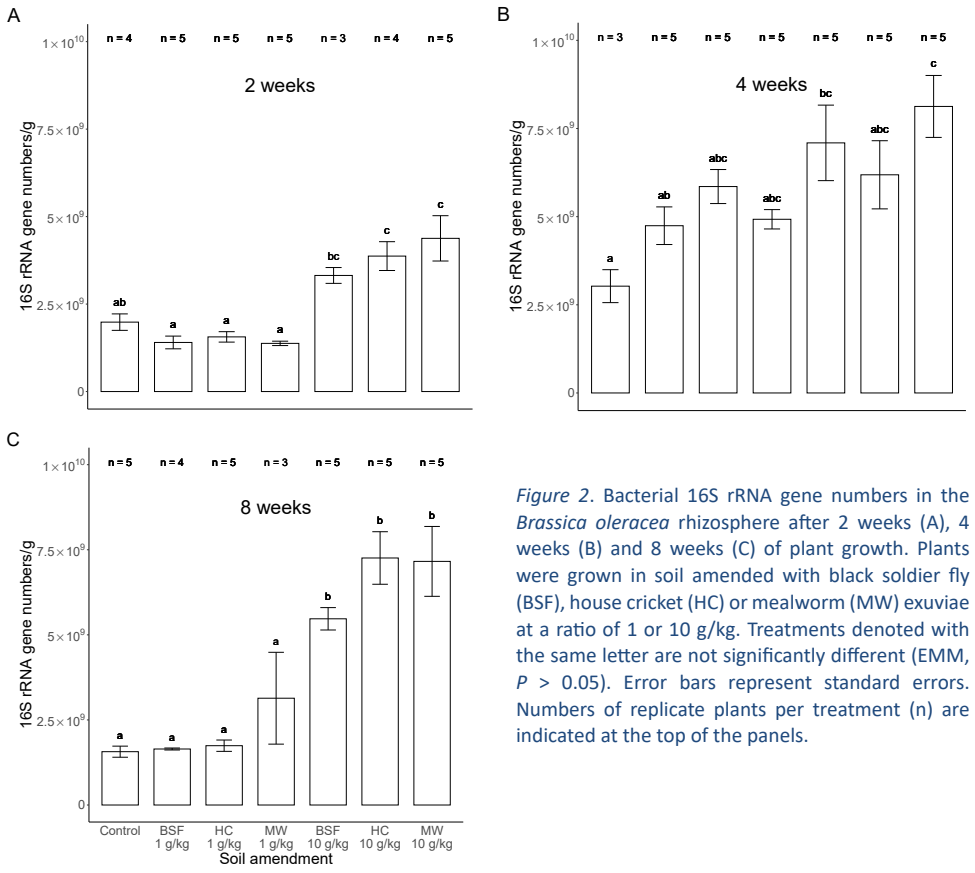


Figure 2. Bacterial 16S rRNA gene numbers in the *Brassica oleracea* rhizosphere after 2 weeks (A), 4 weeks (B) and 8 weeks (C) of plant growth. Plants were grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 1 or 10 g/kg. Treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Error bars represent standard errors. Numbers of replicate plants per treatment (n) are indicated at the top of the panels.

PCoAs using Bray-Curtis metrics showed that bacterial communities in the *B. oleracea* rhizosphere separated by soil amendment (Figures 3B, D and F). After 2 weeks of plant growth, rhizosphere communities that were exposed to the different amendments separated from the control (Figure 3B). After 4 weeks, the rhizosphere communities of control plants and of plants grown in soil amended with black soldier fly exuviae formed distinct clusters; the communities of plants grown in soil amended with house cricket or mealworm exuviae were also distinct from the control and from the black soldier fly treatment; yet they were similar to each other (Figure 3D). After 8 weeks, the rhizosphere communities of control plants and of plants grown in soil amended with house cricket exuviae clustered separately, whereas the communities of plants grown in soil amended with black soldier fly or mealworm exuviae were similar to each other; yet the latter two also separated clearly from the communities of the control and house cricket treatment (Figure 3F). PERMANOVA based on Bray-Curtis dissimilarity matrices confirmed that soil amendment with insect exuviae had a significant effect on the composition of rhizosphere bacterial communities after

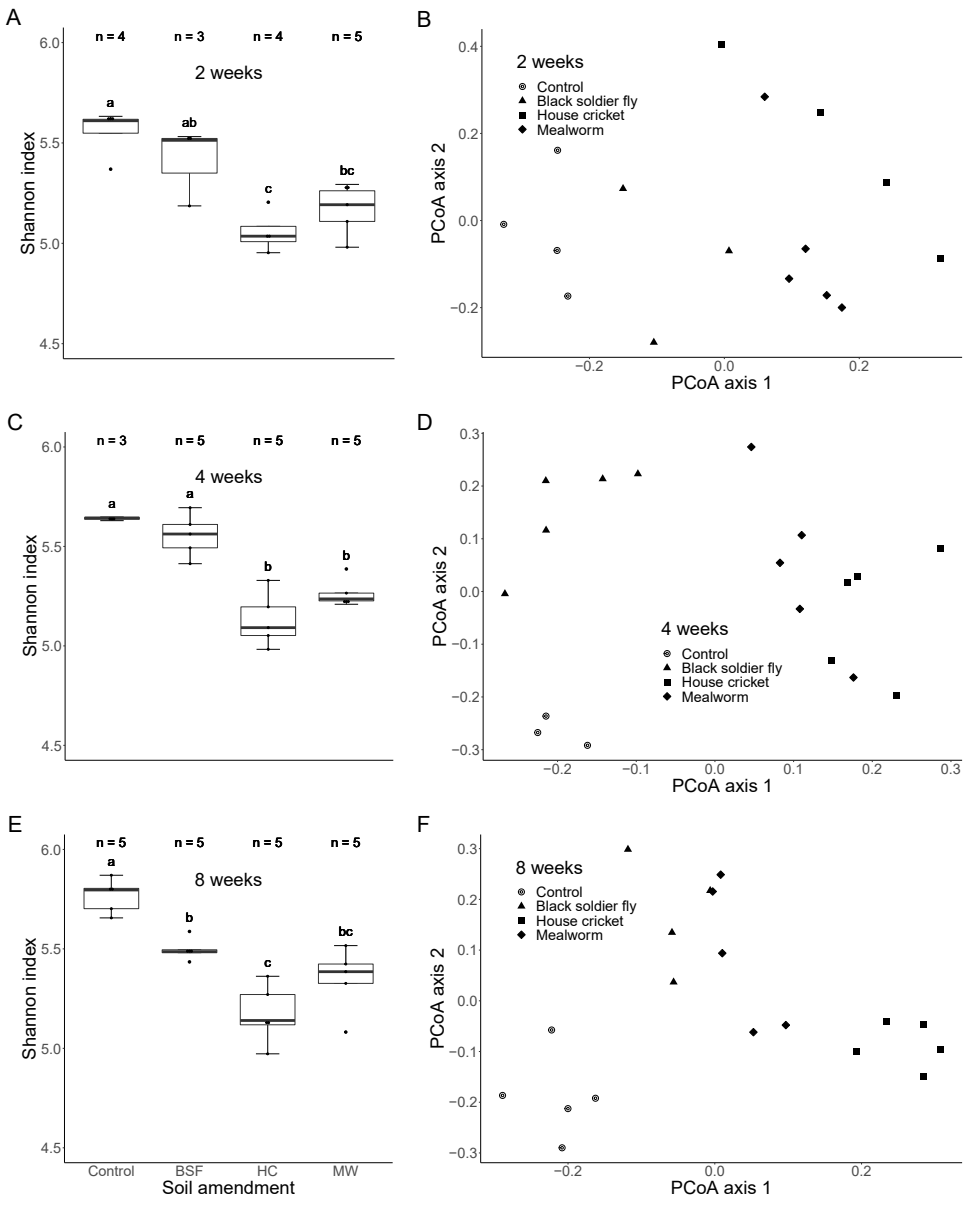


Figure 3. Shannon indices and PCoAs of bacterial communities in the *Brassica oleracea* rhizosphere after 2 weeks (A and B), 4 weeks (C and D) and 8 weeks (E and F) of plant growth. Plants were grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 10 g/kg. Shannon indices of treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Box plot whiskers represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants per treatment (n) are indicated at the top of the panels.

2 weeks (22.21% of the variation, $P < 0.001$), 4 weeks (20.27% of the variation, $P < 0.001$) and 8 weeks of plant growth (18.95% of the variation, $P < 0.001$).

Bacterial community composition

Significant differences in the proportions of bacterial taxa in the *B. oleracea* rhizosphere between the control and at least one of the soil amendments were observed for nine phyla (Table 1), 37 families (Table S1) and 34 genera (Table S2). In the heatmap presenting relative abundances of families that were significantly different after 2 weeks, samples grouped according to soil amendment (Figure 4A). Samples grouped similarly after 4 weeks, with the exception of outlying samples BSF6 and MW8 (Figure 4B), and 8 weeks, with the exception of outlier MW14 (Figure 4C). In the heatmaps presenting relative abundances of genera that were significantly different between the control and at least one of the soil amendments after 2 weeks and 4 weeks, samples largely grouped according to soil amendment, with the only outliers being samples MW2 (Figure 5A) and MW9 (Figure 5B), respectively. After 8 weeks, samples grouped similarly as after 2 and 4 weeks, with samples BSF10, MW14 and MW15 as the only outliers (Figure 5C).

At the phylum level, soil amendment with exuviae of any of the three insect species significantly increased the relative abundance of Bacteroidetes after 8 weeks and of Proteobacteria after 2 and 8 weeks of plant growth as compared to the control (EMM: $P < 0.05$; Table 1). Amendment with house cricket exuviae caused a significantly greater increase in relative abundance of Bacteroidetes than either of the other soil amendments (EMM: $P < 0.05$; Table 1). It was the only amendment that significantly increased the relative abundance of Firmicutes after 2 weeks and caused a significantly greater increase in relative abundance of this phylum than amendment with mealworm exuviae after 4 weeks (EMM: $P < 0.001$; Table 1). Soil amendment with black soldier fly exuviae caused a significantly greater increase in relative abundance of Proteobacteria than either of the other amendments after 2 weeks (EMM: $P < 0.05$; Table 1). All amendments significantly reduced the relative abundances of Chloroflexi and Acidobacteria at all time points (EMM: $P < 0.05$; Table 1), whereas the relative abundance of Cyanobacteria was significantly reduced only after 4 weeks (EMM: $P < 0.001$; Table 1). Soil amendment with house cricket exuviae caused a significantly greater reduction in relative abundance of Chloroflexi and Acidobacteria than either of the other amendments after 8 weeks and 2 weeks, respectively (EMM: $P < 0.05$; Table 1). It was the only amendment that significantly reduced the relative abundance of Armatimonadetes, whereas only amendment with black soldier fly exuviae significantly reduced the relative abundance of Firmicutes (EMM: $P < 0.001$; Table 1). Both of these reductions occurred after 8 weeks and were also significant compared to both other soil amendments (EMM: $P < 0.05$; Table 1).

Table 1

Relative abundances of bacterial phyla in the Brassica oleracea *rhizosphere that were significantly different from the control after 2, 4 or 8 weeks of plant growth following soil amendment with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at 10 g/kg (% ± standard error)*

Phylum	2 weeks				4 weeks				8 weeks			
	Control (n = 4)	BSF (n = 3)	HC (n = 4)	MW (n = 5)	Control (n = 3)	BSF (n = 5)	HC (n = 5)	MW (n = 5)	Control (n = 5)	BSF (n = 5)	HC (n = 5)	MW (n = 5)
Actinobacteria	24.31 ±1.92^{a}	22.44 ±1.20^{a}	**30.04 ±0.92^{b}**	**31.50 ±0.89^{b}**	21.58 ±0.96^{a}	25.59 ±1.30^{a}	26.06 ±2.49^{a}	29.35 ±1.48^{a}	26.26 ±1.12^{a}	32.07 ±1.29^{ab}	29.04 ±2.36^{ab}	**32.65 ±1.18^{b}**
Firmicutes	10.56 ±0.65^{a}	8.72 ±0.63^{a}	**17.64 ±0.58^{b}**	11.02 ±0.85^{a}	8.82 ±0.87^{a}	8.93 ±0.42^{a}	**18.58 ±0.62^{c}**	**12.72 ±0.98^{b}**	13.28 ±0.45^{ab}	7.36 ±0.48^{c}	15.76 ±0.77^{a}	10.81 ±1.02^{b}
Proteobacteria	24.81 ±0.74^{a}	**38.35 ±0.31^{c}**	**30.46 ±0.61^{b}**	**32.46 ±0.96^{b}**	32.30 ±1.60^{ab}	37.37 ±1.55^{b}	28.74 ±1.29^{a}	32.03 ±2.06^{ab}	22.48 ±0.94^{a}	**31.22 ±1.38^{b}**	**32.30 ±1.88^{b}**	**30.24 ±1.59^{b}**
Verrucomicrobia	3.53 ±0.18^{ab}	4.19 ±0.48^{a}	2.59 ±0.20^{bc}	2.34 ±0.25^{c}	3.47 ±0.71^{a}	3.65 ±0.44^{a}	2.55 ±0.23^{a}	2.81 ±0.20^{a}	3.58 ±0.35^{a}	2.98 ±0.21^{ab}	2.06 ±0.13^{b}	2.79 ±0.21^{ab}
Chloroflexi	11.00 ±0.87^{a}	7.41 ±0.45^{b}	5.04 ±0.29^{b}	6.61 ±0.37^{b}	9.67 ±0.62^{a}	6.86 ±0.48^{b}	5.71 ±0.40^{b}	5.90 ±0.72^{b}	11.98 ±0.39^{a}	8.39 ±0.27^{b}	4.14 ±0.34^{c}	6.55 ±0.79^{b}
Acidobacteria	8.90 ±0.28^{a}	5.52 ±0.45^{b}	3.05 ±0.40^{c}	5.85 ±0.62^{b}	8.41 ±0.06^{a}	4.46 ±0.57^{b}	2.72 ±0.57^{b}	4.56 ±0.21^{b}	8.12 ±0.74^{a}	4.33 ±0.35^{b}	1.27 ±0.37^{c}	3.31 ±0.73^{bc}
Bacteroidetes	2.61 ±0.31^{ab}	4.14 ±0.28^{a}	3.73 ±0.62^{a}	1.88 ±0.32^{b}	3.02 ±0.43^{a}	2.65 ±0.38^{a}	4.20 ±0.77^{a}	2.81± 0.46^{a}	1.11 ±0.17^{a}	**2.28 ±0.19^{b}**	**6.00 ±0.95^{c}**	**2.77 ±0.42^{b}**
Cyanobacteria	4.65 ±2.45^{a}	0.26 ±0.14^{a}	0.43 ±0.16^{a}	0.20 ±0.10^{a}	2.14 ±0.25^{a}	0.33 ±0.07^{b}	0.53 ±0.10^{b}	0.38 ±0.15^{b}	0.89 ±0.29^{a}	0.52 ±0.11^{a}	0.64 ±0.18^{a}	0.52 ±0.10^{a}
Armatimonadetes	0.73 ±0.27^{a}	0.37 ±0.05^{a}	0.51 ±0.18^{a}	0.52 ±0.26^{a}	0.92 ±0.33^{a}	0.66 ±0.13^{a}	0.30 ±0.04^{a}	0.81 ±0.17^{a}	1.09 ±0.23^{a}	0.75 ±0.15^{a}	0.06 ±0.04^{b}	0.83 ±0.05^{a}

Note. Treatments denoted with the same letter are not significantly different (EMM/Dunn's test, $P > 0.05$). Bold relative abundances are significantly higher than in the control. Underlined relative abundances are significantly lower than in the control.

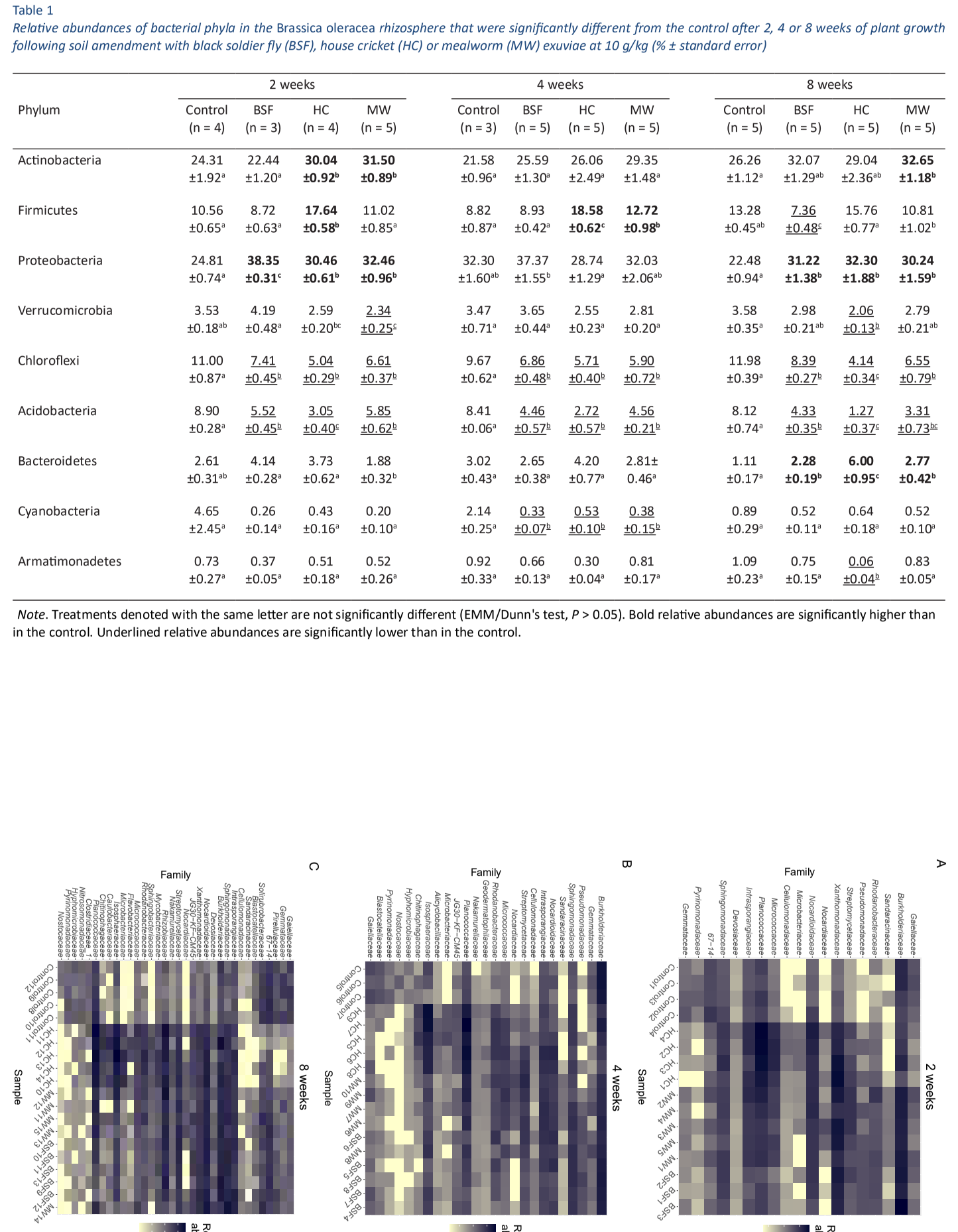
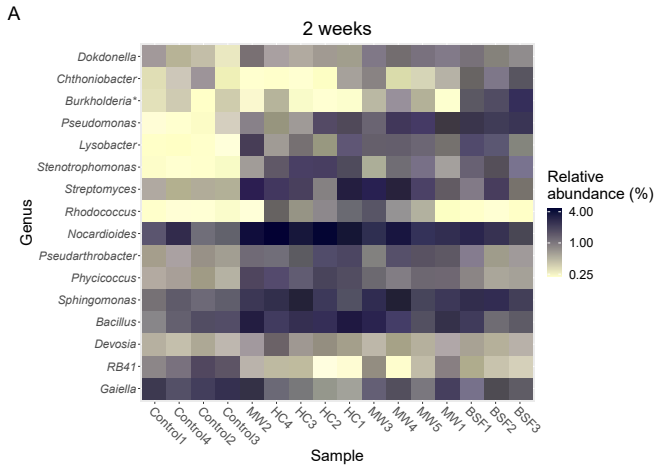
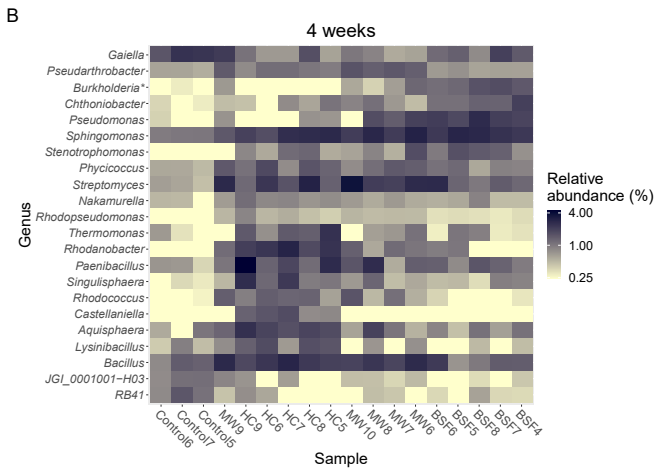


Figure 4. Relative abundances of bacterial families in the *Brassica oleracea* rhizosphere that were significantly different from the control after 2 weeks (A), 4 weeks (B) and 8 weeks (C) of plant growth (EMM/Dunn's test: $P < 0.05$). Plants were grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 10 g/kg. Families and individual samples are shown in rows and columns, respectively, and are ordered based on PCoA ordination.





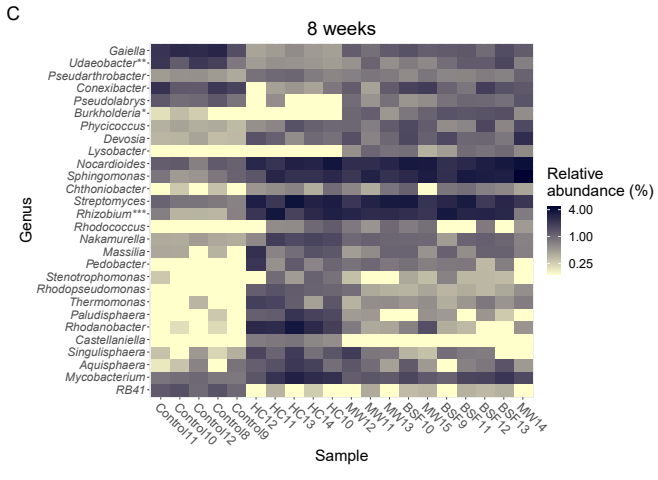


Figure 5. Relative abundances of bacterial genera in the *Brassica oleracea* rhizosphere that were significantly different from the control after 2 weeks (A), 4 weeks (B) and 8 weeks (C) of plant growth (EMM/Dunn's test: $P < 0.05$). Plants were grown in soil amended with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at a ratio of 10 g/kg. Genera and individual samples are shown in rows and columns, respectively, and are ordered based on PCoA ordination.
*Including *Caballeronia* and *Paraburkholderia*.
***Candidatus_Udaeobacter*.
***Including *Allorhizobium*, *Neorhizobium* and *Pararhizobium*.

At the family level, soil amendment with any of the insect exuviae significantly increased the relative abundances of *Xanthomonadaceae*, *Sphingomonadaceae*, *Rhizobiaceae*, *Rhodanobacteraceae*, *Pseudomonadaceae*, *Nocardioidaceae*, *Intrasporangiaceae*, *Streptomycetaceae*, *Cellulomonadaceae*, *Isosphaeraceae* and *Sphingobacteriaceae* at one or more time points (EMM: $P < 0.05$; Table S1). Amendment with house cricket exuviae caused a significantly greater increase in relative abundance of *Isosphaeraceae* than either of the other soil amendments after 8 weeks (EMM: $P < 0.05$; Table S1) and was the only amendment that significantly increased the relative abundance of this family already after 4 weeks (EMM: $P < 0.001$; Table S1). Similarly, it caused a significantly greater increase in relative abundance of *Planococcaceae* than amendment with mealworm exuviae after 4 weeks and was the only soil amendment that significantly increased the relative abundance of this family after 2 weeks and 8 weeks (EMM: $P < 0.001$; Table S1). Only amendment with house cricket exuviae significantly increased the relative abundances of *Caulobacteraceae*, *Nakamurellaceae*, *Microbacteriaceae*, *Alicyclobacillaceae*, *Chitinophagaceae* and *Flavobacteriaceae* at one or more time points (EMM/Dunn's test: $P < 0.05$; Table S1). These increases were also significant compared to both other soil amendments for *Microbacteriaceae* (EMM: $P < 0.05$; Figure 6A), *Alicyclobacillaceae* (EMM: $P < 0.05$; Figure 6B), *Chitinophagaceae* (EMM: $P < 0.05$; Figure 6C) and *Caulobacteraceae* (EMM: $P < 0.001$; Figure 6D). Soil amendment with black soldier fly exuviae was the only amendment that significantly increased the relative abundance of *Pseudomonadaceae* both after 2 weeks and 4 weeks (EMM: $P < 0.001$; Table S1). It was the only amendment that significantly increased the relative abundance of *Burkholderiaceae*, with increases occurring after 2 weeks and 8 weeks (EMM: $P < 0.05$; Table S1). Amendment with mealworm exuviae caused a significantly greater increase in relative abundance of *Nocardiaceae* than amendment with house cricket exuviae after 8 weeks (EMM: $P = 0.036$; Table S1) and was the only soil amendment that significantly increased the relative abundance of this family already after 2 weeks (EMM: $P = 0.014$; Table S1). At each time point, it was the only amendment that significantly increased the relative abundance of *Sandaracinaceae* (EMM: $P < 0.001$; Table S1). This increase was also significant compared to both other soil amendments (EMM: $P < 0.05$; Figure 6E). All amendments significantly reduced the relative abundances of *Nitrosomonadaceae*, *Hyphomicrobiaceae*, *Gaiellaceae*, *Blastocatellaceae*, *Pyrinomonadaceae*, *Gemmataceae*, *Nostocaceae* and an unclassified family (67-14) belonging to the Actinobacteria at one or more time points (EMM/Dunn's test: $P < 0.05$; Table S1). Only amendment with house cricket exuviae significantly reduced the relative abundances of *Blastocatellaceae* and *Nostocaceae* both after 4 and 8 weeks (EMM/Dunn's test: $P < 0.05$; Table S1). It caused a significantly greater reduction in relative abundance of *Gaiellaceae* than either of the other soil amendments after 8 weeks (EMM: $P < 0.001$; Table S1) and was the only amendment that significantly reduced the relative abundance of this family already after 2 weeks (EMM: $P = 0.008$; Table S1) and 4

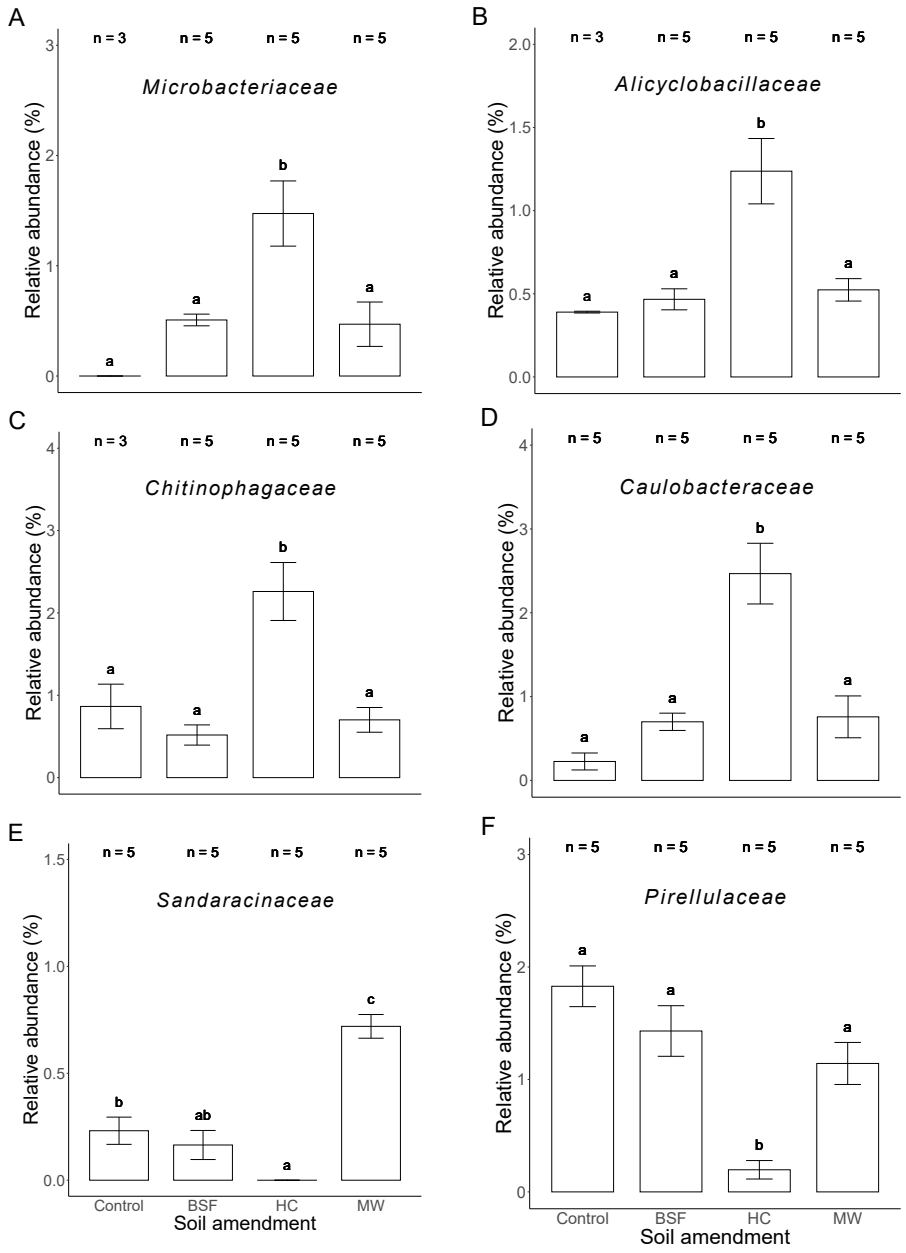


Figure 6. Relative abundances of bacterial families in the *Brassica oleracea* rhizosphere that were differentially enriched or depleted by soil amendment with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae after 4 weeks (A, B and C) or 8 weeks (D, E and F) of plant growth. Soil was amended with exuviae at a ratio of 10 g/kg. Treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Error bars represent standard errors. Numbers of replicate plants per treatment (n) are indicated at the top of the panels.

weeks (EMM: $P = 0.037$; Table S1). Soil amendment with house cricket exuviae was the only amendment that significantly reduced the relative abundances of *Sandaracinaceae*, *Solirubrobacteraceae* and *Pirellulaceae*, in all cases after 8 weeks (EMM: $P < 0.05$; Table S1). The reduction in relative abundance of *Pirellulaceae* was also significant compared to both other soil amendments (EMM: $P < 0.05$; Figure 6F). There were no families of which relative abundances were significantly reduced only by amendment with black soldier fly or mealworm exuviae.

Soil amendment with the exuviae of any insect species significantly increased the relative abundances of the genera *Stenotrophomonas*, *Lysobacter*, *Sphingomonas*, *Pseudomonas*, *Devosia*, *Nocardioides*, *Phycicoccus*, *Streptomyces* and a group of different *Rhizobiaceae* (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*) at one or more time points (EMM: $P < 0.05$; Table S2). Only amendment with house cricket exuviae significantly increased the relative abundances of the genera *Stenotrophomonas*, *Rhodopseudomonas* and *Rhodanobacter* not only after 2 weeks or 4 weeks but also after 8 weeks and of the genus *Rhodococcus* already after 2 weeks (EMM/Dunn's test: $P < 0.05$; Table S2). It caused a significantly greater increase in relative abundance of the genus *Rhodanobacter* than amendment with mealworm exuviae after 4 weeks (EMM: $P = 0.002$; Table S2). Soil amendment with house cricket exuviae was the only amendment that significantly increased the relative abundances of the genera *Castellaniella*, *Thermomonas*, *Mycobacterium*, *Nakamurella*, *Lysinibacillus*, *Paenibacillus*, *Aquisphaera*, *Singulisphaera*, *Paludisphaera* and *Pedobacter* at one or more time points (EMM/Dunn's test: $P < 0.05$; Table S2). These increases were also significant compared to both other soil amendments for the genera *Thermomonas* (EMM: $P < 0.05$; Figure 7A), *Lysinibacillus* (EMM: $P < 0.05$; Figure 7B), *Aquisphaera* (EMM: $P < 0.05$; Figure 7C), *Singulisphaera* (EMM: $P < 0.05$; Figure 7D), *Castellaniella* (Dunn's test: $P < 0.05$; Figure 7E) and *Paludisphaera* (EMM: $P < 0.001$; Figure 7F). Only amendment with black soldier fly exuviae significantly increased the relative abundance of the genus *Pseudomonas* after 2 and after 4 weeks (EMM: $P < 0.001$; Table S2). It caused a significantly greater increase in relative abundance of a group of different *Burkholderiaceae* (*Burkholderia-Caballeronia-Paraburkholderia*) than amendment with mealworm exuviae after 4 weeks (EMM: $P < 0.001$; Table S2) and 8 weeks (EMM: $P = 0.046$; Table S2) and was the only soil amendment that significantly increased the relative abundance of these genera already after 2 weeks (EMM: $P < 0.001$; Table S2). Amendment with mealworm exuviae caused a significantly greater increase in relative abundance of the genus *Pseudarthrobacter* than amendment with house cricket exuviae after 4 weeks (EMM: $P < 0.001$; Table S2). There were no genera of which relative abundances were significantly increased only by amendment with black soldier fly or mealworm exuviae. All soil amendments significantly reduced the relative abundances of the genus *Gaiella* after 8 weeks (EMM: $P < 0.001$; Table S2) and of an unclassified genus

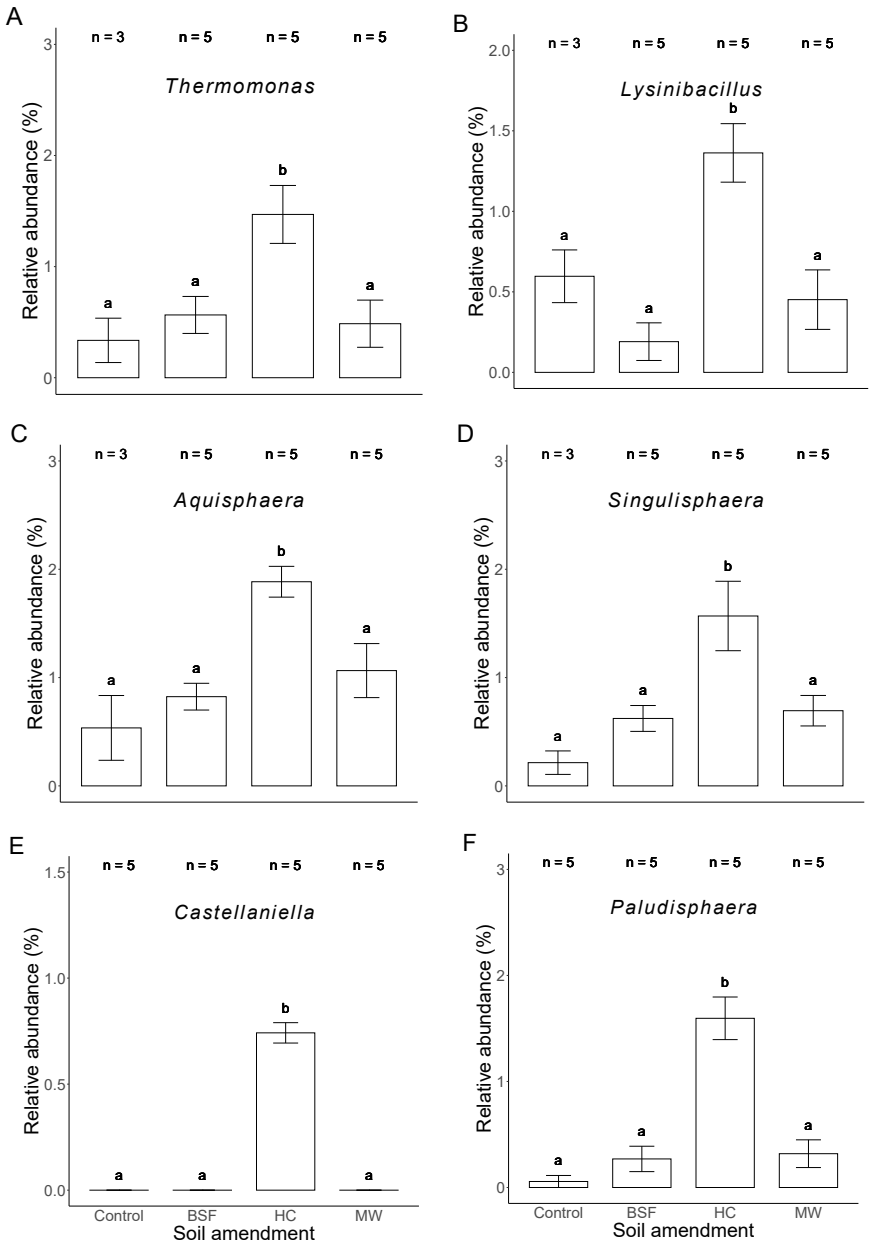


Figure 7. Relative abundances of bacterial genera in the *Brassica oleracea* rhizosphere that were differentially enriched by soil amendment with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae after 4 (A, B, C and D) or 8 weeks of plant growth (E and F). Soil was amended with exuviae at a ratio of 10 g/kg. Treatments denoted with the same letter are not significantly different (EMM/Dunn's test, $P > 0.05$). Error bars represent standard errors. Numbers of replicate plants per treatment (n) are indicated at the top of the panels.

belonging to the *Pyrinomonadaceae* (RB41) at all time points (EMM: $P < 0.05$; Table S2). Amendment with house cricket exuviae caused a significantly greater reduction in relative abundance of the genus *Gaiella* than either of the other soil amendments (EMM: $P < 0.001$; Table S2) and was the only amendment that significantly reduced the relative abundance of this genus already after 2 weeks (EMM: $P = 0.008$; Table S2) and 4 weeks (EMM: $P = 0.037$; Table S2). It was the only soil amendment that significantly reduced the relative abundances of the genera *Pseudolabrys* and *Conexibacter*, in both cases after 8 weeks (EMM: $P < 0.05$; Table S2). The reduction in relative abundance of the genus *Pseudolabrys* was also significant compared to both other soil amendments (EMM: $P = 0.001$; Table S2). There were no genera whose relative abundances were significantly reduced only by amendment with black soldier fly or mealworm exuviae.

Discussion

The exuviae of all three insect species tested here positively affected *B. oleracea* growth in amended soil. Shoot fresh biomass was increased by a factor of 1.7-2.2 when soil was amended with 1 g/kg and by a factor of 7-9.5 when soil was amended with 10 g/kg. A previous study that used the same insect exuviae and soil showed that the effect of exuviae on *B. oleracea* biomass is comparable to that of synthetic fertilizer (Wantulla et al., 2022). As control plants did not receive any fertilizer in the present study, nutrient shortage in the soil might have limited plant growth and thus resulted in more pronounced effects of the soil amendments. Insect exuviae are rich in plant nutrients and may also stimulate plant growth-promoting bacteria (Barragán-Fonseca et al., 2022). It remains unknown to which extent the enhanced plant growth observed here can be attributed to bacterial plant growth promotion or whether it might have been the result of nutrient addition alone. Compared with shrimp-derived chitin, black soldier fly exuviae have been reported to contain more phosphate and potassium but less nitrogen (Postma et al., 2022). This nitrogen is partially present in the form of chitin and so only available to the plant upon bacterial degradation of the chitin. Soil amendment with chitin, a major component of exuviae, has previously been shown to increase the biomass of lettuce plants (Debode et al., 2016). Interestingly, differences in effects on plant growth between soil amendments with exuviae from different insect species do not seem to be related to their chitin content. House cricket exuviae, which by far had the strongest effect on plant biomass, have an intermediate chitin content between that of mealworm exuviae and the exuviae of black soldier fly larvae (Nurfikari and de Boer, 2021). Therefore, it is unlikely that the observed differences in plant growth are solely due to different chitin contents. Nonetheless, the contents of other components, such as proteins and lipids, might contribute to the nutrient content of insect exuviae. Compounds other than chitin may

also improve plant growth, either directly via nutrient supply or indirectly by stimulating plant-beneficial bacteria.

Bacterial growth in the *B. oleracea* rhizosphere was stimulated by the exuviae of each insect species when soil was amended with 10 g/kg. At a ratio of 1 g/kg, however, no significant changes in bacterial abundance occurred. Positive effects on the abundance of bacteria are consistent with research on chitin amendments, which have been shown to increase bacterial population densities in soil (Cretoiu et al., 2013; Kielak et al., 2013). While total abundance increased, bacterial diversity in the rhizosphere was diminished by soil amendment with any of the insect exuviae. This indicates that only specific groups of bacteria were able to use exuviae as a substrate and to proliferate in response to the soil amendments. Each amendment clearly shifted the structure of the bacterial community, which differed from the control in all cases. These findings are largely in line with what has been observed in chitin-enriched soil. Although amendment with chitin was not found to affect bacterial diversity in the rhizosphere of lettuce (Debode et al., 2016), it has been reported to reduce the diversity of bacteria in bulk soil (Andreo-Jimenez et al., 2021; Ootsuka et al., 2021). Furthermore, chitin amendment has been shown to alter microbial community structures in soil and in the plant rhizosphere (Andreo-Jimenez et al., 2021; Cretoiu et al., 2013; Debode et al., 2016). Nonetheless, it seems that the effects of insect exuviae on rhizosphere bacterial communities cannot be explained by chitin alone. Soil amendment with house cricket or mealworm exuviae resulted in lower community diversity than amendment with the exuviae of black soldier fly larvae, which contain a larger proportion of chitin. Among the exuviae tested here, those of mealworms and black soldier fly larvae reportedly have the lowest and highest chitin content, respectively (Nurfikari & Boer, 2021). However, their addition to soil resulted in rhizosphere communities that clustered close to each other after 8 weeks of plant growth, while those in soil amended with house cricket exuviae formed a distinct cluster. The influence of chitin-containing substrates on soil bacterial communities has been shown to depend on the nature of the chitin resource and is thought to be determined by co-occurring structural compounds (Bai, 2015). While so far only arthropod and fungal chitin resources had been described to affect bacterial communities differently, such differences also appear to exist between the exuviae of different insect species. Although soil amendment with exuviae affected bacterial community structure at all measured time points, separation of the communities by amendment became clearer for individual treatments after 4 and 8 weeks. Interestingly, the proportion of variation between communities that is attributed to soil amendment decreased over time. On the other hand, the number of bacterial families and genera that were enriched or depleted by at least one amendment increased with each time point. This indicates that rhizosphere communities in soil amended with exuviae continued to differentiate from those in unamended soil over a period of at least 8 weeks.

Most bacterial taxa that were enriched in the *B. oleracea* rhizosphere following soil amendment with any of the insect exuviae are known to be stimulated by chitin. Among them are the phyla Bacteroidetes and Proteobacteria (Cretoiu et al., 2014; Debode et al., 2016) as well as the families *Xanthomonadaceae* (Crocker et al., 2019; Ootsuka et al., 2021), *Pseudomonadaceae* (Crocker et al., 2019), *Streptomycetaceae* (Ootsuka et al., 2021; Shimoi et al., 2020), *Cellulomonadaceae* (Crocker et al., 2019) and *Sphingobacteriaceae* (Cretoiu et al., 2014). Genera that were enriched by each of the amendments and that have also been reported to respond positively to chitin are *Stenotrophomonas* (Jacquiod et al., 2013), *Lysobacter* (Iwasaki et al., 2020; Jacquiod et al., 2013), *Pseudomonas* (Andreo-Jimenez et al., 2021), *Devosia* (Andreo-Jimenez et al., 2021), *Nocardioides* (Jacquiod et al., 2013), *Streptomyces* (Debode et al., 2016; Iwasaki et al., 2020) and *Rhizobium* (Andreo-Jimenez et al., 2021). Indeed, all of these genera include species with plant growth-promoting or biocontrol properties (Chhetri et al., 2022; Gopalakrishnan et al., 2015; Hayward et al., 2010; Mercado-Blanco & Bakker, 2007; Nafis et al., 2019; Rey & Dumas, 2017; Ryan et al., 2009). This suggests that improved plant growth as a result of soil amendment with insect exuviae is, at least partly, mediated by beneficial soil bacteria that are involved in chitin degradation. The enrichment of several bacterial families and genera only following amendment with house cricket exuviae is particularly interesting, given the outstanding effect of this amendment on *B. oleracea* growth. Notable examples of genera that were stimulated by house cricket exuviae but that did not respond to black soldier fly or mealworm exuviae are *Lysinibacillus* and *Paenibacillus*, both of which are well-known for comprising various plant-beneficial species (Ahsan & Shimizu, 2021; Grady et al., 2016). Other genera that were only enriched by soil amendment with house cricket exuviae such as *Singulisphaera* or *Pedobacter* have sporadically been associated with enhanced plant growth (Gu et al., 2020; Morais et al., 2019). Moreover, some genera that only responded to house cricket exuviae, such as *Castellaniella* or *Thermomonas*, have been reported to play important roles in denitrification processes (McIlroy et al., 2016; Wu et al., 2022). Thus, soil nitrate levels might have been considerably higher after amendment with house cricket exuviae than after amendment with black soldier fly or mealworm exuviae. Among the taxa that were enriched by more than one soil amendment, certain bacterial families and genera were stimulated earlier or longer by house cricket exuviae than by the exuviae of either of the other insect species. This was the case for the genus *Stenotrophomonas* as well as for other genera that reportedly contain plant growth-promoting species such as *Rhodopseudomonas* or *Rhodococcus* (Francis & Vereecke, 2019; Hsu et al., 2021). In the same way, soil amendment with black soldier fly exuviae resulted in longer-lasting enrichment of the genus *Pseudomonas* and earlier as well as stronger enrichment of different *Burkholderiaceae* than either of the other amendments. Several *Pseudomonas* species do not only promote plant growth, but are also able to control insect pests or suppress plant diseases (Kupferschmied et al., 2013).

Similarly, many members of the genus *Burkholderia* exhibit biocontrol activity against plant pathogens, while others have been found to display insecticidal activity (Cordova-Kreylos et al., 2013; Eberl & Vandamme, 2016). More effective stimulation of these genera in the soil might explain the negative effects of black soldier fly exuviae on cabbage root maggots that were observed in a previous study (Wantulla et al., 2022).

In view of their effects on the composition of rhizosphere bacterial communities, insect exuviae seem to have an enormous potential for the promotion of plant growth and health. When added to soil, the exuviae of black soldier fly larvae, house cricket or mealworms all stimulated bacteria that have been demonstrated to improve plant growth and are likely to benefit plant health. Although various potentially beneficial bacteria were commonly enriched by these soil amendments, there were striking differences regarding the enrichment of important genera such as *Lysinibacillus, Paenibacillus* or *Pseudomonas*. The exuviae of each insect species might thus have a potential in their own right to be used in crop production. Depending on the crop, its pathogens and pests as well as soil quality, exuviae of particular species might be more or less useful. It is therefore essential to study the effects of insect exuviae on other plant species and their rhizosphere communities but also to compare them in different agricultural soils. In addition, more extensive research on the impact of soil amendment with exuviae on plant pathogens and pests is needed. While the promotion of naturally occurring beneficial bacteria is promising, an integrated application of insect exuviae with commercial microbial agents should also be considered. As introduced agents often suffer from poor field persistence, the use of organic amendments to aid their establishment in the soil has often been suggested. The results presented here provide a basis for testing the combined application of exuviae from certain insect species and selected beneficial bacteria.

Acknowledgements

This work was supported by the Dutch Research Council, NWO (grant number ALWGK.2016.010). We are grateful to Azkia Nurfikari for providing the *Bacillus circulans* DNA and her advice on processing and analyzing the amplicon sequencing data.

Data availability statement

The raw sequencing data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB58190 (https://www.ebi.ac.uk/ena/browser/view/PRJEB58190).

Supporting information

Table S1

Relative abundances of bacterial families in the Brassica oleracea *rhizosphere that were significantly different from the control after 2, 4 or 8 weeks of plant growth following soil amendment with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at 10 g/kg (% ± standard error)*

		2 weeks				4 weeks				8 weeks			
Phylum	Family	Control (n = 4)	BSF (n = 3)	HC (n = 4)	MW (n = 5)	Control (n = 3)	BSF (n = 5)	HC (n = 5)	MW (n = 5)	Control (n = 5)	BSF (n = 5)	HC (n = 5)	MW (n = 5)
Actino-bacteria	*Micrococcaceae*	1.22 ± 0.07^{a}	1.47 ± 0.18^{a}	**4.61 ± 0.33^{c}**	**2.95 ± 0.18^{b}**	1.04 ± 0.02^{a}	1.12 ± 0.04^{ab}	2.65 ± 0.32^{ab}	**2.97 ± 0.17^{b}**	0.86 ± 0.04^{a}	1.27 ± 0.06^{ab}	**2.37 ± 0.35^{b}**	**2.15 ± 0.13^{b}**
	Intrasporangia-ceae	2.19 ± 0.10^{a}	2.14 ± 0.20^{a}	**4.02 ± 0.38^{b}**	**3.72 ± 0.24^{b}**	1.97 ± 0.08^{a}	2.82 ± 0.29^{ab}	3.24 ± 0.41^{ab}	**3.62 ± 0.22^{b}**	1.68 ± 0.11^{a}	**3.38 ± 0.46^{b}**	2.73 ± 0.46^{ab}	**3.24 ± 0.27^{b}**
	Streptomyceta-ceae	0.55 ± 0.01^{a}	**2.21 ± 0.38^{b}**	**2.62 ± 0.29^{b}**	**3.35 ± 0.34^{b}**	0.57 ± 0.06^{a}	2.20 ± 0.30^{ab}	2.14 ± 0.51^{ab}	**3.40 ± 0.33^{b}**	0.87 ± 0.06^{a}	**3.57 ± 0.28^{b}**	**3.47 ± 0.21^{b}**	**3.32 ± 0.27^{b}**
	Mycobacteriaceae	1.00 ± 0.18^{a}	1.04 ± 0.24^{a}	0.69 ± 0.30^{a}	1.42 ± 0.07^{a}	1.07 ± 0.11^{a}	1.17 ± 0.06^{a}	1.39 ± 0.06^{a}	1.26 ± 0.13^{a}	0.89 ± 0.04^{a}	1.67 ± 0.17^{ab}	**2.31 ± 0.28^{b}**	1.32 ± 0.07^{ab}
	Nocardioidaceae	1.97 ± 0.36^{a}	3.20 ± 0.46^{ab}	**4.92 ± 0.42^{c}**	**4.10 ± 0.23^{bc}**	1.39 ± 0.34^{a}	**2.99 ± 0.45^{b}**	2.87 ± 0.60^{ab}	**3.00 ± 0.10^{b}**	1.39 ± 0.17^{a}	**4.49 ± 0.32^{b}**	**4.37 ± 0.38^{b}**	**4.42 ± 0.34^{b}**
	Solirubrobacter-aceae	2.30 ± 0.53^{ab}	1.71 ± 0.12^{b}	2.01 ± 0.28^{b}	3.39 ± 0.12^{a}	2.15 ± 0.37^{ab}	1.91 ± 0.17^{ab}	1.30 ± 0.30^{b}	2.40 ± 0.15^{a}	2.52 ± 0.16^{a}	1.93 ± 0.21^{ab}	0.90 ± 0.32^{b}	2.59 ± 0.38^{a}
	Nakamurellaceae	0.50 ± 0.19^{a}	0.60 ± 0.08^{a}	0.54 ± 0.19^{a}	0.59 ± 0.15^{a}	0.33 ± 0.17^{a}	0.59 ± 0.08^{ab}	**0.87 ± 0.07^{b}**	0.63 ± 0.03^{ab}	0.43 ± 0.02^{a}	0.96 ± 0.12^{ab}	**1.50 ± 0.22^{b}**	0.98 ± 0.07^{ab}
	Geodermatophi-laceae	0.43 ± 0.16^{a}	0.64 ± 0.08^{a}	0.73 ± 0.04^{a}	0.81 ± 0.18^{a}	0.38 ± 0.03^{a}	0.47 ± 0.03^{ab}	**0.85 ± 0.11^{c}**	**0.72 ± 0.13^{bc}**	0.50 ± 0.10^{a}	0.52 ± 0.02^{a}	1.05 ± 0.27^{a}	0.66 ± 0.09^{a}
	Cellulomonad-aceae	0.10 ± 0.10^{a}	0.49 ± 0.07^{ab}	**1.24 ± 0.19^{c}**	**0.62 ± 0.03^{b}**	0.21 ± 0.11^{a}	**0.91 ± 0.04^{c}**	**0.73 ± 0.12^{bc}**	0.46 ± 0.06^{ab}	0.21 ± 0.05^{a}	**0.63 ± 0.10^{b}**	0.08 ± 0.08^{a}	0.29 ± 0.13^{ab}
	67-14	2.86 ± 0.28^{a}	1.30 ± 0.17^{b}	1.46 ± 0.21^{b}	1.81 ± 0.27^{b}	2.25 ± 0.20^{ab}	2.28 ± 0.18^{ab}	1.65 ± 0.17^{b}	2.37 ± 0.19^{a}	3.30 ± 0.20^{a}	3.03 ± 0.14^{a}	1.29 ± 0.24^{b}	2.53 ± 0.32^{a}
	Microbacteriaceae	0.11 ± 0.11^{a}	0.48 ± 0.26^{ab}	**1.29 ± 0.18^{b}**	0.52 ± 0.22^{ab}	0.00 ± 0.00^{a}	0.51 ± 0.05^{a}	**1.47 ± 0.30^{b}**	0.47 ± 0.20^{a}	0.00 ± 0.00^{a}	0.55 ± 0.05^{ab}	**2.87 ± 0.40^{b}**	0.71 ± 0.12^{ab}
	Gaiellaceae	2.11 ± 0.18^{a}	1.42 ± 0.21^{ab}	0.90 ± 0.14^{b}	1.70 ± 0.25^{ab}	2.04 ± 0.27^{a}	1.33 ± 0.19^{ab}	0.94 ± 0.19^{b}	1.03 ± 0.28^{ab}	2.33 ± 0.24^{a}	1.27 ± 0.09^{b}	0.51 ± 0.03^{c}	1.15 ± 0.06^{b}
	Nocardiaceae	0.06 ± 0.06^{a}	0.11 ± 0.11^{a}	1.09 ± 0.20^{ab}	**1.31 ± 0.35^{b}**	0.09 ± 0.09^{a}	0.13 ± 0.08^{a}	**1.18 ± 0.19^{b}**	**1.74 ± 0.20^{b}**	0.00 ± 0.00^{a}	0.31 ± 0.19^{ab}	**0.75 ± 0.23^{b}**	**1.43 ± 0.10^{c}**
Firmicutes	*Planococcaceae*	3.02 ± 0.24^{a}	2.79 ± 0.30^{a}	**8.01 ± 0.49^{b}**	4.31 ± 0.34^{a}	2.26 ± 0.14^{a}	2.25 ± 0.22^{a}	**7.34 ± 0.58^{c}**	**4.33 ± 0.38^{b}**	2.93 ± 0.36^{ab}	2.00 ± 0.37^{a}	**6.81 ± 0.39^{c}**	3.95 ± 0.29^{b}
	Alicyclobacillaceae	0.57 ± 0.05^{a}	0.41 ± 0.28^{a}	0.66 ± 0.06^{a}	0.41 ± 0.11^{a}	0.39 ± 0.01^{a}	0.47 ± 0.06^{a}	**1.24 ± 0.20^{b}**	0.52 ± 0.07^{a}	0.71 ± 0.14^{a}	0.30 ± 0.07^{a}	0.52 ± 0.15^{a}	0.40 ± 0.12^{a}
	Clostridiaceae_1	0.53 ± 0.10^{a}	0.08 ± 0.08^{a}	0.21 ± 0.12^{a}	0.49 ± 0.12^{a}	1.01 ± 0.19^{a}	0.49 ± 0.20^{a}	0.60 ± 0.20^{a}	0.52 ± 0.24^{a}	1.08 ± 0.09^{a}	0.26 ± 0.13^{b}	0.61 ± 0.21^{ab}	0.20 ± 0.09^{b}

Table S1 continued

Phylum	Family	2 weeks Control (n = 4)	2 weeks BSF (n = 3)	2 weeks HC (n = 4)	2 weeks MW (n = 5)	4 weeks Control (n = 3)	4 weeks BSF (n = 5)	4 weeks HC (n = 5)	4 weeks MW (n = 5)	8 weeks Control (n = 5)	8 weeks BSF (n = 5)	8 weeks HC (n = 5)	8 weeks MW (n = 5)
Proteobacteria	*Sphingomonadaceae*	2.19 ± 0.16^{a}	3.00 ± 0.30^{ab}	**3.56 ± 0.35^{b}**	3.03 ± 0.20^{ab}	1.68 ± 0.15^{a}	**3.54 ± 0.16^{b}**	2.89 ± 0.19^{ab}	**3.07 ± 0.41^{b}**	1.14 ± 0.15^{a}	**3.98 ± 0.09^{b}**	**3.18 ± 0.15^{b}**	**4.08 ± 0.67^{b}**
	Burkholderiaceae	3.89 ± 0.54^{ab}	**7.04 ± 1.10^{c}**	3.46 ± 0.58^{a}	5.83 ± 0.15^{bc}	6.77 ± 0.15^{a}	5.41 ± 0.29^{ab}	3.67 ± 0.60^{c}	4.42 ± 0.20^{bc}	2.15 ± 0.26^{a}	**4.61 ± 0.39^{b}**	3.99 ± 0.75^{ab}	3.85 ± 0.27^{ab}
	Devosiaceae	0.51 ± 0.04^{a}	0.56 ± 0.04^{ab}	**0.87 ± 0.16^{b}**	0.58 ± 0.03^{ab}	0.53 ± 0.01^{a}	0.82 ± 0.12^{a}	0.72 ± 0.06^{a}	0.64 ± 0.02^{a}	0.37 ± 0.02^{a}	**1.23 ± 0.16^{b}**	1.07 ± 0.15^{ab}	**1.35 ± 0.31^{b}**
	Rhizobiaceae	2.22 ± 0.65^{a}	2.47 ± 0.81^{a}	2.03 ± 0.31^{a}	1.36 ± 0.36^{a}	3.41 ± 0.26^{a}	3.26 ± 0.30^{a}	1.90 ± 0.28^{a}	3.02 ± 0.48^{a}	1.14 ± 0.16^{a}	**4.11 ± 0.35^{b}**	**5.39 ± 0.46^{b}**	**3.85 ± 0.72^{b}**
	Xanthomonadaceae	1.65 ± 0.15^{a}	**5.59 ± 0.35^{b}**	**6.36 ± 0.82^{b}**	**5.68 ± 0.60^{b}**	1.61 ± 0.46^{a}	4.24 ± 0.56^{a}	4.13 ± 0.28^{a}	4.09 ± 0.90^{a}	0.72 ± 0.08^{a}	**3.04 ± 0.23^{b}**	**2.71 ± 0.18^{b}**	**2.70 ± 0.20^{b}**
	Pseudomonadaceae	0.09 ± 0.09^{a}	**2.28 ± 0.02^{b}**	**1.22 ± 0.30^{b}**	**1.76 ± 0.29^{b}**	0.13 ± 0.13^{a}	**2.04 ± 0.14^{b}**	0.29 ± 0.18^{a}	1.14 ± 0.35^{ab}	0.10 ± 0.06^{a}	0.41 ± 0.20^{a}	0.61 ± 0.17^{a}	0.30 ± 0.12^{a}
	Rhodanobacteraceae	0.64 ± 0.18^{a}	**2.43 ± 0.11^{b}**	**1.54 ± 0.13^{b}**	**2.58 ± 0.31^{b}**	0.82 ± 0.18^{a}	2.22 ± 0.45^{ab}	**3.07 ± 0.25^{b}**	2.20 ± 0.30^{ab}	0.55 ± 0.12^{a}	1.56 ± 0.09^{ab}	**3.03 ± 0.37^{c}**	**1.96 ± 0.36^{bc}**
	Hyphomicrobiaceae	0.65 ± 0.06^{a}	0.26 ± 0.13^{a}	0.35 ± 0.13^{a}	0.26 ± 0.11^{a}	0.85 ± 0.14^{a}	0.23 ± 0.10^{c}	0.53 ± 0.04^{ab}	0.47 ± 0.01^{bc}	1.10 ± 0.15^{a}	0.37 ± 0.10^{b}	0.43 ± 0.18^{b}	0.45 ± 0.02^{b}
	Sandaracinaceae	0.32 ± 0.17^{a}	0.52 ± 0.13^{a}	0.14 ± 0.08^{a}	**1.16 ± 0.02^{b}**	0.07 ± 0.07^{a}	0.28 ± 0.01^{a}	0.14 ± 0.08^{a}	**0.98 ± 0.05^{b}**	0.23 ± 0.06^{b}	0.17 ± 0.07^{ab}	0.00 ± 0.00^{a}	**0.72 ± 0.06^{c}**
	Caulobacteraceae	0.36 ± 0.17^{ab}	1.36 ± 0.25^{a}	1.11 ± 0.38^{ab}	0.30 ± 0.13^{b}	0.71 ± 0.01^{a}	1.26 ± 0.10^{a}	1.25 ± 0.23^{a}	0.84 ± 0.16^{a}	0.23 ± 0.10^{a}	0.70 ± 0.10^{a}	**2.47 ± 0.36^{b}**	0.76 ± 0.25^{a}
	Nitrosomonadaceae	0.45 ± 0.16^{a}	0.75 ± 0.32^{a}	0.57 ± 0.24^{a}	0.46 ± 0.21^{a}	0.76 ± 0.22^{a}	0.59 ± 0.17^{a}	0.55 ± 0.08^{a}	0.26 ± 0.11^{a}	1.11 ± 0.08^{a}	0.39 ± 0.06^{bc}	0.58 ± 0.07^{b}	0.24 ± 0.10^{c}
Chloroflexi	*JG30-KF-CM45*	1.51 ± 0.26^{a}	1.23 ± 0.26^{a}	1.74 ± 0.28^{a}	1.24 ± 0.18^{a}	0.75 ± 0.19^{a}	1.41 ± 0.12^{ab}	**1.81 ± 0.08^{b}**	0.97 ± 0.22^{a}	1.00 ± 0.10^{a}	**2.08 ± 0.30^{b}**	**2.16 ± 0.06^{b}**	1.77 ± 0.30^{ab}
Planctomycetes	*Isosphaeraceae*	1.35 ± 0.27^{a}	2.31 ± 0.48^{a}	1.52 ± 0.34^{a}	1.75 ± 0.25^{a}	1.61 ± 0.54^{a}	2.46 ± 0.30^{a}	**6.19 ± 0.67^{b}**	2.50 ± 0.34^{a}	1.11 ± 0.20^{a}	**2.45 ± 0.10^{b}**	**6.38 ± 0.62^{c}**	**2.41 ± 0.62^{b}**
	Pirellulaceae	1.09 ± 0.40^{a}	0.61 ± 0.18^{a}	0.47 ± 0.23^{a}	0.66 ± 0.06^{a}	1.05 ± 0.18^{a}	0.92 ± 0.20^{a}	0.50 ± 0.22^{a}	0.79 ± 0.08^{a}	1.83 ± 0.18^{a}	1.43 ± 0.23^{a}	0.20 ± 0.08^{b}	1.14 ± 0.19^{a}
	Gemmataceae	1.69 ± 0.17^{a}	0.87 ± 0.17^{b}	0.56 ± 0.19^{b}	1.08 ± 0.09^{b}	1.61 ± 0.16^{a}	1.12 ± 0.07^{ab}	0.61 ± 0.11^{b}	1.46 ± 0.36^{ab}	1.99 ± 0.40^{a}	0.95 ± 0.11^{b}	0.29 ± 0.11^{b}	1.09 ± 0.23^{ab}

Table S1 continued

Phylum	Family	2 weeks Control (n = 4)	2 weeks BSF (n = 3)	2 weeks HC (n = 4)	2 weeks MW (n = 5)	4 weeks Control (n = 3)	4 weeks BSF (n = 5)	4 weeks HC (n = 5)	4 weeks MW (n = 5)	8 weeks Control (n = 5)	8 weeks BSF (n = 5)	8 weeks HC (n = 5)	8 weeks MW (n = 5)
Acidobacteria	*Pyrinomonadaceae*	1.31 ± 0.22^{a}	0.45 ± 0.05^{b}	0.27 ± 0.16^{b}	0.54 ± 0.16^{b}	1.12 ± 0.19^{a}	0.34 ± 0.10^{b}	0.27 ± 0.17^{b}	0.27 ± 0.11^{b}	1.22 ± 0.09^{a}	0.29 ± 0.07^{b}	0.13 ± 0.08^{b}	0.15 ± 0.09^{b}
	Blastocatellaceae	1.35 ± 0.31^{a}	1.20 ± 0.48^{a}	0.66 ± 0.15^{a}	1.05 ± 0.26^{a}	1.79 ± 0.10^{a}	0.75 ± 0.13^{b}	0.34 ± 0.21^{b}	0.65 ± 0.08^{b}	1.04 ± 0.16^{a}	0.94 ± 0.21^{a}	0.19 ± 0.11^{b}	0.85 ± 0.13^{a}
Bacteroidetes	*Sphingobacteriaceae*	0.43 ± 0.07^{a}	0.72 ± 0.10^{a}	0.40 ± 0.17^{a}	0.20 ± 0.12^{a}	0.35 ± 0.16^{a}	0.65 ± 0.19^{a}	0.80 ± 0.23^{a}	0.90 ± 0.25^{a}	0.01 ± 0.01^{a}	**0.98 ± 0.13^{bc}**	**1.55 ± 0.16^{c}**	**0.83 ± 0.23^{b}**
	Chitinophagaceae	0.31 ± 0.22^{a}	1.39 ± 0.09^{a}	1.17 ± 0.37^{a}	0.51 ± 0.10^{a}	0.87 ± 0.27^{a}	0.52 ± 0.12^{a}	**2.26 ± 0.35^{b}**	0.70 ± 0.15^{a}	0.32 ± 0.09^{a}	0.49 ± 0.11^{a}	**2.42 ± 0.27^{b}**	0.83 ± 0.25^{a}
	Flavobacteriaceae	0.55 ± 0.15^{a}	0.67 ± 0.04^{a}	0.67 ± 0.39^{a}	0.42 ± 0.18^{a}	0.59 ± 0.05^{a}	0.29 ± 0.14^{a}	0.34 ± 0.09^{a}	0.35 ± 0.16^{a}	0.06 ± 0.06^{a}	0.33 ± 0.05^{ab}	**1.11 ± 0.41^{b}**	0.50 ± 0.16^{ab}
Cyanobacteria	*Nostocaceae*	1.23 ± 0.85^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.03 ± 0.03^{a}	0.90 ± 0.31^{a}	0.03 ± 0.03^{b}	0.04 ± 0.04^{b}	0.05 ± 0.05^{b}	0.52 ± 0.18^{a}	0.04 ± 0.04^{ab}	0.00 ± 0.00^{b}	0.10 ± 0.04^{ab}

Note. Treatments denoted with the same letter are not significantly different (EMM/Dunn's test, $P > 0.05$). Bold relative abundances are significantly higher than in the control. Underlined relative abundances are significantly lower than in the control.

Table S2

Relative abundances of bacterial genera in the Brassica oleracea *rhizosphere that were significantly different from the control after 2, 4 or 8 weeks of plant growth following soil amendment with black soldier fly (BSF), house cricket (HC) or mealworm (MW) exuviae at 10 g/kg (% ± standard error)*

Family	Genus	2 weeks Control (n = 4)	2 weeks BSF (n = 3)	2 weeks HC (n = 4)	2 weeks MW (n = 5)	4 weeks Control (n = 3)	4 weeks BSF (n = 5)	4 weeks HC (n = 5)	4 weeks MW (n = 5)	8 weeks Control (n = 5)	8 weeks BSF (n = 5)	8 weeks HC (n = 5)	8 weeks MW (n = 5)
Micrococcaceae	*Pseudarthrobacter*	0.65 ± 0.03^{a}	0.76 ± 0.09^{a}	**1.53 ± 0.16^{b}**	**1.38 ± 0.13^{b}**	0.59 ± 0.02^{a}	0.66 ± 0.03^{a}	**1.01 ± 0.06^{b}**	**1.42 ± 0.04^{c}**	0.52 ± 0.03^{a}	0.70 ± 0.02^{ab}	**0.88 ± 0.07^{b}**	**0.86 ± 0.06^{b}**
Intrasporangiaceae	*Phycicoccus*	0.60 ± 0.04^{a}	0.68 ± 0.06^{a}	**1.68 ± 0.10^{b}**	**1.29 ± 0.14^{b}**	0.55 ± 0.03^{a}	0.92 ± 0.10^{ab}	**1.28 ± 0.17^{b}**	**1.22 ± 0.12^{b}**	0.40 ± 0.02^{a}	**1.04 ± 0.24^{b}**	0.96 ± 0.15^{ab}	**1.16 ± 0.14^{b}**
Streptomycetaceae	*Streptomyces*	0.55 ± 0.01^{a}	1.35 ± 0.34^{ab}	**2.06 ± 0.46^{b}**	**2.48 ± 0.32^{b}**	0.57 ± 0.06^{a}	**1.49 ± 0.29^{b}**	**1.83 ± 0.33^{b}**	**2.57 ± 0.30^{b}**	0.87 ± 0.06^{a}	**2.86 ± 0.26^{b}**	**3.07 ± 0.29^{b}**	**2.58 ± 0.26^{b}**
Mycobacteriaceae	*Mycobacterium*	1.00 ± 0.18^{a}	1.04 ± 0.24^{a}	0.69 ± 0.30^{a}	1.42 ± 0.07^{a}	1.07 ± 0.11^{a}	1.17 ± 0.06^{a}	1.39 ± 0.06^{a}	1.26 ± 0.13^{a}	0.89 ± 0.04^{a}	1.67 ± 0.17^{ab}	**2.31 ± 0.28^{b}**	1.32 ± 0.07^{ab}
Nocardioidaceae	*Nocardioides*	1.66 ± 0.32^{a}	2.39 ± 0.29^{ab}	**4.04 ± 0.26^{c}**	**2.98 ± 0.27^{bc}**	1.12 ± 0.34^{a}	2.08 ± 0.25^{a}	2.47 ± 0.63^{a}	2.59 ± 0.18^{a}	1.10 ± 0.10^{a}	**3.05 ± 0.22^{b}**	**2.93 ± 0.23^{b}**	**3.03 ± 0.30^{b}**

Table S2 continued

Family	Genus	2 weeks				4 weeks				8 weeks			
		Control (n = 4)	BSF (n = 3)	HC (n = 4)	MW (n = 5)	Control (n = 3)	BSF (n = 5)	HC (n = 5)	MW (n = 5)	Control (n = 5)	BSF (n = 5)	HC (n = 5)	MW (n = 5)
Solirubrobacteraceae	*Conexibacter*	1.62 ± 0.43^{a}	1.13 ± 0.10^{a}	1.14 ± 0.16^{a}	1.59 ± 0.14^{a}	1.53 ± 0.27^{a}	1.22 ± 0.22^{a}	0.62 ± 0.29^{a}	0.75 ± 0.13^{a}	1.71 ± 0.21^{a}	1.39 ± 0.20^{a}	0.45 ± 0.13^{b}	1.22 ± 0.24^{ab}
Nakamurellaceae	*Nakamurella*	0.50 ± 0.19^{a}	0.60 ± 0.08^{a}	0.54 ± 0.19^{a}	0.59 ± 0.15^{a}	0.33 ± 0.17^{a}	0.59 ± 0.08^{ab}	**0.87 ± 0.07^{b}**	0.63 ± 0.03^{ab}	0.43 ± 0.02^{a}	0.96 ± 0.12^{ab}	**1.50 ± 0.22^{b}**	0.98 ± 0.07^{ab}
Gaiellaceae	*Gaiella*	2.11 ± 0.18^{a}	1.42 ± 0.21^{ab}	0.90 ± 0.14^{b}	1.70 ± 0.25^{ab}	2.04 ± 0.27^{a}	1.33 ± 0.19^{ab}	0.94 ± 0.19^{b}	1.03 ± 0.28^{ab}	2.33 ± 0.24^{a}	1.27 ± 0.09^{b}	0.51 ± 0.03^{c}	1.15 ± 0.06^{b}
Nocardiaceae	*Rhodococcus*	0.06 ± 0.06^{a}	0.00 ± 0.00^{a}	**1.02 ± 0.15^{b}**	0.56 ± 0.28^{ab}	0.09 ± 0.09^{a}	0.13 ± 0.08^{a}	**1.08 ± 0.14^{b}**	**1.00 ± 0.21^{b}**	0.00 ± 0.00^{a}	0.31 ± 0.19^{ab}	**0.70 ± 0.21^{b}**	**0.76 ± 0.10^{b}**
Planococcaceae	*Lysinibacillus*	0.86 ± 0.15^{ab}	0.13 ± 0.13^{a}	1.54 ± 0.18^{b}	0.90 ± 0.17^{b}	0.60 ± 0.16^{a}	0.19 ± 0.12^{a}	**1.36 ± 0.18^{b}**	0.45 ± 0.19^{a}	0.79 ± 0.19^{ab}	0.42 ± 0.20^{a}	1.55 ± 0.26^{b}	0.62 ± 0.17^{a}
Bacillaceae	*Bacillus*	1.41 ± 0.19^{a}	1.59 ± 0.30^{ab}	**2.61 ± 0.24^{b}**	**2.44 ± 0.27^{b}**	1.16 ± 0.18^{a}	1.32 ± 0.23^{a}	**2.17 ± 0.17^{b}**	**2.24 ± 0.17^{b}**	1.59 ± 0.10^{ab}	1.04 ± 0.01^{a}	2.27 ± 0.22^{b}	2.17 ± 0.31^{b}
Paenibacillaceae	*Paenibacillus*	0.70 ± 0.16^{ab}	0.89 ± 0.45^{ab}	1.28 ± 0.26^{b}	0.20 ± 0.12^{a}	0.59 ± 0.12^{a}	1.17 ± 0.13^{ab}	**2.23 ± 0.58^{b}**	1.40 ± 0.33^{ab}	0.89 ± 0.30^{a}	0.80 ± 0.21^{a}	1.34 ± 0.26^{a}	0.65 ± 0.29^{a}
Sphingomonadaceae	*Sphingomonas*	1.28 ± 0.07^{a}	**2.45 ± 0.22^{b}**	**2.35 ± 0.29^{b}**	**2.47 ± 0.23^{b}**	0.99 ± 0.02^{a}	**2.42 ± 0.13^{b}**	**2.25 ± 0.20^{b}**	**2.23 ± 0.26^{b}**	0.77 ± 0.11^{a}	**2.97 ± 0.19^{b}**	**2.29 ± 0.26^{b}**	**3.09 ± 0.47^{b}**
Xanthobacteraceae	*Pseudolabrys*	1.01 ± 0.18^{a}	0.79 ± 0.27^{a}	0.70 ± 0.02^{a}	0.71 ± 0.03^{a}	0.94 ± 0.25^{a}	0.90 ± 0.11^{a}	0.86 ± 0.11^{a}	0.79 ± 0.13^{a}	1.08 ± 0.09^{a}	0.88 ± 0.09^{a}	0.12 ± 0.12^{b}	0.88 ± 0.14^{a}
	Rhodopseudomonas	0.00 ± 0.00^{a}	0.09 ± 0.09^{a}	0.21 ± 0.12^{a}	0.15 ± 0.09^{a}	0.00 ± 0.00^{a}	0.32 ± 0.01^{ab}	**0.55 ± 0.08^{b}**	**0.49 ± 0.01^{b}**	0.00 ± 0.00^{a}	0.38 ± 0.02^{ab}	**1.18 ± 0.06^{b}**	0.56 ± 0.10^{ab}
Burkholderiaceae	*Massilia*	0.67 ± 0.12^{a}	1.45 ± 0.13^{a}	1.01 ± 0.17^{a}	1.14 ± 0.20^{a}	0.92 ± 0.20^{a}	1.32 ± 0.23^{a}	0.97 ± 0.18^{a}	1.17 ± 0.16^{a}	0.25 ± 0.10^{a}	**1.01 ± 0.10^{b}**	**1.33 ± 0.28^{b}**	0.70 ± 0.10^{ab}
	Burkholderia-Caballeronia-Paraburkholderia	0.28 ± 0.10^{a}	**1.91 ± 0.36^{b}**	0.13 ± 0.13^{a}	0.36 ± 0.15^{a}	0.10 ± 0.10^{a}	**1.39 ± 0.10^{c}**	0.00 ± 0.00^{a}	**0.70 ± 0.14^{b}**	0.16 ± 0.07^{a}	**1.27 ± 0.11^{c}**	0.00 ± 0.00^{a}	**0.89 ± 0.13^{b}**
	Castellaniella	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	**1.22 ± 0.18^{b}**	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	**0.74 ± 0.05^{b}**	0.00 ± 0.00^{a}

Table S2 continued

Devosiaceae	*Devosia*	0.51 ± 0.04^{a}	0.56 ± 0.04^{ab}	**0.87 ± 0.16^{b}**	0.58 ± 0.03^{ab}	0.53 ± 0.01^{a}	0.82 ± 0.12^{a}	0.65 ± 0.04^{a}	0.64 ± 0.02^{a}	0.37 ± 0.02^{a}	**1.23 ± 0.16^{b}**	1.00 ± 0.12^{ab}	**1.35 ± 0.31^{b}**
Rhizobiaceae	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	1.30 ± 0.28^{a}	1.81 ± 0.47^{a}	0.78 ± 0.32^{a}	1.00 ± 0.21^{a}	2.09 ± 0.16^{a}	2.14 ± 0.33^{a}	0.92 ± 0.11^{a}	1.66 ± 0.44^{a}	0.51 ± 0.09^{a}	**2.88 ± 0.18^{b}**	**2.67 ± 0.34^{b}**	**2.19 ± 0.37^{b}**
Xanthomonadaceae	*Thermomonas*	0.35 ± 0.12^{a}	0.67 ± 0.34^{a}	1.56 ± 0.29^{a}	1.33 ± 0.37^{a}	0.34 ± 0.20^{a}	0.57 ± 0.17^{a}	**1.47 ± 0.26^{b}**	0.49 ± 0.21^{a}	0.07 ± 0.07^{a}	0.57 ± 0.07^{ab}	**1.31 ± 0.24^{b}**	0.59 ± 0.06^{ab}
	Stenotrophomonas	0.00 ± 0.00^{a}	**1.33 ± 0.16^{c}**	**1.79 ± 0.11^{c}**	**0.82 ± 0.12^{b}**	0.00 ± 0.00^{a}	**1.21 ± 0.15^{b}**	**0.87 ± 0.14^{b}**	0.74 ± 0.27^{ab}	0.06 ± 0.06^{a}	0.44 ± 0.07^{ab}	**0.70 ± 0.20^{b}**	0.13 ± 0.08^{ab}
	Lysobacter	0.00 ± 0.00^{a}	**1.39 ± 0.26^{b}**	**1.00 ± 0.19^{b}**	**1.44 ± 0.18^{b}**	0.16 ± 0.08^{a}	0.98 ± 0.20^{a}	0.56 ± 0.18^{a}	1.17 ± 0.30^{a}	0.00 ± 0.00^{a}	**0.85 ± 0.09^{b}**	0.00 ± 0.00^{a}	**0.71 ± 0.12^{b}**
Pseudomonadaceae	*Pseudomonas*	0.09 ± 0.09^{a}	**2.28 ± 0.02^{b}**	**1.22 ± 0.30^{b}**	**1.73 ± 0.28^{b}**	0.13 ± 0.13^{a}	**2.04 ± 0.14^{b}**	0.29 ± 0.18^{a}	1.14 ± 0.35^{ab}	0.10 ± 0.06^{a}	0.41 ± 0.20^{a}	0.61 ± 0.17^{a}	0.30 ± 0.12^{a}
Rhodanobacteraceae	*Rhodanobacter*	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.25 ± 0.16^{a}	0.00 ± 0.00^{a}	0.39 ± 0.24^{ab}	**2.22 ± 0.19^{c}**	**1.05 ± 0.13^{b}**	0.09 ± 0.05^{a}	0.29 ± 0.14^{a}	**2.66 ± 0.29^{b}**	0.73 ± 0.19^{a}
	Dokdonella	0.48 ± 0.08^{a}	**0.93 ± 0.09^{b}**	0.62 ± 0.03^{a}	**1.07 ± 0.04^{b}**	0.31 ± 0.16^{a}	0.78 ± 0.05^{a}	0.21 ± 0.13^{a}	0.41 ± 0.19^{a}	0.31 ± 0.09^{ab}	0.67 ± 0.08^{b}	0.00 ± 0.00^{a}	0.61 ± 0.16^{b}
Chthoniobacteraceae	*Candidatus_Udaeobacter*	1.36 ± 0.25^{a}	1.13 ± 0.12^{a}	1.05 ± 0.17^{a}	1.03 ± 0.17^{a}	1.32 ± 0.47^{a}	0.94 ± 0.09^{a}	0.87 ± 0.12^{a}	0.94 ± 0.14^{a}	1.61 ± 0.26^{a}	1.16 ± 0.15^{ab}	0.53 ± 0.02^{b}	0.73 ± 0.09^{b}
	Chthoniobacter	0.42 ± 0.10^{a}	**1.31 ± 0.13^{b}**	0.15 ± 0.15^{a}	0.41 ± 0.14^{a}	0.22 ± 0.11^{a}	**1.34 ± 0.16^{b}**	0.58 ± 0.18^{a}	0.72 ± 0.12^{a}	0.15 ± 0.07^{a}	**0.86 ± 0.08^{b}**	**0.64 ± 0.10^{b}**	0.51 ± 0.12^{ab}
Isosphaeraceae	*Aquisphaera*	0.43 ± 0.05^{a}	0.72 ± 0.04^{a}	0.19 ± 0.19^{a}	0.27 ± 0.18^{a}	0.54 ± 0.30^{a}	0.82 ± 0.12^{a}	**1.89 ± 0.14^{b}**	1.07 ± 0.25^{a}	0.41 ± 0.16^{a}	0.83 ± 0.24^{ab}	**1.47 ± 0.21^{b}**	0.76 ± 0.17^{ab}
	Paludisphaera	0.00 ± 0.00^{a}	0.15 ± 0.15^{a}	0.25 ± 0.20^{a}	0.00 ± 0.00^{a}	0.11 ± 0.11^{ab}	0.29 ± 0.19^{ab}	1.07 ± 0.36^{b}	0.00 ± 0.00^{a}	0.06 ± 0.06^{a}	0.27 ± 0.12^{a}	**1.60 ± 0.20^{b}**	0.32 ± 0.13^{a}
	Singulisphaera	0.37 ± 0.21^{a}	0.60 ± 0.30^{a}	0.29 ± 0.17^{a}	0.77 ± 0.11^{a}	0.22 ± 0.11^{a}	0.62 ± 0.12^{a}	**1.57 ± 0.32^{b}**	0.69 ± 0.14^{a}	0.29 ± 0.09^{a}	0.57 ± 0.17^{ab}	**1.40 ± 0.19^{b}**	0.78 ± 0.33^{ab}
Pyrinomonadaceae	*RB41*	1.31 ± 0.22^{a}	0.45 ± 0.05^{b}	0.24 ± 0.14^{b}	0.54 ± 0.16^{b}	1.12 ± 0.19^{a}	0.34 ± 0.10^{b}	0.27 ± 0.17^{b}	0.27 ± 0.11^{b}	1.22 ± 0.09^{a}	0.29 ± 0.07^{b}	0.13 ± 0.08^{b}	0.15 ± 0.09^{b}
Blastocatellaceae	*JGI_0001001-H03*	0.74 ± 0.26^{a}	0.65 ± 0.34^{a}	0.33 ± 0.19^{a}	0.60 ± 0.17^{a}	1.01 ± 0.10^{a}	0.20 ± 0.09^{b}	0.28 ± 0.17^{b}	0.52 ± 0.09^{ab}	0.49 ± 0.16^{a}	0.48 ± 0.14^{a}	0.09 ± 0.09^{a}	0.47 ± 0.14^{a}
Sphingobacteriaceae	*Pedobacter*	0.37 ± 0.01^{a}	0.65 ± 0.12^{a}	0.14 ± 0.14^{a}	0.20 ± 0.12^{a}	0.20 ± 0.10^{a}	0.50 ± 0.23^{a}	0.60 ± 0.19^{a}	0.73 ± 0.13^{a}	0.00 ± 0.00^{a}	0.72 ± 0.10^{ab}	**1.14 ± 0.28^{b}**	0.69 ± 0.18^{ab}

Note. Treatments denoted with the same letter are not significantly different (EMM/Dunn's test, $P > 0.05$). Bold relative abundances are significantly higher than in the control. Underlined relative abundances are significantly lower than in the control.

Chapter 5

Effects of amending soil with black soldier fly frass on survival and growth of the cabbage root fly (*Delia radicum*) depend on soil type

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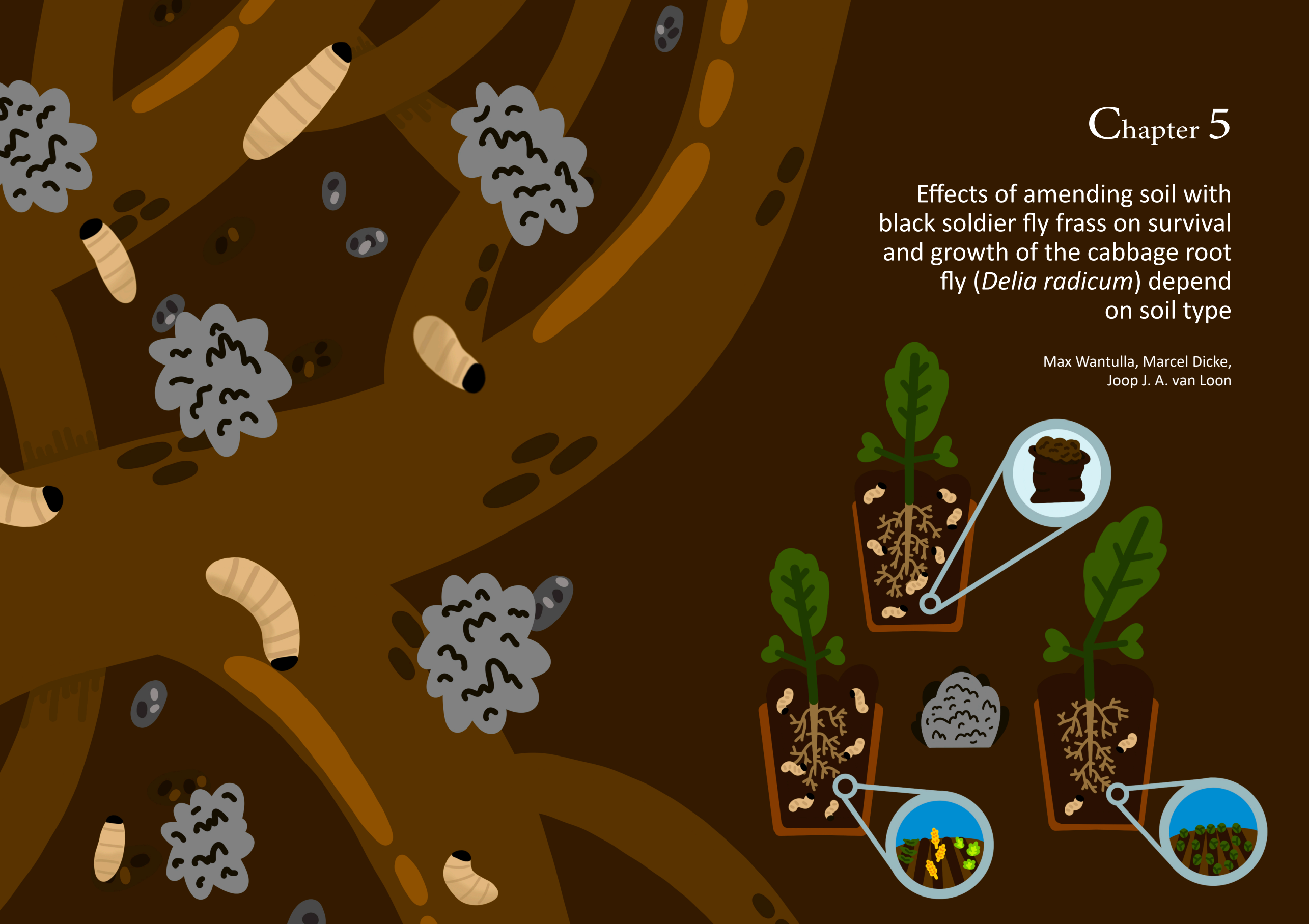

Abstract

New approaches to managing the cabbage root fly (*Delia radicum* L.) are needed because pesticide regulations continue to limit the availability of effective control products. Soil amendment with black soldier fly (*Hermetia illucens* L.) frass has recently been shown to reduce *D. radicum* survival. In a greenhouse experiment, soil from a field on which brassicaceous plant species had repeatedly been grown (brassica field soil) was mixed with frass at ratios of 1, 2 or 5 g/kg. In a second greenhouse experiment, 5 g/kg were added to (a) brassica field soil, (b) soil from a different field on which non-brassicaceous species had been rotated (crop rotation field soil) or (c) blocks of potting soil that were later transplanted to unamended field soil. Brussels sprouts (*Brassica oleracea* L.) plants were grown in amended soil and were infested with *D. radicum* larvae after 4 weeks. While amendment with 1 or 2 g/kg did not affect *D. radicum* performance compared with unamended soil, 5 g/kg reduced *D. radicum* survival and pupal biomass in brassica field soil. In crop rotation field soil, amendment with 5 g/kg reduced pupal biomass but did not reduce *D. radicum* survival. Amendment with 5 g/kg had no effect on *D. radicum* performance in potting soil. In general, *D. radicum* survival was lower in brassica field soil than in either other soil, irrespective of soil amendment. The effects of black soldier fly frass on *D. radicum* appear to depend on soil type.

Introduction

One of the major threats to the production of cruciferous crops in temperate climates is the cabbage root fly or cabbage maggot, *Delia radicum* L. (Diptera: Anthomyiidae). Infestations can cause crop losses of up to 100%, notably if young plants are affected (Ferry et al., 2009). Female flies lay their eggs around the plant stem and hatched larvae burrow into the soil to feed on the roots. Feeding damage and the facilitated entry of pathogens into injured roots often result in slow growth, yellowing, stunting and eventually plant death (Santolamazza-Carbone et al., 2017).

As the availability of effective insecticides to control *D. radicum* has been limited by stricter legislation in many European countries, growers find themselves forced to use alternative management approaches. However, methods such as the deployment of physical barriers, predators or microbial agents are often considered impractical or provide insufficient or inconsistent control (Collier et al., 2020). Moreover, there is a lack of brassica crops with effective resistance to *D. radicum* (Santolamazza-Carbone et al., 2017; Shuhang et al., 2016). It is therefore necessary to combine different control methods that are not sufficiently effective when applied separately. In addition, new tools for *D. radicum* control that can be used in an integrated pest management approach are needed.

Soil amendment with the by-products of insect farming has been suggested to stimulate naturally occurring beneficial microbes that could contribute to the control of plant pests (Barragán-Fonseca et al., 2022). Insect production for food and feed is a rapidly growing industry that generates residual streams consisting of insect feces, exuviae (molted exoskeletons) and unconsumed substrate. This mixture is commonly referred to as insect 'frass' and potential applications in agriculture have been the subject of extensive research in recent years (Poveda, 2021; Schmitt & de Vries, 2020; Torgerson et al., 2021). One of the most important species for the commercial production of insects is the black soldier fly, *Hermetia illucens* L., as its larvae are particularly versatile and can be reared on various organic residual streams (van Huis, 2021). Interestingly, unlike the residual streams of other insect species, exuviae and frass of black soldier fly larvae were recently found to negatively affect the performance of *D. radicum* when added to the soil (Wantulla et al., 2022).

Insect exuviae, which are present in frass in variable proportions, have been shown to stimulate a variety of bacteria with biocontrol potential in the cabbage rhizosphere. In particular, the exuviae of black soldier fly larvae appeared to stimulate the abundance of bacterial species in the genus *Pseudomonas* and different members of the *Burkholderiaceae* more effectively than the exuviae of other insect species (Wantulla

et al., 2023). Certain species in the genera *Pseudomonas* and *Burkholderia* have been shown to exhibit insecticidal activity (Cordova-Kreylos et al., 2013; Kupferschmied et al., 2013). Furthermore, soil amendment with black soldier fly frass was reported to promote the fungal genus *Mortierella* in the rhizosphere of red clover and Italian ryegrass (Fuhrmann et al., 2022). Insecticidal activity of some *Mortierella* species has been demonstrated, e.g. by soil inoculation (Edgington et al., 2014). Bacteria and fungi belonging to these different groups might thus be responsible for negative effects of black soldier fly residual streams on *D. radicum*.

Amending soil with residues from black soldier fly rearing has been proposed as a new method for *D. radicum* management. While soil amendment with 5 g/kg of exuviae of black soldier fly larvae was previously found to reduce *D. radicum* biomass, amendment with frass at a ratio of 10 g/kg resulted in lower larval survival (Wantulla et al., 2022). Since the actual by-product of insect rearing is frass rather than separated exuviae, the present study aimed to further investigate the potential of black soldier fly frass for controlling *D. radicum*. To reduce the amount of frass that was used in a previous study, a lower effective soil amendment ratio was determined. As the stimulation of native soil microbes is thought to be vital to the effectiveness of black soldier fly frass, effects of the amendment on *D. radicum* were subsequently compared between different types of soil.

Materials and methods

Plants and growth conditions

Brassica oleracea L. var. *gemmifera* cv. Cyrus (Brussels sprouts) plants were kept in a greenhouse compartment at 20 ± 3 °C, 60-80% relative humidity and 16 h light/8 h dark photoperiod for Experiment 1. In Experiment 2 temperature in the greenhouse fluctuated between a minimum of 13 °C (at night) and maximum of 32 °C (during the day) before plant infestation with *D. radicum* and between 12 °C and 30 °C after plants were infested under ambient humidity and light conditions.

Insect rearing and plant infestation

The *D. radicum* population used was collected in Zeewolde (Flevoland, the Netherlands) in 2013. All life stages were kept in a climate cabinet at 20 ± 1 °C and 16 h light/8 h dark photoperiod. Larvae were kept on *B. napus* L. subsp. *rapifera* (swede) roots of 10-week-old plants until pupation. Eclosed adult root flies were kept in gauze cages and were fed with a 1:1:1 mixture of sugar, milk powder and yeast. In addition, a solution of

honey in tap water was offered in a Petri dish and tap water was offered in a Petri dish with moist filter paper on top of wet cotton wool. Oviposition was stimulated by providing slices of swede in Petri dishes to the flies in the cages. Eggs were collected and placed on a new swede prior to larval hatching.

To obtain larvae for plant infestation, eggs were incubated in Petri dishes with moist filter paper. Larvae hatched from the eggs after 4 days and plants were infested by carefully placing five neonate larvae on plastic plant labels that were inserted into the soil surface close to the stem. Labels were checked after 30 min and remaining larvae were replaced. This was repeated until five larvae had moved into the soil in every pot.

Insect residual stream

Frass of larvae of black soldier flies, *H. illucens*, used in this study was provided by Bestico (Berkel en Rodenrijs, the Netherlands). The material was first inspected for the presence of insects or insect fragments, which were removed, and was then oven-dried at 60 °C for 24 h. The dried material was subsequently ground to a powder with an SM 100 cutting mill (Retsch, Haan, Germany).

Soil

Potting soil was provided by Unifarm (Wageningen, the Netherlands). Agricultural soil was collected from the topsoil layers of two organically managed fields in Wageningen and Lelystad, the Netherlands, in 2020. The field in Wageningen had been used to grow various brassicaceous plant species since 2011 and black mustard (*Brassica nigra* L.) had recently been grown at the location selected for soil collection. In the field in Lelystad, sugar beet (*Beta vulgaris* L.), onion (*Allium cepa* L.), parsley (*Petroselinum crispum* Mill.), wheat (*Triticum aestivum* L.) and potato (*Solanum tuberosum* L.) had been grown consecutively from 2016 to 2020. The composition of each field soil as assessed by Eurofins Agro (Wageningen, the Netherlands) is shown in Table 1. Both field soils were homogenized by sieving (particle size < 5 mm). Soil was stored at ambient temperature for 10 months before being used in Experiment 1 and for a maximum of 1 month before being used in Experiment 2.

Table 1
Field soil composition

Field	Sand (%)	Silt (%)	Clay (%)	SOM* (%)
Wageningen	81	14	2	3.2
Lelystad	47	34	13	1.6

Note. Analyzed by Eurofins Agro (Wageningen, the Netherlands).
*Soil organic matter.

Experiment 1: effects of soil amendment with black soldier fly frass at different ratios on *D. radicum* performance

Brassica field soil was mixed with dried black soldier fly frass at a ratio of 1, 2 or 5 g/kg of dry soil or left unamended. In a completely randomized design, 20 plants per treatment were grown in 1 L plastic pots, which were individually placed in saucers. Three seeds were sown per pot and gently pressed down. If more than one seed germinated, one or two seedlings were randomly removed from each pot after 1 week. Excess seedlings were transplanted to pots of the same treatment in which no seeds had germinated or were discarded together with ungerminated seeds. Plants were watered three times per week by filling saucers and emptying them after 2 h. Starting 1 week after sowing, plants were fertilized for 3 weeks with an optimized fertilizer solution (Table S1). Each plant received 160 ml of fertilizer per week. Fertilizer amounts per 1 L of field soil were based on a nitrogen fertilization advice for cabbage provided by Eurofins Agro (Wageningen, the Netherlands). Plants were grown for 4 weeks before being infested with *D. radicum* larvae and were uprooted 3 weeks after infestation. Roots were rinsed to remove adhering soil and were checked for remaining larvae before washing all soil through a 1 mm aperture sieve to collect larvae and pupae. Living larvae and pupae were counted and pupae were weighed on a CP2P-F micro balance (Sartorius, Göttingen, Germany). Plants were oven-dried at 105 °C for 24 h before measuring shoot and root dry biomass.

Experiment 2: effects of soil amendment with black soldier fly frass on *D. radicum* performance in different soils

In a full factorial design with soil type and soil amendment as factors, potting soil, brassica field soil and crop rotation field soil were each mixed with black soldier fly frass at a ratio of 5 g/kg or left unamended. Amended and unamended potting soil was pressed into 5 cm^3 blocks using a soil block machine (Visser NM71, Visser Tuinbouwtechniek, 's Gravendeel, the Netherlands). Eighty blocks of each treatment were placed in separate plastic trays. One *B. oleracea* seed was sown per soil block and gently pressed down. Field soils were filled into 1 L plastic pots, which were individually placed in saucers. Brassica field soil was used to prepare 20 pots with amended soil and 60 pots with unamended soil. Two seeds were sown per pot in 20 pots of each treatment, while 40 pots containing unamended soil were left without seeds in order to plant seedlings growing in blocks of potting soil later on. Crop rotation field soil was used to prepare 20 pots with amended and unamended soil, respectively, and two seeds were sown per pot. All seeds were gently pressed down. If both seeds in a pot germinated, one seedling was randomly removed after 1 week. Excess seedlings from pots were transplanted to pots of the same treatment in

which seeds had not germinated or were discarded together with ungerminated seeds. Due to the low germination rate in amended brassica field soil, two pots had to be planted with seedlings from unamended brassica field soil.

All plants were watered three times per week by filling saucers or flooding trays and emptying them after 2 h. Starting 1 week after sowing, field soils were fertilized for 3 weeks with an optimized fertilizer solution (Table S1). Pots containing brassica field soil received 160 ml of fertilizer per week, while pots containing crop rotation field soil received 60 ml of fertilizer per week. Fertilizer amounts per 1 L of field soil were based on a nitrogen fertilization advice for cabbage provided by Eurofins Agro (Wageningen, the Netherlands). After 3 weeks of plant growth, 20 soil blocks with seedlings were randomly selected from amended and unamended blocks of potting soil, respectively, and planted into pots containing unamended brassica field soil. Plants were grown for a total of 4 weeks before being infested with *D. radicum* larvae and were uprooted 3 weeks after infestation. Roots were rinsed to remove adhering soil and were checked for remaining larvae before washing all soil through a 1 mm aperture sieve to collect larvae and pupae. Living larvae and pupae were counted and pupae were weighed on a CP2P-F micro balance (Sartorius, Göttingen, Germany). Plants were oven-dried at 105 °C for 24 h before measuring root dry biomass. Shoot biomass was not included in the evaluation, as a natural infestation with *Pieris rapae* L. caterpillars had led to feeding damage on most plants during the last week of the experiment.

Statistical analysis

All statistical analyses were performed using R (Version 4.1.0; R Core Team, 2021) and the packages car (Fox & Weisberg, 2019), dunn.test (Dinno, 2017), emmeans (Lenth, 2021), rcompanion (Mangiafico, 2022) and stats (R Core Team, 2021). Statistical models for Experiment 1 only included the factor soil amendment, whereas models for Experiment 2 included the factors soil type, soil amendment and their interaction. Generalized linear models (GLM) with binomial distributions were used to analyze seed germination and *D. radicum* survival, while a linear model (LM) was used to analyze root biomass. Pairwise comparisons were performed using estimated marginal means (EMM). Models were validated by plotting residuals and, if necessary, homoscedasticity and normality were confirmed using Levene's test and the Shapiro-Wilk test, respectively. If model assumptions were violated, nonparametric tests were conducted instead. The Kruskal–Wallis test was used to analyze shoot biomass and average pupal biomass per plant in Experiment 1. For Experiment 2, the Scheirer–Ray–Hare (SRH) test was used to analyze seed germination, root biomass and average pupal biomass per plant. Dunn's test was used for pairwise comparisons.

Results

Experiment 1: effects of soil amendment with black soldier fly frass at different ratios on *D. radicum* performance

D. radicum performance

Amendment of brassica soil with black soldier fly frass significantly affected *D. radicum* survival (GLM: χ^2 = 26.97, *df* = 3, *P* < 0.001). When soil was amended with 5 g/kg, survival was significantly reduced compared with the control (EMM: *P* = 0.002; Figure 1A) and compared with amendment with 1 g/kg (EMM: *P* = 0.002; Figure 1A) or 2 g/kg (EMM: *P* < 0.001; Figure 1A). Soil amendment had a significant effect on pupal fresh biomass (Kruskal–Wallis test: *H* = 13.9428, *df* = 3, *P* < 0.001) and amendment with 5 g/kg significantly reduced pupal biomass compared with the control (Dunn's test: *P* = 0.002; Figure 1B).

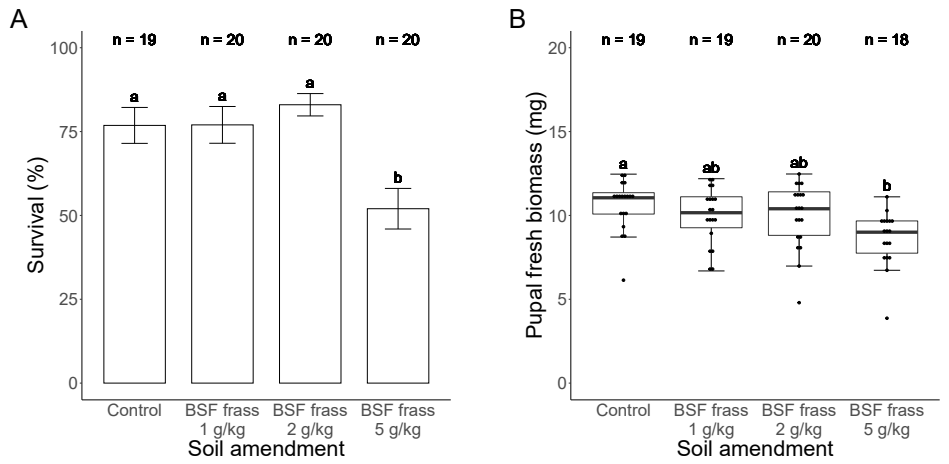

Figure 1. Survival (A) and pupal fresh biomass (B) of *Delia radicum* after feeding on *Brassica oleracea* plants growing in brassica soil amended with black soldier fly (BSF) frass at different ratios. Treatments denoted with the same letter are not significantly different (EMM/Dunn's test, *P* > 0.05). Error bars (A) represent standard errors. Box plot whiskers (B) represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants are indicated at the top of the panels by n.

Plant performance

While the main effect of amendment of brassica soil with black soldier fly frass on *B. oleracea* seed germination was significant (GLM: χ^2 = 12.32, *df* = 3, *P* = 0.006), there were no significant differences in germination between the treatments (EMM: *P* > 0.05; Figure 2). Amendment had no significant effect on shoot dry biomass (Kruskal–Wallis test:

H = 4.2442, *df* = 3, *P* = 0.24; Figure 3A) or root dry biomass (LM: *F* = 0.7681, *df* = 3, *P* = 0.516; Figure 3B) of *B. oleracea* plants.

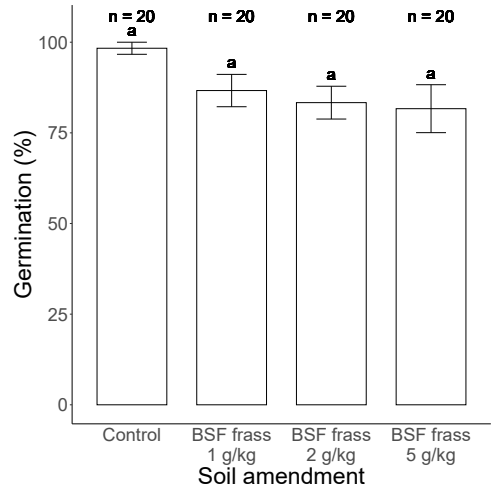

Figure 2. Seed germination of *Brassica oleracea* in brassica soil amended with black soldier fly (BSF) frass at different ratios. Germination did not differ significantly among treatments (EMM, *P* > 0.05). Error bars represent standard errors. Numbers of replicate pots are indicated at the top of the panels by n.

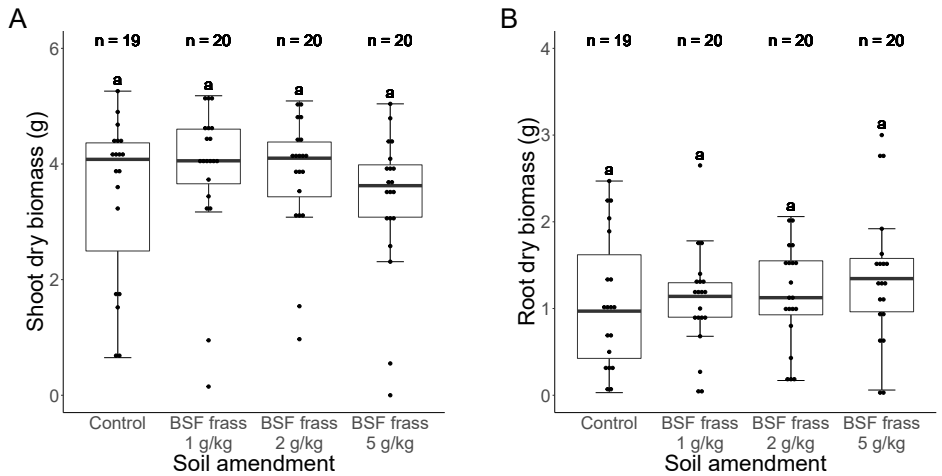

Figure 3. Shoot (A) and root (B) dry biomass of *Brassica oleracea* plants grown in brassica soil amended with black soldier fly (BSF) frass at different ratios 8 weeks after planting and 3 weeks after infestation with *Delia radicum* larvae. Shoot and root biomass did not differ significantly among treatments (Kruskal–Wallis test/LM, *P* > 0.05). Box plot whiskers represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants are indicated at the top of the panels by n.

Experiment 2: effects of soil amendment with black soldier fly frass on *D. radicum* performance in different soils

D. radicum performance

Survival of *D. radicum* was significantly affected by soil type (GLM: χ^2 = 77.051, *df* = 2, *P* < 0.001) and soil amendment with black soldier fly frass (GLM: χ^2 = 5.182, *df* = 1, *P* = 0.023), while the interaction of the two factors was marginally insignificant (GLM: χ^2 = 5.442, *df* = 2, *P* = 0.066). In brassica field soil, survival was significantly lower than in crop rotation field soil (EMM: *P* = 0.006; Figure 4A) or than in blocks of potting soil that had been transplanted to brassica field soil 3 weeks after sowing (EMM: *P* < 0.001; Figure 4A). Soil amendment significantly reduced survival only in brassica field soil (EMM: *P* = 0.027; Figure 4A), with survival being significantly lower than when either of the other soils was amended (EMM: *P* < 0.001; Figure 4A). Pupal fresh biomass was significantly affected by soil type (SRH test: *H* = 8.5535, *df* = 2, *P* = 0.014), soil amendment (SRH test: *H* = 6.9195, *df* = 1, *P* = 0.009) and the interaction of both factors (SRH test: *H* = 7.4527, *df* = 2, *P* = 0.024). Amendment significantly reduced pupal biomass only in crop rotation field soil (Dunn's test: *P* = 0.007; Figure 4B), with pupal biomass being significantly lower than when potting soil was amended (Dunn's test: *P* = 0.020; Figure 4B).

Plant performance

Germination of *B. oleracea* seeds was significantly affected by soil type (SRH test: *H* = 71.862, *df* = 2, *P* < 0.001) and soil amendment with black soldier fly frass (SRH test: *H* = 8.374, *df* = 1, *P* = 0.004) but not by the interaction of the two factors (SRH test: *H* = 5.544, *df* = 2, *P* = 0.063). Amendment of crop rotation field soil resulted in a significantly lower seed germination rate than in amended or unamended potting soil (Dunn's test: *P* < 0.001; Figure 5A). Seed germination rates in brassica field soil were significantly lower than in potting soil, irrespective of soil amendment (Dunn's test: *P* < 0.001; Figure 5A). Root dry biomass of *B. oleracea* plants was significantly affected by soil type (SRH test: *H* = 15.8027, *df* = 2, *P* < 0.001), soil amendment (SRH test: *H* = 6.1019, *df* = 1, *P* = 0.014) and the interaction of both factors (SRH test: *H* = 6.0678, *df* = 2, *P* = 0.048). Plants grown in unamended brassica field soil had a significantly higher root biomass than plants grown in blocks of potting soil that were transplanted to brassica field soil 3 weeks after sowing (Dunn's test: *P* < 0.001; Figure 5B). Soil amendment significantly reduced root biomass only in brassica field soil (Dunn's test: *P* = 0.013; Figure 5B).

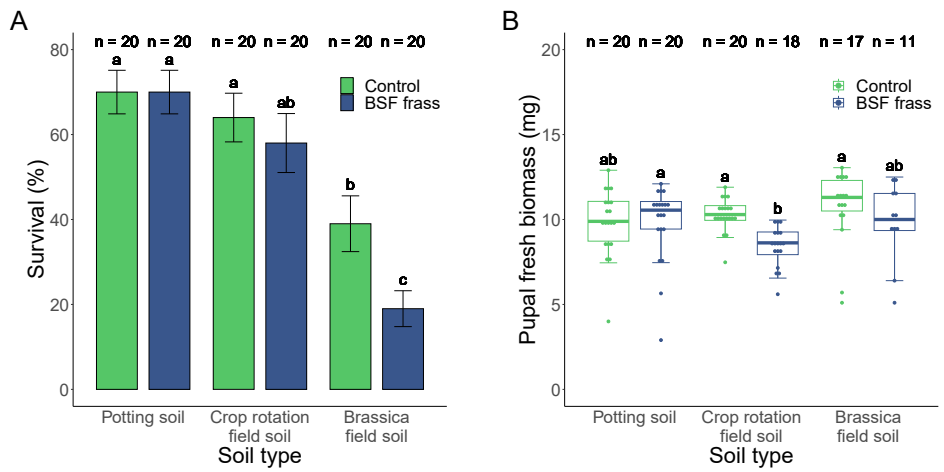

Figure 4. Survival (A) and pupal fresh biomass (B) of *Delia radicum* after feeding on *Brassica oleracea* plants growing in different soils amended with black soldier fly (BSF) frass (5 g/kg) or control soil (unamended). Treatments denoted with the same letter are not significantly different (EMM/Dunn's test, *P* > 0.05). Error bars (A) represent standard errors. Box plot whiskers (B) represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants are indicated at the top of the panels by n.

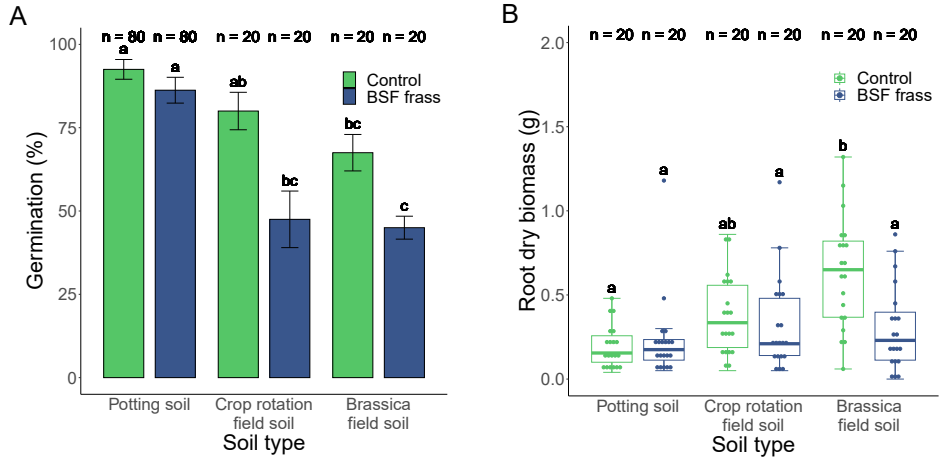

Figure 5. Seed germination (A) and root dry biomass (B) of *Brassica oleracea* 8 weeks after planting and 3 weeks after infestation with *Delia radicum* larvae in different soils amended with black soldier fly (BSF) frass (5 g/kg) or control soil (unamended). Treatments denoted with the same letter are not significantly different (Dunn's test, *P* > 0.05). Error bars (A) represent standard errors. Box plot whiskers (B) represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate pots (A) or plants (B) are indicated at the top of the panels by n.

Discussion

In both experiments reported here, the negative effect of soil amendment with black soldier fly frass on *D. radicum* performance observed in a previous study was confirmed (Wantulla et al., 2022). Similar to the previously tested amendment ratio of 10 g/kg, amending brassica field soil with frass at a ratio of 5 g/kg reduced *D. radicum* survival by 32% in Experiment 1 and by 50% in Experiment 2. In addition, amendment of brassica field soil reduced pupal biomass by 19% in Experiment 1 while amendment with crop rotation field soil reduced pupal biomass by 18% in Experiment 2. At a ratio of 1 or 2 g/kg, however, *D. radicum* performance was not significantly affected by soil amendment with black soldier fly frass. This suggests that a minimum amendment ratio of 5 g/kg might be necessary to reduce *D. radicum* survival and growth, though ratios between 2 and 5 g/kg were not tested and may also prove to be sufficient.

Brassica field soil also proved to be suppressive to *D. radicum* without soil amendment. Larval survival in brassica field soil was lower than in potting soil or in crop rotation field soil and was reduced further by amendment with black soldier fly frass. Although the addition of frass did not significantly affect survival in crop rotation field soil, it did appear to have sublethal effects on *D. radicum* as pupal biomass was reduced. Soil amendment with black soldier fly frass was previously found to have no effect on the biomass of adult *D. radicum*, suggesting that pupae with reduced biomass are less likely to develop into flies (Wantulla et al., 2022).

The inherent suppressiveness of brassica field soil to *D. radicum* may be caused by native microbes whose suppressive activity is enhanced by the addition of black soldier fly frass. Soil amendment with frass possibly promoted the growth of responsible microorganisms by providing nutrients to these microbes (Barragán-Fonseca et al., 2022; Mazzola & Freilich, 2017). Although some of these microbes might also be present in crop rotation field soil, they are presumably less abundant since amendment of this soil only had a minor effect on *D. radicum* performance. Such differences in the native soil microbiome might be due to the different cropping histories of the two agricultural fields. While brassicaceous plants had been grown in the field in Wageningen for years, various crops had been rotated on the field in Lelystad, none of which belonged to the Brassicaceae. Furthermore, *D. radicum* infestations had been a common occurrence in the brassica field in Wageningen. Therefore, the prolonged presence of brassicaceous plants might have led to the development of a *D. radicum*-suppressive soil microbiome, e.g., via plant–soil or plant–soil–insect feedback (Pineda et al., 2017). Alternatively, the presence of *D. radicum* might have stimulated an increased abundance of antagonistic soil microbes

independently of its host plants. Root-feeding by *D. radicum* has been reported to induce specific changes in the rhizosphere microbial community of cabbage plants and reduce *D. radicum* performance on plants subsequently grown in the same soil (Friman et al., 2021a). Interestingly, *D. radicum* feeding was found to increase the abundance of bacteria from the genus *Pseudomonas*, a taxon that also responds positively to soil amendment with black soldier fly exuviae (Wantulla et al., 2023).

Several *Pseudomonas* species have been identified in *D. radicum* larvae and may be beneficial to them, whereas others exhibit varying degrees of pathogenicity to *D. radicum* (Flury et al., 2019; van den Bosch & Welte, 2020). Pseudomonads are well-known for their plant-protective properties and different root-colonizing species can control insect pests by inducing systemic plant resistance or via insecticidal activity (Kupferschmied et al., 2013). Induced systemic resistance (ISR), however, works primarily against generalist herbivores and soil inoculation with an ISR-eliciting *Pseudomonas* strain was found to positively affect *D. radicum* performance (Friman et al., 2021b; Pineda et al., 2013). On the other hand, plant–soil–insect feedback effects associated with *D. radicum* infestation were shown to reduce *D. radicum* performance but did not seem to enhance plant defense (Friman et al., 2021a). Rather than mediating ISR, bacteria that are enriched in the cabbage rhizosphere by *D. radicum* feeding or by soil amendment with black soldier fly residues might thus be pathogenic to *D. radicum*. A root-colonizing *Pseudomonas* strain with insecticidal activity was demonstrated to persist in *D. radicum* after ingestion by root-feeding larvae and to be transmitted to other plants by adult flies. Although the bacterium only had minor effects on *D. radicum*, persistence throughout different life stages was observed in highly susceptible insect species, too (Flury et al., 2019). This suggests that more virulent strains could also be dispersed by *D. radicum*, e.g., if individuals that have been exposed to lower doses survive an infection. The introduction of such *D. radicum* pathogens to soil would be increasingly likely as *D. radicum* infestations repeatedly occur in a field.

Despite the suppression of *D. radicum*, brassica field soil and amendment with black soldier fly frass also had negative effects on *B. oleracea*. Seed germination in brassica field soil was lower than in potting soil. Similarly, the main effect of frass addition was a reduction in *B. oleracea* seed germination. In brassica field soil in Experiment 2 soil amendment with frass reduced root biomass. This is in line with a previous study in which a negative effect of soil amendment with black soldier fly frass on *B. oleracea* shoot biomass was found (Wantulla et al., 2022). While amendment with frass did not affect plant biomass in Experiment 1, it also had a less pronounced effect on *D. radicum* than in Experiment 2. Whereas amendment of brassica field soil halved *D. radicum* survival in Experiment 2 and did not affect pupal biomass, it only reduced survival by a third in Experiment 1 but

reduced pupal biomass, too. Furthermore, control survival in brassica field soil was much higher in Experiment 1 than in Experiment 2. It should be noted that the field soil used in Experiment 1 had been stored for 10 months, whereas in Experiment 2 soil was used only 1 month after collection in the field. Longer storage might have caused changes in the soil microbiome, possibly resulting in the depletion of *D. radicum*-suppressive or plant growth-inhibiting microbes.

Overall, the present study corroborates that soil amendment with black soldier fly frass can negatively affect both *D. radicum* and *B. oleracea* performance. Intriguingly, the extent of these effects depends on the soil type and appears to be influenced by factors such as cropping history, the native microbiome and inherent suppressiveness of the soil. While this context-dependence may pose a challenge to the application of black soldier fly frass for *D. radicum* control, it might also provide new opportunities for its use in crop production. Since it is likely that the negative effects of soil amendment with frass on *D. radicum* and *B. oleracea* are caused by different microorganisms, it might be possible to disentangle these effects. In unamended brassica field soil, *D. radicum* survival was lower than in other soils while *B. oleracea* biomass was higher. This suggests that *D. radicum* suppression is not necessarily associated with inhibited plant growth.

If it is possible to isolate insect pathogens from *D. radicum*-suppressive soil, coapplying them with black soldier fly frass might be an effective method to control *D. radicum* in other soils. Ideally, such an approach would circumvent microbes that are detrimental to crops and that could also be stimulated by the soil amendment. Therefore, future research should focus on isolating entomopathogenic microbes from *D. radicum*-suppressive environments, e.g., fields that have frequently been cultivated with brassicaceous plants. Amendment with black soldier fly frass might result in enrichment of *D. radicum* pathogens in the soil and potentially facilitate their isolation. Addressing these topics may lead to novel approaches in the control of this important pest of brassicaceous crops.

Acknowledgements

This work was supported by the Dutch Research Council, NWO (grant number ALWGK.2016.010). We thank Esther Kangha for her help in conducting one of the experiments, Klaas van Rozen for providing field soil from Lelystad, Andre Ramaker and Melissa Spoor for helping to maintain plants and Sarah Kalisvaart and Els van de Zande for their assistance in collecting *D. radicum* larvae and pupae.

Supporting information

Table S1

Composition of the optimized fertilizer solution

Macronutrients	(mmol/L)	Micronutrients	(µmol/L)	EC*(S/m)	pH
NH_4^+	1.2	Fe	35.0	2.0	5.5
K	7.2	Mn	8.0		
Ca	4.0	Zn	5.0		
Mg	1.8	B	20.0		
NO_3^-	12.4	Cu	0.5		
SO_4^{2-}	3.3	Mo	0.5		
P	1.0				

*Electrical conductivity.

Chapter 6

Insecticidal activity of a *Pseudomonas* sp. isolated from *Delia radicum* exposed to *D. radicum*-suppressive soil amended with black soldier fly frass

Max Wantulla, Marcel Dicke, Joop J. A. van Loon

Abstract

As a result of increasingly restrictive pesticide regulations, there is a need for alternative methods to control the cabbage root fly (*Delia radicum* L.). Survival of *D. radicum* larvae has recently been found to be reduced in soil from a field that had a brassica cropping history compared with two other soils. Furthermore, soil amendment with black soldier fly (*Hermetia illucens* L.) frass has been demonstrated to enhance suppressiveness to *D. radicum*. Bacteria were isolated from dead *D. radicum* larvae that had been exposed to *D. radicum*-suppressive soil amended with black soldier fly frass and their insecticidal activity was assessed. In laboratory bioassays, larvae were allowed to feed on swede slices treated with bacterial suspensions. Treatment with a *Pseudomonas* isolate reduced larval survival and pupation and its capacity to control *D. radicum* was subsequently tested in the greenhouse. The isolate was applied to soil at 10^{10}, 10^{9} and 10^{8} CFU/kg and Brussels sprouts (*Brassica oleracea* L.) plants were grown in treated soil for 5 weeks before being infested with *D. radicum* larvae. Soil application at 10^{10} CFU/kg resulted in a significant reduction in larval survival. The *Pseudomonas* isolate tested here has the potential to be a new biological control agent for *D. radicum* and its insecticidal activity will need to be compared directly with that of other *Pseudomonas* strains. Future studies should moreover investigate the potential role of black soldier fly frass as a substrate for the isolate and consider combined applications as a means to improve *D. radicum* control.

Introduction

Larvae of the cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae), feed on the roots of a wide range of brassicaceous plants and are among the most destructive pests of brassica crops in northern temperate regions (Joseph & Iudice, 2020; Shuhang et al., 2016). Damage can be particularly severe on brassicas sown or planted early in the season as infestations of young plants may result in up to 100% crop loss (Ferry et al., 2009). Adult *D. radicum* females oviposit on the soil surface close to the plant stem. After hatching, the larvae tunnel into the roots and feed for ca. 3 weeks before pupating in the soil (Joseph & Iudice, 2020). Feeding injury can lead to secondary invasion by pathogens and thus increase the susceptibility of plants to root diseases. Plants infested with *D. radicum* may show symptoms such as slow growth, yellowing or stunting (Santolamazza-Carbone et al., 2017).

Diminishing numbers of available insecticides and low levels of crop resistance to *D. radicum* are increasingly forcing European growers to use cultural or biological control methods instead (Collier et al., 2020; Santolamazza-Carbone et al., 2017). As these measures are generally not effective enough to be viable alternatives to chemical treatments, there is a need for additional methods that can help to provide sufficient *D. radicum* control. A rather new approach is the use of by-products from insect mass-rearing as soil amendments in order to stimulate beneficial soil microbes (Barragán-Fonseca et al., 2022). Different residues from the commercial production of black soldier fly larvae, *Hermetia illucens* L., have been shown to reduce *D. radicum* performance when added to soil (Wantulla et al., 2022). The prospects of such applications for frass, the main by-product of black soldier fly rearing, are considered to be particularly promising (Schmitt & de Vries, 2020). However, recent research suggests that the effectiveness of these amendments against *D. radicum* is limited to soils that already exhibit a certain degree of inherent *D. radicum* suppressiveness (Chapter 5). To enable reliable use of black soldier fly frass in *D. radicum* management, it is necessary to identify which properties of *D. radicum*-suppressive soils are responsible for effective control of this pest.

Whereas relatively little research has addressed insect suppressiveness, disease-suppressive soils are a well-known phenomenon (Schlatter et al., 2017). Disease suppression in these soils largely depends on the activity of native soil microbes, which can be promoted by the addition of organic substrates (Mazzola & Freilich, 2017). The development of suppressive microbiomes can be driven by soil feedback processes, such as plant–soil or plant–soil–insect feedback, which may also contribute to the suppression of insect pests (Johnson et al., 2016; Pineda et al., 2017). Interestingly, the performance of *D. radicum* larvae was reduced in soil that had previously been conditioned by *D. radicum*-infested

plants, presumably due to the enrichment of specific microbes (Friman et al., 2021a). In line with this, differences in *D. radicum* suppressiveness between agricultural soils have been suggested to depend on brassica cropping history and occurrence of infestation with *D. radicum* in previous years (Chapter 5).

Suppression of *D. radicum* in certain soils might be the result of increased abundance of beneficial microbes such as entomopathogenic bacteria. Bacteria with the ability to infect *D. radicum* and colonize plant roots can be transmitted from plant to plant by adult flies after ingestion by root-feeding larvae (Flury et al., 2019). The continuous cultivation of potential host crops of *D. radicum* and repeated *D. radicum* infestations might thus promote insect pathogens and their establishment in the soil. Indeed, soil from a field that had been used to grow brassicaceous plants for multiple years was found to be suppressive to *D. radicum*, whereas other soils were not (Chapter 5). The fact that *D. radicum* suppressiveness can be enhanced by soil amendment with black soldier fly frass has led to the assumption that the material might serve as a substrate for already established *D. radicum* pathogens.

Soils that exhibit *D. radicum* suppressiveness can provide new opportunities for the management of this economically relevant pest. Possibly harboring high densities of *D. radicum*-suppressive microbes, they are a potential source of new microbial agents that could be used for *D. radicum* control. The aim of the present study was to isolate insecticidal bacteria from *D. radicum* larvae after exposure to *D. radicum*-suppressive soil. For this purpose, soil was mixed with black soldier fly frass, which had previously been found to enhance suppressiveness. The effects of selected isolates on *D. radicum* performance were assessed in the laboratory and in the greenhouse.

Materials and methods

Insect rearing

The *D. radicum* population used was collected in Zeewolde (Flevoland, the Netherlands) in 2013. All life stages were kept in a climate cabinet at 20 ± 1 °C and 16 h light/8 h dark photoperiod. Larvae were kept on *Brassica napus* L. subsp. *rapifera* (swede) roots of 10-week-old plants until pupation. Eclosed adult root flies were kept in gauze cages and were fed with a 1:1:1 mixture of sugar, milk powder and yeast. In addition, a solution of honey in tap water was offered in a Petri dish and tap water was offered in a Petri dish with moist filter paper on top of wet cotton wool. Oviposition was stimulated by providing slices of swede in Petri dishes to the flies in the cages. Eggs were collected and placed on a new swede prior to larval hatching. To obtain larvae for

experiments, eggs were incubated in Petri dishes with moist filter paper. Larvae hatched from the eggs after 4 days.

Soil

Agricultural soil was collected from the topsoil layer of an organically managed field in Wageningen, the Netherlands in 2019 for the isolation of bacteria and in 2021 for the greenhouse experiment. The field had been used to grow various brassicaceous plants since 2011 and soil was collected from locations at which black mustard (*Brassica nigra* L.) had recently been grown. Soil composition as assessed for the same field by Eurofins Agro (Wageningen, the Netherlands) in 2018 was 81% sand, 14% silt and 2% clay, with a soil organic matter content of 3.2%. The soil was homogenized by sieving (particle size < 5 mm) and stored at ambient temperature in a non-heated warehouse for 5 - 8 months before being used.

Black soldier fly frass

Frass of larvae of black soldier flies, *H. illucens*, was provided by Bestico (Berkel en Rodenrijs, the Netherlands). The material was first inspected for the presence of insects or insect fragments, which were removed, and was then heat-treated and dried in an oven at 60 °C for 24 h. The dried material was subsequently ground to a powder with an SM 100 cutting mill (Retsch, Haan, Germany).

Isolation and identification of bacteria

The bacteria used in this study were isolated from *D. radicum* larvae that had been exposed to agricultural soil. Soil was mixed with heat-treated frass of larvae of black soldier flies, *H. illucens*, at a ratio of 10 g/kg. Neonate *D. radicum* larvae were carefully placed in Petri dishes (9 cm diameter) containing 1.5% water agar and were gently covered with soil before adding a slice of swede root on the surface. Petri dishes were sealed with Parafilm and kept in the dark at 25 ± 1 °C. After 2 weeks, dead larvae were collected and stored individually in sterile 1.5-ml microcentrifuge tubes at 4 °C for 1 - 4 weeks. Four intact specimens with atypical appearance were selected for the extraction of putative pathogens. Signs of potential disease included brownish or reddish coloration, milky hemolymph or softness of the cadaver. Larvae were surface-sterilized in 70% ethanol, rinsed and homogenized in sterile water before plating serial dilutions on lysogeny broth (LB) agar. Plates were incubated at 30 °C and single bacterial colonies were isolated after 24 h.

For identification using PCR and sequencing of 16S rRNA genes, single colonies were suspended in 100 µl of water. PCR reaction mixes contained 1 µl of suspended bacteria, 5 µl of GoTaq Reaction Buffer (Promega, Madison, Wisconsin, USA), 1.5 µl of $MgCl_2$ solution (25 mM), 0.5 µl of deoxynucleoside triphosphates (10 mM each), 0.5 µl of each primer (10 µM; Eurofins Genomics, Ebersberg, Germany), 0.125 µl of GoTaq DNA polymerase (Promega, Madison, Wisconsin, USA) and 15.875 µl of H_2O with a total volume of 25 µl. General bacterial primers used were 27f/1495r and reaction conditions were 1 cycle of 5 min at 94 °C, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 3 min, and 1 cycle of 72 °C for 20 min (Majerus & Majerus, 2010). Amplified DNA was sent to Eurofins Genomics Germany (Ebersberg, Germany) for sequencing and sequences were compared to the NCBI 16S rRNA database using BLAST.

Bacterial culture conditions

Bacteria were grown in tubes containing 30 ml of LB medium that were incubated while shaking at 250 rpm at 30 °C. Cultures were centrifuged after 24 h and pellets were washed and resuspended in sterile 0.9% NaCl solution before diluting to the desired cell density based on OD_{600}. The number of cells present in suspensions used for experiments was evaluated using the plate count method. Dilution series were plated on LB agar in triplicate and plates were incubated at 30 °C overnight.

Laboratory bioassays of different bacterial isolates against *D. radicum*

Bacterial suspensions of three isolates identified as *Priestia* sp., *Pseudomonas* sp. and *Serratia* sp. were prepared as described above and diluted to ca. 10^7 CFU/ml. Numbers of viable cells present in suspensions as determined by the plate count method are shown in Table S1. Slices of swede roots were immersed in bacterial suspension or 0.9% NaCl solution and were individually placed in Petri dishes (9 cm diameter) containing 1.5% water agar. Ten neonate *D. radicum* larvae were added to each Petri dish by carefully transferring them to plastic labels that were placed on top of the root slices. Labels were checked after 15 min and remaining larvae were replaced. This was repeated until ten larvae had moved onto the root slice in every Petri dish. Each treatment was replicated five times. Petri dishes were arranged in a completely randomized design and kept in the dark at 20 ± 1 °C. After 3 weeks, living larvae and pupae were counted and pupae were weighed on a CP2P-F micro balance (Sartorius, Göttingen, Germany). The bioassay was repeated three times.

Greenhouse experiment to test the efficacy of a *Pseudomonas* isolate against *D. radicum*

A suspension of the isolate identified as *Pseudomonas* sp. was prepared as described above and diluted to ca. 2×10^8, 2×10^7 and 2×10^6 CFU/ml. Numbers of viable cells present in suspensions as determined by the plate count method are shown in Table S1. Each dilution was mixed into soil at 50 ml/kg of dry soil, resulting in doses of ca. 10^{10}, 10^9 and 10^8 CFU/kg. Control soil was mixed with the same volume of 0.9% NaCl solution. Per treatment, 22 *Brassica oleracea* L. var. *gemmifera* cv. Cyrus (Brussels sprouts) plants were grown in 1 L plastic pots and kept in a greenhouse compartment at 20 ± 3 °C, 60-80% relative humidity and 16 h light/8 h dark photoperiod. Pots were individually placed in saucers and arranged in a completely randomized design. Two seeds were sown per pot and gently pressed down. If both seeds germinated, one seedling was randomly removed from each pot after 1 week. Excess seedlings were transplanted to pots of the same treatment in which no seeds had germinated or were discarded together with ungerminated seeds.

Plants were watered three times per week by filling saucers and emptying them after 2 h. Starting 1 week after sowing, plants were fertilized for 3 weeks with an optimized fertilizer solution (Table S2). Each plant received 160 ml of fertilizer per week. Fertilizer amounts per 1 L of field soil were based on a nitrogen fertilization advice for cabbage provided by Eurofins Agro (Wageningen, the Netherlands). Plants were grown for 5 weeks before being infested with *D. radicum* by carefully placing five neonate larvae on plastic labels that were inserted into the soil surface close to the stem. Labels were checked after 30 min and remaining larvae were replaced. This was repeated until five larvae had moved into the soil in every pot. Plants were uprooted 3 weeks after infestation and roots were rinsed to remove adhering soil. Roots were checked for remaining larvae and all soil was washed through a 1 mm aperture sieve to collect larvae and pupae. Living larvae and pupae were counted and pupae were weighed on a CP2P-F micro balance (Sartorius, Göttingen, Germany). Plants were oven-dried at 105 °C for 24 h to quantify shoot and root dry biomass.

Statistical analysis

Statistical tests were performed using R (Version 4.2.2; R Core Team, 2022) and the packages car (Fox & Weisberg, 2019), dunn.test (Dinno, 2017), emmeans (Lenth, 2022), lme4 (Bates, Maechler, Bolker & Walker, 2015), nlme (Pinheiro, Bates & R Core Team, 2022) and stats (R Core Team, 2022). Data from the three bioassays were combined and analyzed using mixed effects models with bioassay as a random factor. Generalized linear mixed effects models (GLMM) with binomial distributions were used to analyze *D. radicum* survival

and pupation, while a linear mixed effects model (LMM) was used to analyze average pupal biomass per Petri dish. Pairwise comparisons were performed using estimated marginal means (EMM). Models were validated by plotting residuals and, where necessary, homoscedasticity and normality were confirmed using Levene's test and the Shapiro-Wilk test, respectively. For the greenhouse experiment, seed germination, *D. radicum* survival and pupation rates were analyzed using generalized linear models (GLM) with binomial distributions and pairwise comparisons of EMMs. Quasi-GLMs were used to analyze survival and pupation as overdispersion was detected in the data. Average pupal biomass per plant was analyzed using a linear model (LM) and pairwise comparisons of EMMs, whereas shoot and root biomass were analyzed with the Kruskal–Wallis test and Dunn's test due to the violation of model assumptions.

Results

16S rRNA gene sequences of bacteria isolated from *D. radicum*

Per *D. radicum* larva, one or two morphologically different types of bacterial colonies were obtained. In total, seven colonies were isolated. The 16S rRNA gene sequences of the only isolate from Larva 1 and of one of two isolates from Larva 2 were most similar to *Serratia fonticola* (accession number AJ233429), showing 99.7% and 99.5% sequence identity, respectively. The other isolate from Larva 2 exhibited 99.8% sequence identity with *Pseudomonas protegens* (accession number AJ278812). While one of two bacteria isolated from Larva 3 showed 99.9% sequence identity with *Priestia megaterium* (accession number AB271751), the sequence of the other isolate shared 99.9% identity with that of *Priestia aryabhattai* (accession number EF114313). One of two isolates from Larva 4 exhibited 99.7% sequence identity with *Serratia marcescens* (accession number AB681729), while the other was most similar to *Providencia burhodogranariea* (accession number HM038004), showing 99.6% sequence identity. The three isolates with high similarity to *Pseudomonas protegens*, *Priestia megaterium* and *Serratia marcescens* were selected for assessing insecticidal activity against *D. radicum*.

Insecticidal activity of bacterial isolates in laboratory bioassays

Treatment of swede root slices with bacterial suspensions significantly affected *D. radicum* survival (GLMM: $\chi^2 = 14.374$, $df = 3$, $P = 0.002$) and pupation (GLMM: $\chi^2 = 33.617$, $df = 3$, $P < 0.001$). The tested *Pseudomonas* isolate significantly reduced survival compared with the control (EMM: $P = 0.002$; Figure 1A) and reduced percentage pupation compared with the control and both other bacterial isolates (EMM: $P < 0.001$; Figure 1B). Treatment with

bacterial suspensions had no significant effect on pupal fresh biomass (LMM: $\chi^2 = 4.0093$, $df = 3$, $P = 0.261$; Figure 2).

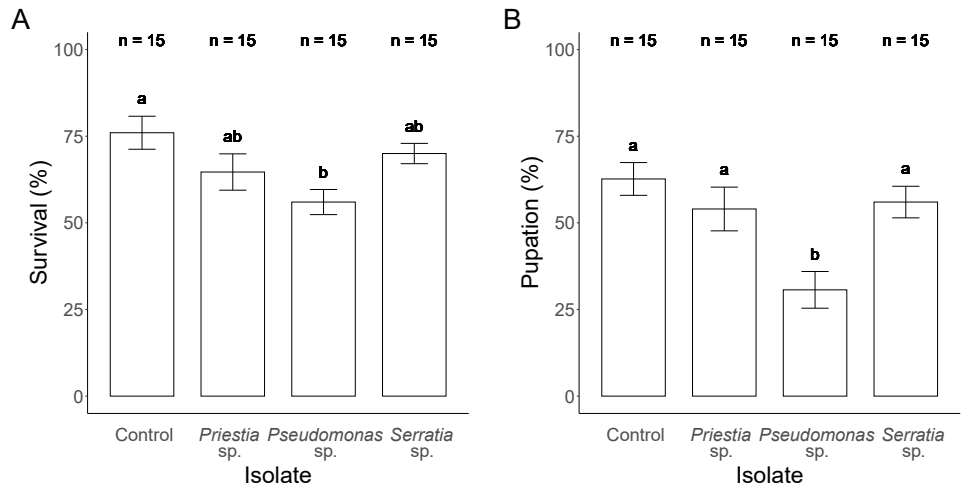

Figure 1. Survival (A) and pupation (B) of *Delia radicum* after 3 weeks of feeding on *Brassica napus* (swede) root slices treated with suspensions of different bacterial isolates containing ca. 10^7 CFU/ml. Treatments denoted with the same letter are not significantly different (EMM, $P > 0.05$). Error bars represent standard errors. Numbers of replicate Petri dishes are indicated at the top of the panels by n.

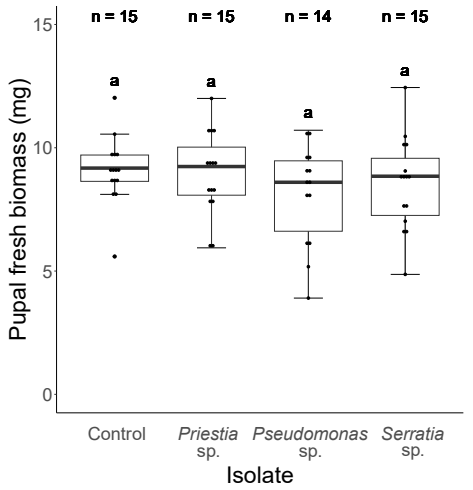

Figure 2. Pupal fresh biomass of *Delia radicum* after 3 weeks of feeding on *Brassica napus* (swede) root slices treated with suspensions of different bacterial isolates containing ca. 10^7 CFU/ml. Biomass did not differ significantly among treatments (LMM, $P = 0.261$). Box plot whiskers represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate Petri dishes are indicated at the top of the panels by n.

Control of *D. radicum* by a *Pseudomonas* isolate under greenhouse conditions

Soil application of the *Pseudomonas* isolate had a significant effect on *D. radicum* survival (GLM: χ^2 = 14.208, *df* = 3, *P* = 0.003). When soil was mixed with bacterial suspension at 10^{10} CFU/kg, survival was significantly reduced compared with the control and compared with an application rate of 10^8 CFU/kg (EMM: *P* = 0.047; Figure 3A). Application to soil significantly affected *D. radicum* pupation (GLM: χ^2 = 11.847, *df* = 3, *P* = 0.008) and at 10^{10} CFU/kg significantly reduced pupation compared with the control (EMM: *P* = 0.041; Figure 3B).

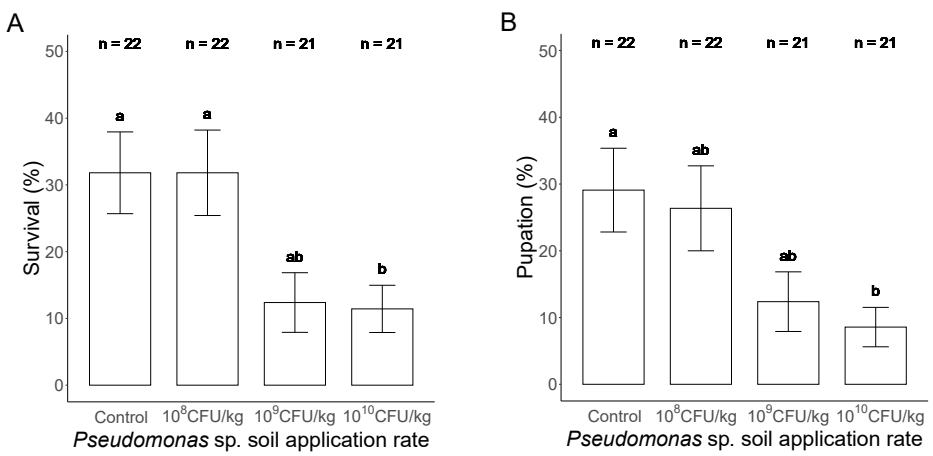

Figure 3. Survival (A) and pupation (B) of *Delia radicum* after 3 weeks of feeding on roots of *Brassica oleracea* plants growing in field soil inoculated with three densities of a *Pseudomonas* isolate in a greenhouse experiment. Treatments denoted with the same letter are not significantly different (EMM, *P* > 0.05). Error bars represent standard errors. Numbers of replicate plants are indicated at the top of the panels by n.

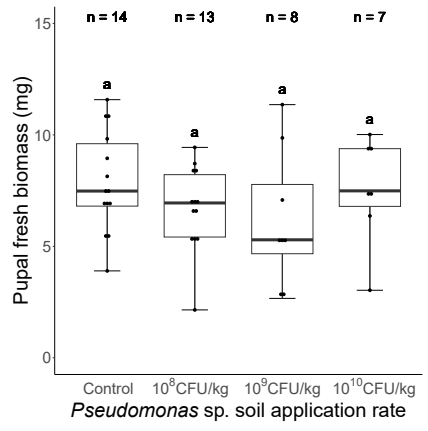

Figure 4. Pupal fresh biomass of *Delia radicum* after 3 weeks of feeding on roots of *Brassica oleracea* plants growing in field soil inoculated with three densities of a *Pseudomonas* isolate in a greenhouse experiment. Biomass did not differ significantly among treatments (LM, *P* = 0.353). Box plot whiskers represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants are indicated at the top of the panels by n.

Pupal fresh biomass was not significantly affected by soil application of the *Pseudomonas* isolate (LM: *F* = 1.1216, *df* = 3, *P* = 0.353; Figure 4). Soil application of the isolate had no significant effect on *B. oleracea* seed germination (GLM: χ^2 = 1.3421, *df* = 3, *P* = 0.719; Figure 5), shoot dry biomass (Kruskal–Wallis test: *H* = 3.2857, *df* = 3, *P* = 0.35; Figure 6A) or root dry biomass (Kruskal–Wallis test: *H* = 0.2326, *df* = 3, *P* = 0.97; Figure 6B) of *B. oleracea* plants.

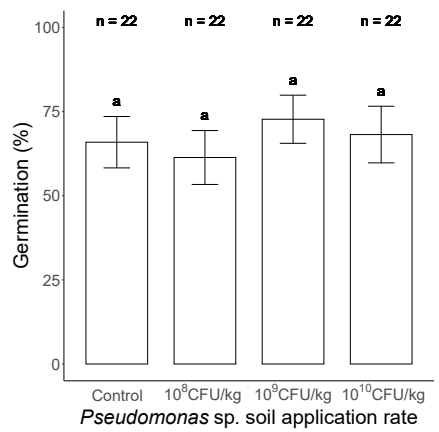

Figure 5. Germination of *Brassica oleracea* seeds in field soil inoculated with three densities of a *Pseudomonas* isolate. Germination did not differ significantly among treatments (GLM, *P* = 0.719). Error bars represent standard errors. Numbers of replicate pots are indicated at the top of the panels by n.

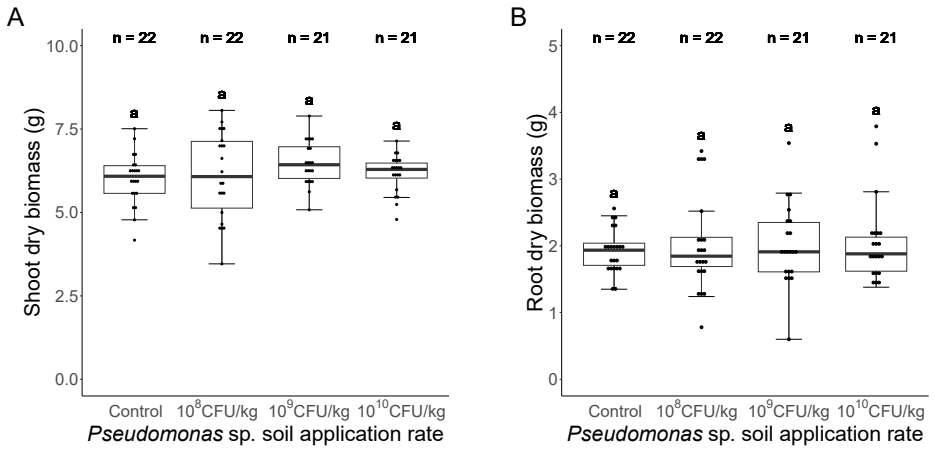

Figure 6. Shoot (A) and root (B) dry biomass of *Brassica oleracea* plants grown in field soil inoculated with three densities of a *Pseudomonas* isolate 8 weeks after planting and 3 weeks after infestation with *Delia radicum* larvae. Shoot and root biomass did not differ significantly among treatments (Kruskal–Wallis test, *P* > 0.05). Box plot whiskers represent largest values within 75% quantiles + 1.5 × interquartile range (IQR) and smallest values within 25% quantiles - 1.5 × IQR. Numbers of replicate plants are indicated at the top of the panels by n.

Discussion

All bacterial isolates examined in this study could be assigned to genera that have previously been associated with *D. radicum* or brassica crops. Whereas *P. megaterium* (previously *Bacillus megaterium*) has been isolated from *B. napus* L. roots, the genera *Providencia*, *Pseudomonas* and *Serratia* have repeatedly been found in *D. radicum* larvae (Lukwinski et al., 2006; Ribeiro et al., 2021; van den Bosch & Welte, 2020; Welte et al., 2016). Although *Pseudomonas* and *Serratia* species identified in larvae have been suggested to benefit *D. radicum*, both genera are well-known for comprising various entomopathogenic strains, too (Jackson et al., 2001; Kupferschmied et al., 2013; Petersen & Tisa, 2013). Among the bacteria tested here, however, only the *Pseudomonas* isolate was found to have insecticidal activity against *D. radicum*, reducing larval survival and pupation by 26% and 51%, respectively. Remarkably, both *D. radicum* feeding and soil amendment with black soldier fly residues have been shown to increase the abundance of bacteria from the genus *Pseudomonas* in the *B. oleracea* rhizosphere (Friman et al., 2021a; Wantulla et al., 2023). These findings suggest that insecticidal pseudomonads may be responsible for the suppressiveness of soil to *D. radicum* after continuous brassica cultivation and for the enhancement of *D. radicum* suppressiveness by soil amendment with black soldier fly frass.

Many bacteria from the genus *Pseudomonas* are known for being excellent root colonizers that can suppress plant diseases (Berendsen et al., 2012; Friman et al., 2021c). Due to their activity against a range of pathogens, different species are marketed as biocontrol agents (Kupferschmied et al., 2013). In addition, certain root-colonizing *P. protegens* and *P. chloroaphis* strains exhibit oral toxicity to insects, especially to lepidopteran larvae (Ruffner et al., 2013). However, few strains have been found to affect *D. radicum*, with their impact on larval performance being variable. Although two *Pseudomonas* strains were shown to reduce the survival of larvae that were feeding on treated radishes, each strain only caused a significant reduction in one of two reported experiments (Flury et al., 2019). Radishes were immersed in bacterial suspensions with an OD_{600} of 0.47, which should contain ca. 3.8×10^8 CFU/ml and thus considerably higher cell densities than the suspensions used in the present study. Although such comparisons need to be drawn with caution, not least due to substantial differences between experimental setups, the *Pseudomonas* isolate tested here might thus be more toxic to *D. radicum* than previously tested strains. As the isolate inhibited larval development, significantly reducing pupation after 3 weeks, it is possible that other effects on *D. radicum* performance would have been more pronounced in bioassays lasting longer. On the one hand, allowing slowly developing larvae to pupate might have shown a potential effect on pupal biomass. On the other hand, larvae that had not pupated after 3 weeks might have eventually died, possibly resulting in a still greater reduction in larval survival.

When applied to field soil, the *Pseudomonas* isolate reduced *D. radicum* survival by 64% under greenhouse conditions. At a rate of 10^{10} CFU/kg, it proved to provide effective control for at least 5 weeks after application. Although lower application rates did not significantly reduce *D. radicum* performance, they might be effective when infestations occur sooner after application. Furthermore, it should be noted that low control survival due to *D. radicum* suppressiveness of the field soil used may have caused effects on *D. radicum* to be less noticeable. While soil application of the *Pseudomonas* isolate did not negatively affect *B. oleracea* seed germination or plant growth, it was not found to improve plant performance, despite reducing *D. radicum* survival. It is possible that five larvae per plant were not sufficient to cause significant feeding damage in any of the treatments. Other greenhouse studies have used twice or four times as many *D. radicum* larvae or eggs to infest plants of similar ages and sizes (Friman et al., 2021a; Joseph & Iudice, 2020; Shuhang et al., 2016). Considering the relatively low infestation pressure in the present study, possible consequences of *D. radicum* feeding or of a reduction therein might have become apparent only after a longer growing period.

The *Pseudomonas* isolate tested here could be a promising new control agent for *D. radicum*. While its insecticidal activity toward *D. radicum* larvae is not as strong as the activity of other strains against lepidopterans, applying pseudomonads to control root pests is likely to have practical advantages. The ability of various members of the genus, including insecticidal species, to colonize roots and persist in the rhizosphere has important implications for long-term plant protection (Kupferschmied et al., 2013). Furthermore, because pests such as *D. radicum* are known to facilitate infections by root pathogens, additional disease suppressive properties of a control agent could be particularly useful. Several examples show that insecticidal and antimicrobial activity of *Pseudomonas* species are not mutually exclusive (Ruffner et al., 2013). Nonetheless, the potential of the new isolate to control plant diseases and its rhizosphere competence remain to be investigated. In order to put its insecticidal activity into perspective, direct comparisons with other *Pseudomonas* strains and comparative phylogenetic analyses will be necessary. Finally, another focus of future research should be to elucidate the potential role of black soldier fly frass as a substrate for the new isolate. Investigating possible additive or synergistic effects on *D. radicum* performance could help to improve the practicability of both control measures and advance their implementation in *D. radicum* management.

Overall, the controlled exposure of insect pests to suppressive soil appears to be a promising approach for the isolation of new microbial control agents that deserves further exploration. Methods that are more specialized than the one described here may be more suitable for exposing insects to specific groups of insecticidal microbes. For

example, allowing insects to feed on the roots of plants that were grown in suppressive soil should result in more effective exposure to root-colonizing bacteria that exhibit oral toxicity. Such approaches arguably represent a more goal-oriented strategy than the common practice of isolating entomopathogens from non-suppressive environments using non-target species as bait.

Acknowledgements

This work was supported by the Dutch Research Council, NWO (grant number ALWGK.2016.010). We thank Kristian van Zadelhoff for collecting dead *D. radicum* larvae and his assistance in isolating bacteria and Tamara Darvas for her help in performing the greenhouse experiment.

Supporting information

Table S1

Cell quantities present in bacterial suspensions tested in different experiments as determined by the plate count method

	Number of viable cells (× 10^7 CFU/ml)*			
Isolate	Bioassay 1	Bioassay 2	Bioassay 3	Greenhouse
Priestia sp.	1.4	2.0	1.8	-
Pseudomonas sp.	1.0	0.2	0.8	2.1
Serratia sp.	0.4	1.2	1.5	-

*× 10^6 and × 10^8 CFU/ml for respective dilutions used in the greenhouse experiment.

Table S2

Composition of the optimized fertilizer solution

Macronutrients	(mmol/L)	Micronutrients	(µmol/L)	EC*(S/m)	pH
NH_4^+	1.2	Fe	35.0	2.0	5.5
K	7.2	Mn	8.0		
Ca	4.0	Zn	5.0		
Mg	1.8	B	20.0		
NO_3^-	12.4	Cu	0.5		
SO_4^{2-}	3.3	Mo	0.5		
P	1.0				

*Electrical conductivity.

Chapter 7

General discussion

By transforming organic residual streams into high-quality animal protein, commercial insect farming has enormous potential to contribute to both sustainable food production and a circular economy (van Huis, 2021). As the insect production industry is growing, however, it is faced with the issue of dealing with increasing amounts of its own residual streams. Finding valuable uses for these materials, such as possible applications in crop production, can help to maximize the circularity of agriculture and increase the profitability of insect farming. While there has been a surge of interest in utilizing insect frass as fertilizer, potential applications in crop protection have received relatively little attention (Fuhrmann et al., 2022; Houben et al., 2020; Poveda et al., 2019; Watson et al., 2021). Nonetheless, such applications could be instrumental in valorizing residues of insect production, especially in view of increasing limitations on pesticide use in many countries.

Soil amendment with organic substrates can promote the activity of naturally occurring microbes with biocontrol properties (Mazzola & Freilich, 2017). However, the influence of insect residual streams on soil microbes is largely unknown. Although effects of insect frass on soil microbial communities have been examined, most studies do not focus on the plant rhizosphere, i.e., the soil region directly around the roots (Houben et al., 2020; Watson et al., 2021). As the development of rhizosphere communities is strongly influenced by root exudates, they are generally distinct from microbial communities that are not exposed to these compounds (Paterson et al., 2006). Furthermore, there is a lack of studies investigating the impact of soil amendment with insect residual streams on soil pests. In the research presented here, the effects of insect frass and exuviae on an important root-feeding pest, *Delia radicum* L. (Diptera: Anthomyiidae), and on the bacterial community in the rhizosphere of its host crop, *Brassica oleracea* L., were investigated for the first time.

Factors determining the effects of insect-derived soil amendments

The research presented in this thesis was based on the underlying assumption that soil amendment with insect frass or exuviae could stimulate microbes with the potential to control crop pests (Chapter 2). Remarkably, residual streams derived from different insect species differentially affected the performance of root-feeding *D. radicum* larvae (Chapter 3). Whereas soil amendment with black soldier fly exuviae inhibited *D. radicum* growth, amendment with mealworm exuviae did not affect larval biomass and resulted in higher survival. Furthermore, amendment with the exuviae of different insect species had differential effects on the rhizosphere bacterial community of *B. oleracea* plants (Chapter 4). For example, exuviae of black soldier fly larvae and mealworms caused distinct shifts in bacterial community structure, most notably after 4 weeks of plant growth. In the *D. radicum* performance experiments, plants were infested with larvae after 4 or 5 weeks,

which suggests that bacterial community changes had already taken place and thereby could be involved in the effects on *D. radicum* that were observed. In any case, insect species seems to be a critical factor that determines the impact of insect-derived soil amendments on both rhizosphere microbial communities and root-feeding pests. Incubation time is likely to influence these effects, especially since decomposition rates of insect residual streams in soil can differ substantially between species (Nurfikari, 2022). Over time, rhizosphere communities in soil amended with the exuviae of different species were found to diverge in some cases but to become more similar in others (Chapter 4).

Differences in the composition of residual streams from different insect species are a plausible explanation for their specific effects on rhizosphere bacterial communities and on *D. radicum*. Exuviae of black soldier fly larvae, house crickets and mealworms have been reported to differ in their chitin and lipid contents and contain different proportions of carbon and nitrogen (Nurfikari, 2022; Nurfikari and de Boer, 2021). However, contents of these components could not conclusively be related to the differences between rhizosphere communities associated with respective soil amendments (Chapter 4). Other compounds, such as specific proteins, may play an important role in how amendment with insect residual streams affects microbial communities and other organisms in the soil. While the amounts of exuviae in industrially produced frass are unknown, they too are likely to differ between insect species. Although generally considered a minor component of frass, exuviae are thought to be crucial to the promotion of plant-protective soil microbes, not least due to their high chitin content (Chapter 2). Nonetheless, the effects of soil amendment with black soldier fly frass on *D. radicum* performance seemed to be similar to those of amendment with exuviae alone (Chapters 3 and 5).

Insect residual streams might serve as substrates for microbes with biocontrol potential that occur naturally in the soil (Chapter 2). Interestingly, the impact of amendment with black soldier fly frass on *D. radicum* varied between different types of soil, with effects on larval performance being restricted to soil from agricultural fields (Chapter 5). While the amendment did not affect *D. radicum* survival or growth in potting soil, it reduced larval survival in one field soil but only resulted in lower pupal biomass in another. In fact, *D. radicum* survival differed between soils irrespective of soil amendment and black soldier fly frass only reduced survival further if the amended soil was inherently *D. radicum*-suppressive. Soil that was found to be suppressive originated from a field on which brassicaceous plant species had been grown for years and in which *D. radicum* infestations had repeatedly occurred. In contrast, non-suppressive soil originated from a field on which non-brassicaceous crops had been cultivated in rotation. Soil history may thus be a key factor in *D. radicum* suppressiveness and the enhancement thereof by amendment with

black soldier fly frass. It is well-established that disease-suppressive soils can develop as a result of long-term monocultures, often requiring the presence of the respective pathogen (Schlatter et al., 2017). Disease suppression in these soils is generally based on the activity of microbes, which can be stimulated by amendment with organic substrates (Mazzola & Freilich, 2017). Therefore, it seems likely that natural and frass-induced *D. radicum* suppression are mediated by soil microbes as well.

Overall, there is evidence that the effects of insect residual streams on *D. radicum* depend not only on the insect species from which they are derived but also on the soil to which they are added. Taken together with the reasoning given above on the role of microbes in explaining differential effects of amendments derived from different insect species, resident soil microbes may be responsible for both species- and soil specificity of the effects of insect-derived soil amendments on *D. radicum* performance.

Identifying the microbial suspects

Although insect residual streams may be colonized by soil bacteria and fungi, research presented in this thesis focused on bacteria as potential mediators of *D. radicum* suppression. Soil bacteria are often considered to be the main drivers of chitin degradation and respond more quickly and strongly to chitinous amendments than fungi (Cretoiu et al., 2013; Kielak et al., 2013). Indeed, in bulk soil, insect frass and exuviae have been reported to induce more drastic changes in bacterial than in fungal communities, with increases in fungal biomass being described as relatively modest (Nurfikari, 2022). Other studies, by contrast, have found amendment with frass to affect fungal communities in particular, which may be explained by the introduction of fungi to soil due to the use of unhygienized frass (Fuhrmann et al., 2022; Watson et al., 2021). Concerning *D. radicum* control, the stimulation of potentially entomopathogenic soil microbes by insect frass or exuviae is especially interesting. Whereas promising bacterial genera, such as *Bacillus* or *Pseudomonas*, have been found to respond positively to insect residual streams, there are no such reports for well-known genera of fungal entomopathogens. On the contrary, soil amendment with black soldier fly exuviae has been shown to reduce the abundance of the entomopathogenic fungal genus *Metarhizium* (Nurfikari, 2022). Furthermore, fungal growth was never observed on dead *D. radicum* larvae that had been exposed to soil amended with black soldier fly frass, but cadavers often showed signs of bacterial infection (Chapter 6). These different findings suggest that bacteria may indeed be more important colonizers of insect frass and exuviae than fungi, particularly with regard to their role in *D. radicum* control.

The fact that amendment with black soldier fly frass enhanced the natural suppressiveness of soil to *D. radicum* suggests its function as a substrate for *D. radicum*-suppressive microbes (Chapter 5). While insect suppressiveness is an understudied area of research, disease-suppressive soils are mostly attributed to bacteria, with *Pseudomonas*-based suppression being a famous example (Schlatter et al., 2017). Remarkably, an insecticidal *Pseudomonas* strain could be isolated from *D. radicum* after exposure to *D. radicum*-suppressive soil that had been amended with black soldier fly frass (Chapter 6). Species within the genus *Pseudomonas* are known to be exceptionally versatile bacteria that may colonize roots or insects and can have biocontrol activity against plant pathogens and pests (Kupferschmied et al., 2013). It has moreover been demonstrated that root-colonizing species with insecticidal activity can be transmitted between plants by *D. radicum* (Flury et al., 2019). Thus, prolonged cultivation of brassicaceous plant species and repeated *D. radicum* infestations might have resulted in an enrichment of insecticidal pseudomonads in *D. radicum*-suppressive soil. Interestingly, soil amendment with black soldier fly exuviae increased the abundance of the genus *Pseudomonas* in the *B. oleracea* rhizosphere (Chapter 4). Although the genus was also enriched following amendment with house cricket or mealworm exuviae, enrichment by black soldier fly exuviae lasted for a longer period of time. Altogether, it seems that pseudomonads may play an important role in natural *D. radicum* suppressiveness and the enhancement of suppression by soil amendment with black soldier fly residual streams.

While it is possible that the effects of black soldier fly frass and exuviae on *D. radicum* are mediated by select groups of bacteria, such as pseudomonads, a range of microbial agents may be involved. In disease-suppressive soils, pathogen suppression may result from the activity of individual species, numerous taxa or the total microbiome, all of which can potentially be stimulated by soil amendments (Mazzola & Freilich, 2017; Schlatter et al., 2017). Similar to the genus *Pseudomonas*, other genera in the *B. oleracea* rhizosphere, including *Burkholderia*, were enriched more strongly by black soldier fly exuviae than by the exuviae of other species (Chapter 4). These bacteria might also contribute to reduced *D. radicum* performance in soil amended with black soldier fly residual streams. Despite such indications, the potential involvement of certain groups of microbes in *D. radicum* suppression requires further investigation.

Black soldier fly frass in *D. radicum* management

Among the insects that are produced on an industrial scale, larvae of the black soldier fly are especially important. This is mainly due to the fact that they can be reared on residual streams that cannot be used for other insect species, such as fermented

straw, almond hulls, catering waste or even manure (van Huis, 2021). Consequently, black soldier fly larvae mass-rearings are the largest source of insect residual streams and there is great interest in potential uses for black soldier fly frass in agriculture (Schmitt & de Vries, 2020). The capacity of soil amendment with black soldier fly residual streams to enhance suppression of *D. radicum*, a significant agricultural pest, raises hopes for possible applications in crop protection. Due to the lack of available insecticides in several European countries, growers often have to rely on methods that only provide partial *D. radicum* control (Collier et al., 2020; Herbst et al., 2017). Soil amendment with black soldier fly frass could help to improve *D. radicum* management, for example, as part of integrated pest management programs. However, the implementation of control strategies that employ black soldier fly frass ultimately depends on the practicability of its application and on whether or not consistent efficacy can be ensured. Group discussions with farmers have shown that potential difficulties in applying insect frass and inconsistent effectiveness can be major barriers to the adoption of frass in crop protection (Foolen-Torgerson, 2022).

Varying effects of black soldier fly frass in different types of soil arguably represents the greatest challenge to its practical application for *D. radicum* control. The fact that the amendment had no effect on *D. radicum* performance in potting soil is particularly problematic, as brassica crops are often grown in the greenhouse before being transplanted to the field. While amendment with black soldier fly frass negatively affected *D. radicum* performance in two agricultural soils, larval survival was only reduced in soil from one particular field (Chapter 5). Although amendment inhibited larval growth in soil from the other field, such sublethal effects may have little relevance in practice. This suggests that soil amendment with black soldier fly frass cannot reliably control *D. radicum*, even when crops are seeded directly into amended field soil. Furthermore, amending soil with frass at the effective ratio of 5 g/kg would be difficult to apply in the field. Assuming that a 10 cm layer of soil is amended, broadcast field application of dried frass at 5 g/kg would equate to more than 500 kg/ha. Even if black soldier fly frass is a by-product that may be available in large quantities and at a low cost, the use of such quantities might not be feasible from a technical or logistical perspective. Thus, more targeted application methods, such as in-furrow application during sowing, would probably be a more practical option for amendment of field soil. Arguably the most efficient approach, however, would be the addition of frass to blocks of potting soil or seedling substrates that are used for the production of brassica transplants in the greenhouse. Therefore, finding ways to ensure that amendment with black soldier fly frass can reduce *D. radicum* performance in any type of soil, including potting soil, will be crucial to its implementation.

The exuviae present in black soldier fly frass can stimulate the growth of soil microbes with biocontrol potential, such as bacteria from the genera *Pseudomonas* or *Burkholderia* (Chapter 4). Both of these genera include entomopathogenic species, some of which have been shown to exhibit insecticidal activity against *D. radicum* (Cordova-Kreylos et al., 2013; Flury et al., 2019; Kupferschmied et al., 2013). Most notably, an insecticidal *Pseudomonas* strain that was isolated from *D. radicum* following exposure to *D. radicum*-suppressive soil reduced larval survival when applied to field soil in the greenhouse (Chapter 6). As bacteria such as this strain might be responsible for *D. radicum* suppression in certain agricultural soils, their isolation and application could provide a means of transferring suppressiveness. Transferability of disease suppressiveness by inoculating soil with the responsible microbes is a common characteristic of specific disease suppression (Schlatter et al., 2017). For example, it has been shown that take-all, a root disease of wheat caused by *Gaeumannomyces tritici*, can be suppressed in take-all conducive soil by the introduction of selected *Pseudomonas* strains from take-all suppressive soil (Raaijmakers & Weller, 1998). In soils that are not naturally suppressive to *D. radicum*, application of *D. radicum*-suppressive bacteria could potentially serve as a basis for amendment with black soldier fly frass. Soil amendment with frass might then enhance transferred *D. radicum* suppressiveness in a way that is similar to the enhancement of natural suppressiveness (Chapter 5). Since amendment with black soldier fly exuviae enriched the genus *Pseudomonas* in the *B. oleracea* rhizosphere, an insecticidal *Pseudomonas* isolate could be a suitable candidate for such an approach (Chapter 4). Different *Pseudomonas* strains that are used to control plant diseases are available in commercial formulations for seed treatment or for drenching potted plants and vegetable transplants (Kupferschmied et al., 2013). Similar to *Pseudomonas*-based products for the control of root diseases, insecticidal *Pseudomonas* strains would also have to be applied to roots in order to control root-feeding pests. Therefore, these products can provide valuable examples for the formulation and application of pseudomonads to control *D. radicum*. At the same time, inconsistent efficacy of *Pseudomonas*-based products under field conditions illustrates the importance of additional measures to stimulate the activity of microbial control agents.

Although soil amendment with black soldier fly frass may have limited potential to be a stand-alone treatment for *D. radicum* control, it is likely to enhance the *D. radicum*-suppressive activity of soil microbes (Chapter 5). Microbes with insecticidal activity against *D. radicum* may only be present in certain soils, but they can be isolated and used as soil inoculants to reduce *D. radicum* performance (Chapter 6). While neither of these methods is likely to provide consistent control of *D. radicum* on its own, it might be possible to combine both approaches to improve their efficacy and reliability.

Conclusions and further prospects

Despite promising effects in *D. radicum*-suppressive soil, amendment with black soldier fly frass is still far from becoming a reliable method for the control of *D. radicum*. The key to successful implementation in the future might be microbial agents, such as insecticidal bacteria, that can be applied to soil or seeds to provide a baseline of *D. radicum* suppression. As a new *Pseudomonas* isolate reduced *D. radicum* survival when applied to soil, it appears to be an excellent candidate for this (Chapter 6). Nonetheless, its role in natural *D. radicum* suppression and the influence of black soldier fly frass on its insecticidal activity have yet to be investigated. To determine whether the isolate is involved in natural or frass-induced suppression of *D. radicum*, it is essential to detect it in *D. radicum*-suppressive soil. Although soil amendment with black soldier fly exuviae enriched the genus *Pseudomonas* in the *B. oleracea* rhizosphere, the identity of the stimulated pseudomonads is not known (Chapter 4). Methods for the quantification of specific *Pseudomonas* strains have been described and could be used to confirm the presence or absence of the insecticidal isolate in different soils (von Felten et al., 2010). Furthermore, the suitability of black soldier fly frass as a substrate for the *Pseudomonas* isolate can be elucidated by assessing whether soil amendment can sustain or enrich natural and inoculated populations of this bacterium.

While the insecticidal *Pseudomonas* isolate may be important for *D. radicum* suppression, other isolates from dead larvae that had been exposed to suppressive soil still need to be tested for their activity (Chapter 6). These bacteria belong to genera such as *Serratia* or *Providencia*, which include symbionts of *D. radicum* as well as insect pathogens (Galac & Lazzaro, 2011; Jackson et al., 2001; Petersen & Tisa, 2013; van den Bosch & Welte, 2020; Welte et al., 2016). Potential entomopathogens may moreover be isolated from newly collected *D. radicum* larvae that show signs of disease. Since the setup used to expose larvae to soil amended with black soldier fly frass was rather straightforward, it can probably be adjusted to improve exposure to bacteria with oral toxicity (Chapter 6). For example, soil could be used to prepare suspensions, which can be concentrated and applied to slices of turnip. In order to specifically increase exposure to rhizobacteria, suspensions could be prepared from rhizosphere samples of *B. oleracea* plants that were grown in amended soil. Alternatively, the roots of uprooted plants could be fed directly to *D. radicum* larvae. Finding other *D. radicum* suppressive soils could be very helpful in elucidating the potential microbial basis of natural *D. radicum* suppression. By comparing the microbial communities of multiple suppressive soils with those of non-suppressive soils, it might be possible to identify specific taxa that are commonly associated with suppressiveness (Schlatter et al., 2017). Ideally, an initial screening for *D. radicum* suppressiveness should be conducted with different soils that have been under long-term monoculture of host crops of *D. radicum*

without insecticide applications. Although continuous monocultures may be uncommon in organic crop production, such conditions are likely to be found in organic experimental fields for brassicaceous plants.

All in all, the research presented in this thesis has led to several important findings on the potential of different insect residual streams and different soils for *D. radicum* control. In the future, the effects of soil amendment with insect residual streams on belowground pests other than *D. radicum* should be investigated in a similar way. Furthermore, frass and exuviae of insect species that were not considered here but that are also important for the insect production industry, such as the housefly, may hold potential to be examined in future studies. In conclusion, it seems that black soldier fly frass may be most suitable to enhance the suppressiveness of certain soils to *D. radicum*, not least due to its wide availability. When added to naturally suppressive soils, it may facilitate the isolation of new microbial agents that can contribute to *D. radicum* control. In combination with such biocontrol agents, soil amendment with black soldier fly frass has the potential to become an effective and low-cost tool for the management of this significant agricultural pest.

Acknowledgements

I thank Joop van Loon and Marcel Dicke for providing helpful comments on an earlier version of this chapter.

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Summary

The cabbage root fly or cabbage maggot, *Delia radicum* L. (Diptera: Anthomyiidae), is one of the most destructive pests of brassicaceous crops in Europe and North America. As legal restrictions on insecticide use have limited the availability of effective control options, new methods for *D. radicum* management are needed. While microbial control agents, such as entomopathogens, may be used as an alternative to conventional pesticides, they often do not persist long enough in the soil to provide reliable crop protection. To promote the growth of naturally occurring microbes with biocontrol potential, soil can be amended with organic substrates. A relatively new approach to stimulating elements of soil microbiomes that can contribute to crop protection is soil amendment with the by-products of insect farming. The insect production industry is generating large amounts of 'frass', a mixture of insect feces, unconsumed feed and exuviae (molted exoskeletons), which is available for applications in agriculture. Although the utilization of insect frass as fertilizer is often considered, studies investigating its potential for pest control are largely lacking. Therefore, the research presented in this thesis aimed to explore possible applications of different insect residual streams for the control of *D. radicum*. Frass or separated exuviae of black soldier fly larvae, *Hermetia illucens* L., house crickets, *Acheta domesticus* L., and yellow mealworms, *Tenebrio molitor* L., were used as soil amendments to assess the effects on *D. radicum* and on the rhizosphere bacterial community of *Brassica oleracea* L., an important host crop of this pest.

Chapter 2 presents a literature review on the potential of insect-derived soil amendments to improve plant growth and health. Insect frass and exuviae are rich in plant nutrients and contain chitin as well as other compounds that can promote the activity of plant beneficial microbes. Plant growth-promoting or plant-protective soil bacteria, such as *Bacillus* species, respond positively to chitin and may be stimulated more effectively by insect exuviae than by other chitin resources. These bacteria colonize roots and can induce systemic plant resistance to pests and diseases. They may moreover control plant pathogens and insects directly via the production of antimicrobial or insecticidal secondary metabolites. Soil amendment with insect frass or exuviae might help to reduce the use of synthetic fertilizer and pesticides in crop production. Large quantities of insect residual streams are expected to become available in the future as the insect production industry continues to grow.

In Chapter 3, the effects of soil amendment with exuviae or frass of black soldier fly larvae, house crickets and mealworms on the performance of *D. radicum* are reported. Soil from an organically managed field for brassicaceous plant species (brassica field soil) was mixed with different insect residual streams and was used to grow *B. oleracea* plants in the greenhouse. Plants were grown in amended soil for 5 weeks before being infested

with *D. radicum* larvae, which were subsequently retrieved from the roots or soil before pupation or allowed to develop into flies. Only soil amendment with black soldier fly frass or exuviae significantly reduced *D. radicum* survival or biomass. Amendment with mealworm exuviae resulted in significantly higher larval survival than amendment with black soldier fly exuviae. This indicates that the impact of soil amendment with insect residual streams on *D. radicum* depends on the insect species from which they are derived. Whereas *D. radicum* performance was negatively affected by black soldier fly residual streams, *D. radicum* larvae seem to benefit from the addition of mealworm exuviae to soil.

In Chapter 4, the effects of exuviae from black soldier fly larvae, house crickets and mealworms on the rhizosphere bacterial community of *B. oleracea* are reported. Plants were grown in amended brassica field soil in the greenhouse and rhizosphere samples were collected after 2, 4 and 8 weeks. In general, soil amendment with insect exuviae stimulated bacterial growth, diminished community diversity and shifted bacterial community structure in the *B. oleracea* rhizosphere. Differences between bacterial communities by insect species from which the exuviae originated became more distinct over time and communities in soil amended with black soldier fly or house cricket exuviae were most distinct after 4 and 8 weeks, respectively. Black soldier fly exuviae stimulated the genus *Pseudomonas* and different genera belonging to the *Burkholderiaceae* for a longer period of time compared with the exuviae of the other two species. Similarly, soil amendment with house cricket exuviae resulted in the unique enrichment of genera such as *Lysinibacillus* or *Paenibacillus*. These findings suggest that the influence of insect exuviae on rhizosphere bacterial communities depends on the species from which they are derived. Previously observed differences in effects on *D. radicum* might be related to the differential enrichment of specific groups of microbes in the *B. oleracea* rhizosphere.

Since soil amendment with black soldier fly frass reduced *D. radicum* survival, its potential to control *D. radicum* was investigated further as reported in Chapter 5. To determine a minimum effective amendment ratio, brassica field soil was mixed with frass at 1, 2 or 5 g/kg and was used to grow *B. oleracea* plants in the greenhouse. Plants were infested with larvae after 4 weeks and pupae were retrieved to evaluate *D. radicum* performance. Larval survival and pupal biomass were only reduced at a soil amendment ratio of 5 g/kg, which was subsequently tested in different types of soil. Brassica field soil, soil from a different field on which non-brassicaceous plant species had been rotated (crop rotation field soil) and potting soil were mixed with black soldier fly frass at 5 g/kg and used to grow *B. oleracea* plants in the greenhouse. The performance of *D. radicum* was assessed as in the previous experiment. Whereas amendment did not affect *D. radicum* performance in potting soil, it reduced pupal biomass in crop rotation field soil but only reduced *D. radicum* survival significantly in brassica field soil. Irrespective of soil amendment, survival was lower in brassica field soil than in the other soils. This suggests that the effects of black soldier fly frass on *D. radicum* performance depend on inherent *D. radicum* suppressiveness of the amended soil. Natural suppressiveness to *D. radicum* might be related to soil history.

In Chapter 6, the effects of different bacterial isolates on *D. radicum* performance are reported. Bacteria were isolated from dead *D. radicum* larvae that had been exposed to brassica field soil amended with black soldier fly frass. The isolates were applied to swede slices, which were fed to *D. radicum* larvae in laboratory bioassays. An isolate identified as *Pseudomonas* sp. reduced larval survival and pupation and was subsequently applied to brassica field soil in a greenhouse experiment. Soil was treated with 10^{10}, 10^{9} or 10^{8} CFU/kg and was used to grow *B. oleracea* plants, which were infested with *D. radicum* larvae after 5 weeks. Pupae were collected to evaluate *D. radicum* performance. Larval survival was significantly reduced only when the isolate was applied to soil at 10^{10} CFU/kg. This indicates that the new *Pseudomonas* isolate has the potential to control *D. radicum* for several weeks after soil application. The isolate may possibly be involved in the natural and frass-induced suppression of *D. radicum* in brassica field soil.

Taking into account the findings of all previous chapters, three major aspects of the research presented in this thesis are discussed in Chapter 7. The key factors that determine how insect residual streams affect different soil organisms are highlighted with a focus on soil microbes as potential mediators of the effects on *D. radicum*. Consequently, indications of the involvement of specific groups of microbes in *D. radicum* suppression are assessed. Finally, implications of the research presented here for the use of black soldier fly frass in *D. radicum* control are discussed and suggestions for future research are provided.

Zusammenfassung

Die Kleine Kohlfliege, *Delia radicum* L. (Diptera: Anthomyiidae), ist einer der zerstörerischsten Schädlinge an Kreuzblütengewächsen in Europa und Nordamerika. Da gesetzliche Restriktionen für den Einsatz von synthetischen Pflanzenschutzmitteln die Verfügbarkeit effektiver Bekämpfungsmöglichkeiten eingeschränkt haben, werden neue Methoden zur Bekämpfung von *D. radicum* benötigt. Obwohl mikrobielle Pflanzenschutzmittel, wie zum Beispiel Insektenpathogene, als Alternative zu konventionellen Pestiziden benutzt werden können, bleiben sie häufig nicht lange genug im Boden vorhanden, um die Pflanzen zuverlässig zu schützen. Um das Wachstum natürlich vorkommender Mikroorganismen mit Potenzial für die biologische Schädlingsbekämpfung zu begünstigen, kann Boden mit organischen Substraten angereichert werden. Ein relativ neuer Ansatz, um jene Teile von Bodenmikrobiomen zu stimulieren, die zum Pflanzenschutz beitragen können, ist die Bodenanreicherung mit Nebenprodukten der Insektenaufzucht. Die industrielle Produktion von Insekten generiert große Mengen von ‚Fraß', einer Mischung aus Insektenexkrementen, Futterresten und Exuvien (abgestreifte Exoskelette), welche für Anwendungen in der Landwirtschaft zur Verfügung stehen. Während die Verwendung von Insektenfraß als Düngemittel oft in Erwägung gezogen wird, fehlen Studien, die das Potenzial für die Schädlingsbekämpfung untersuchen, weitgehend. Daher war es das Ziel dieser Arbeit, die mögliche Nutzung verschiedener Restströme der Insektenproduktion zur Bekämpfung von *D. radicum* zu erforschen. Fraß oder getrennte Exuvien von Larven der Schwarzen Soldatenfliege, *Hermetia illucens* L., Heimchen, *Acheta domesticus* L., und Mehlwürmern, *Tenebrio molitor* L., wurden zur Bodenanreicherung verwendet, um Auswirkungen auf *D. radicum* und auf die bakterielle Gemeinschaft in der Rhizosphäre von *Brassica oleracea* L., einer wichtigen Wirtspflanze dieses Schädlings, zu bewerten.

Kapitel 2 stellt eine Literaturarbeit über das Potenzial der Bodenanreicherung mit Insekten-Restströmen zur Verbesserung der Boden- und Pflanzengesundheit dar. Insektenfraß und -exuvien sind reich an Pflanzennährstoffen und enthalten Chitin sowie andere Verbindungen, die die Aktivität von nützlichen Mikroorganismen fördern können. Pflanzenwachstumsfördernde oder pflanzenschützende Bodenbakterien, wie etwa *Bacillus*-Arten, reagieren positiv auf Chitin und können möglicherweise effektiver durch Insektenexuvien stimuliert werden als durch andere Chitinquellen. Diese Bakterien besiedeln Wurzeln und können systemische Pflanzenresistenzen gegen Schädlinge und Krankheiten induzieren. Außerdem können sie Pflanzenpathogene und Insekten direkt durch die Produktion antimikrobieller oder insektizider Sekundärmetaboliten bekämpfen. Bodenanreicherung mit Insektenfraß oder -exuvien könnte dazu beitragen, den Einsatz synthetischer Düngemittel und Pestizide im Pflanzenbau zu reduzieren. Da die industrielle Insektenproduktion zunimmt, ist davon auszugehen, dass in Zukunft große Mengen von Insekten-Restströmen verfügbar sein werden.

In Kapitel 3 werden die Auswirkungen der Bodenbehandlung mit Exuvien oder Fraß von Larven der Schwarzen Soldatenfliege, Heimchen und Mehlwürmern auf *D. radicum* beschrieben. Erde von einem ökologisch bewirtschafteten Feld für Kreuzblütengewächse (Brassica-Felderde) wurde im Gewächshaus mit verschiedenen Insekten-Restströmen gemischt und verwendet, um *B. oleracea*-Pflanzen heranzuziehen. Die Pflanzen wurden 5 Wochen lang herangezogen und anschließend mit *D. radicum*-Larven infiziert, welche entweder vor der Verpuppung wieder von den Wurzeln und aus der Erde eingesammelt wurden oder sich zu Fliegen entwickeln konnten. Nur die Behandlungen mit Fraß oder Exuvien von Larven der Schwarzen Soldatenfliege verringerten die Überlebensrate oder das Gewicht von *D. radicum* signifikant. Die Behandlung mit Exuvien von Mehlwürmern führte zu einer signifikant höheren Überlebensrate der Larven, als die Behandlung mit Exuvien von Larven der Schwarzen Soldatenfliege. Dies deutet darauf hin, dass die Auswirkungen der Bodenbehandlung mit Insekten-Restströmen auf *D. radicum* von der Insektenart abhängig sind, von der diese stammen. Während Restströme, die von der Schwarzen Soldatenfliege stammten, negative Auswirkungen auf *D. radicum* hatten, scheint die Bodenbehandlung mit Exuvien von Mehlwürmern sich positiv auf *D. radicum*-Larven auszuwirken.

In Kapitel 4 werden die Effekte von Exuvien von Larven der Schwarzen Soldatenfliege, Heimchen und Mehlwürmern auf die bakterielle Gemeinschaft in der Rhizosphäre von *B. oleracea* beschrieben. Die Pflanzen wurden im Gewächshaus in behandelter Brassica-Felderde herangezogen und Proben der Rhizosphäre wurden nach 2, 4 und 8 Wochen gesammelt. Die Behandlung mit Insektenexuvien stimulierte grundsätzlich das bakterielle Wachstum, verringerte die Diversität und veränderte die Struktur der bakteriellen Gemeinschaft in der *B. oleracea*-Rhizosphäre. Unterschiede in der bakteriellen Gemeinschaft zwischen den verschiedenen Behandlungen wurden mit der Zeit deutlicher und waren in den Behandlungen mit Exuvien von Larven der Schwarzen Soldatenfliege oder von Heimchen jeweils nach 4 und 8 Wochen am ausgeprägtesten. Exuvien von Larven der Schwarzen Soldatenfliege stimulierten die Gattung *Pseudomonas* und verschiedene Gattungen der *Burkholderiaceae* über eine längere Zeit als die Exuvien der zwei anderen Insektenarten. In ähnlicher Weise führte nur die Behandlung mit Exuvien von Heimchen zu einer Anreicherung von Gattungen wie *Lysinibacillus* oder *Paenibacillus*. Diese Ergebnisse legen nahe, dass die Effekte von Insektenexuvien auf bakterielle Gemeinschaften in der Rhizosphäre von der jeweiligen Insektenart abhängen. Die zuvor beobachteten Unterschiede in der Auswirkung auf *D. radicum* könnten mit der unterschiedlichen Anreicherung bestimmter Gruppen von Mikroorganismen in der *B. oleracea*-Rhizosphäre zusammenhängen.

Da die Bodenbehandlung mit Fraß von Larven der Schwarzen Soldatenfliege die Überlebensrate von *D. radicum* verringerte, wurde das Potenzial für die *D. radicum*-

Bekämpfung weiter untersucht, wie in Kapitel 5 beschrieben. Um eine minimale effektive Anwendungsrate zu bestimmen, wurde Fraß mit Brassica-Felderde im Verhältnis von 1, 2 oder 5 g/kg gemischt, welche anschließend im Gewächshaus verwendet wurde, um *B. oleracea*-Pflanzen heranzuziehen. Die Pflanzen wurden nach 4 Wochen mit Larven infiziert und die Auswirkungen auf *D. radicum* anhand wieder eingesammelter Puppen bewertet. Die Überlebensrate der Larven und das Gewicht der Puppen wurden nur bei einer Anwendungsrate von 5 g/kg verringert, welche anschließend in verschiedenen Bodentypen getestet wurde: Brassica-Felderde, Erde von einem anderen Feld, auf dem hintereinander verschiedene Pflanzenarten, die nicht zu den Kreuzblütengewächsen gehören, angebaut worden waren (Fruchtfolge-Felderde) und Blumenerde. Fraß wurde mit der jeweiligen Erde im Verhältnis von 5 g/kg gemischt, welche anschließend im Gewächshaus verwendet wurde, um *B. oleracea*-Pflanzen heranzuziehen. Effekte auf *D. radicum* wurden wie im vorherigen Experiment untersucht. Während die Behandlung der Blumenerde keine Auswirkungen auf *D. radicum* hatte, verringerte sie in der Fruchtfolge-Felderde das Gewicht der Puppen, führte aber nur in Brassica-Felderde zu einer signifikanten Verringerung der Überlebensrate von *D. radicum*. Unabhängig von der Behandlung, war die Überlebensrate in Brassica-Felderde geringer als in den beiden anderen Bodentypen. Dies legt nahe, dass die Effekte von Fraß von Larven der Schwarzen Soldatenfliege auf *D. radicum* von inhärenten, *D. radicum*-unterdrückenden Eigenschaften des behandelten Bodens abhängen. Die natürliche Unterdrückung von *D. radicum* könnte im Zusammenhang mit der Feldhistorie stehen.

In Kapitel 6 werden die Effekte verschiedener Bakterienisolate auf *D. radicum* beschrieben. Die Bakterien wurden aus toten *D. radicum*-Larven isoliert, nachdem diese Brassica-Felderde ausgesetzt worden waren, die mit Fraß von Larven der Schwarzen Soldatenfliege behandelt worden war. Steckrübenscheiben wurden mit den Isolaten behandelt und in Bioassays an *D. radicum*-Larven verfüttert. Ein als *Pseudomonas* sp. identifiziertes Isolat verringerte die Überlebens- und Verpuppungsrate der Larven und wurde anschließend in einem Gewächshausexperiment verwendet, um Brassica-Felderde zu behandeln. Die Erde wurde mit 10^{10}, 10^{9} oder 10^{8} CFU/kg behandelt und anschließend verwendet, um *B. oleracea*-Pflanzen heranzuziehen, welche nach 5 Wochen mit *D. radicum*-Larven infiziert wurden. Um die Auswirkungen auf *D. radicum* zu bewerten, wurden Puppen eingesammelt. Nur bei einer Anwendungsrate von 10^{10} CFU/kg verringerte das Isolat die Überlebensrate der Larven signifikant. Dies deutet darauf hin, dass das neue *Pseudomonas*-Isolat, *D. radicum* potenziell bis zu mehreren Wochen nach der Bodenanwendung bekämpfen kann. Das Isolat könnte möglicherweise an der natürlichen und Fraß-induzierten Unterdrückung von *D. radicum* in Brassica-Felderde beteiligt sein.

Zusammenfassung

Unter Berücksichtigung der Ergebnisse aller vorherigen Kapitel werden in Kapitel 7 drei Schwerpunkte dieser Arbeit diskutiert. Die für die Auswirkungen von Insekten-Restströmen auf unterschiedliche Bodenorganismen entscheidenden Faktoren werden hervorgehoben, mit dem Schwerpunkt auf Bodenmikroorganismen als potenzielle Vermittler von Effekten auf *D. radicum*. Folglich werden Hinweise auf die Beteiligung bestimmter Gruppen von Mikroorganismen an der Unterdrückung von *D. radicum* beurteilt. Anschließend wird die Bedeutung dieser Arbeit für die Verwendung von Fraß von Larven der Schwarzen Soldatenfliege zur Bekämpfung von *D. radicum* diskutiert und es werden Vorschläge für zukünftige Untersuchungen gemacht.

Samenvatting

De koolvlieg, *Delia radicum* L. (Diptera: Anthomyiidae), is een van de meest destructieve plagen van kruisbloemige gewassen in Europa en Noord-Amerika. Aangezien wettelijke restricties op het gebruik van insecticiden de beschikbaarheid van effectieve bestrijdingsmogelijkheden beperkt hebben, is er behoefte aan nieuwe methoden voor de bestrijding van de koolvlieg. Hoewel microben, zoals ziekteverwekkers, alternatieven bieden om het gebruik van conventionele pesticiden te verminderen, blijven ze vaak niet lang genoeg in de bodem aanwezig om betrouwbare gewasbescherming te kunnen bieden. Om de groei van natuurlijk voorkomende microben met potentieel voor biologische bestrijding te bevorderen, kunnen organische substraten aan de bodem toegevoegd worden. Een relatief nieuwe benadering om delen van het bodemmicrobioom te stimuleren die aan gewasbescherming kunnen bijdragen, is het toevoegen van bijproducten van de insectenkweek aan de grond waarop de planten groeien. De insectenindustrie genereert grote hoeveelheden 'frass', een mengsel van insectenuitwerpselen, niet geconsumeerd voer en exuviae (vervellingshuidjes), dat voor toepassing in de landbouw beschikbaar is. Hoewel het gebruik van insectenfrass als mest vaak in overweging genomen wordt, ontbreken studies die het potentieel daarvan voor plaagbestrijding onderzoeken grotendeels. Daarom was het doel van het in dit proefschrift gepresenteerde onderzoek om mogelijke toepassingen van verschillende insectenreststromen voor de bestrijding van *D. radicum* te bestuderen. Frass of daaruit verkregen exuviae van de larven van de zwarte soldatenvlieg, *Hermetia illucens* L., huiskrekels, *Acheta domesticus* L., en meelwormen, *Tenebrio molitor* L., werden aan grond toegevoegd om de effecten op *D. radicum* en op de microbiële gemeenschap in de rhizosfeer van *Brassica oleracea* L., een belangrijke waardplant van *D. radicum*, te bepalen.

Hoofdstuk 2 presenteert een literatuuronderzoek over het potentieel van bodemverbeteraars afkomstig van insecten om plantengroei en -gezondheid te verbeteren. Frass en exuviae van insecten zijn rijk aan nutriënten voor planten en bevatten zowel chitine als ook andere verbindingen die de activiteit van nuttige microben kunnen bevorderen. Plantengroei bevorderende of plantenbeschermende bodembacteriën, zoals *Bacillus* soorten, reageren positief op chitine and kunnen door exuviae van insecten mogelijkerwijze effectiever gestimuleerd worden dan door andere bronnen van chitine. Deze bacteriën koloniseren plantenwortels en kunnen systemische plantenresistentie tegen plagen en ziektes opwekken. Bovendien kunnen ze plantenpathogenen en insecten via de productie van antimicrobiële of insectendodende secundaire metabolieten direct bestrijden. Mogelijkerwijze kan bodemtoepassing van frass of exuviae van insecten bijdragen aan de vermindering van het gebruik van kunstmest en pesticiden in de landbouw. De verwachting is dat in de toekomst grote hoeveelheden insectenreststromen beschikbaar komen wanneer de insectenindustrie verder groeit.

In Hoofdstuk 3 worden de effecten van bodemtoepassing van exuviae of frass van de larven van de zwarte soldatenvlieg, huiskrekels en meelwormen op *D. radicum* gerapporteerd. Grond van een biologisch proefveld voor kruisbloemige plantensoorten (brassica veldgrond) werd in de kas met verschillende insectenreststromen gemengd en gebruikt om *B. oleracea* planten op te kweken. De planten werden 5 weken lang in de behandelde grond gekweekt voordat ze met *D. radicum* larven geïnfecteerd werden, welke vervolgens of voor de verpopping uit de wortels of uit de grond verzameld werden of zich tot vliegen konden ontwikkelen. Alleen bodemtoepassing van frass of exuviae van de zwarte soldatenvlieg verminderde de overleving of het gewicht van *D. radicum* significant. De toepassing van exuviae van meelwormen leidde tot een significant hogere overleving van de larven dan de toepassing van exuviae van de zwarte soldatenvlieg. Dit duidt erop dat het effect van bodemtoepassing van insectenreststromen op *D. radicum* afhankelijk is van de insectensoort waarvan ze afkomstig zijn. Terwijl reststromen afkomstig van de zwarte soldatenvlieg *D. radicum* negatief beinvloedden, had de toevoeging van exuviae van meelwormen een positief effect op *D. radicum* larven.

In Hoofdstuk 4 worden de effecten van exuviae van de larven van de zwarte soldatenvlieg, huiskrekels en meelwormen op de bacteriële gemeenschap in de rhizosfeer van *B. oleracea* gerapporteerd. De planten werden in de kas in behandelde brassica veldgrond opgekweekt en monsters van de rhizosfeer werden na 2, 4 en 8 weken verzameld. Over het algemeen stimuleerde de toevoeging van exuviae van insecten aan de grond bacteriële groei, verminderde de diversiteit en veranderde de structuur van de bacteriële gemeenschap in de rhizosfeer van *B. oleracea*. Verschillen tussen de bacteriële gemeenschappen, afhankelijk van de insectensoort waarvan de exuviae afkomstig waren, werden in de loop van de tijd duidelijker en de verschillen in de microbiële samenstelling in grond gemengd met exuviae van de zwarte soldatenvlieg of huiskrekels waren na 4 en 8 weken het grootst. Vergeleken met de exuviae van de andere twee insectensoorten, stimuleerden exuviae van de zwarte soldatenvlieg het geslacht *Pseudomonas* en verschillende geslachten binnen de *Burkholderiaceae* voor langere tijd. Alleen toevoeging van exuviae van huiskrekels leidde tot verrijking van geslachten zoals *Lysinibacillus* of *Paenibacillus*. Deze bevindingen suggereren dat de invloed van exuviae van insecten op bacteriële gemeenschappen in de rhizosfeer afhankelijk is van de insectensoort waarvan ze afkomstig zijn. De eerder geobserveerde verschillen in effecten op *D. radicum* staan mogelijkerwijze in verband met de differentiële verrijking van specifieke groepen van microben in de rhizosfeer van *B. oleracea*.

Omdat bodemtoepassing van frass van de zwarte soldatenvlieg de overleving van *D. radicum* verminderde, werd het potentieel van deze vliegensoort om *D. radicum* te bestrijden verder onderzocht, zoals in Hoofdstuk 5 gerapporteerd. Om een minimale effectieve toepassingsratio te bepalen werd in de kas frass in een ratio van 1, 2 of 5 g/kg met brassica veldgrond gemengd

en gebruikt om *B. oleracea* planten op te kweken. De planten werden na 4 weken met larven geïnfecteerd en poppen werden verzameld om de effecten op *D. radicum* te evalueren. De overleving van de larven en het popgewicht waren alleen bij een bodemtoepassingsratio van 5 g/kg gereduceerd, welke vervolgens in verschillende grondtypen getest werd. In de kas werd frass van de zwarte soldatenvlieg gemengd met brassica veldgrond, grond van een ander veld waarop verschillende niet-kruisbloemige plantensoorten na elkaar geteeld waren (vruchtwisseling veldgrond) en potgrond in een ratio van 5 g/kg en gebruikt om *B. oleracea* planten op te kweken. De effecten op *D. radicum* werden bestudeerd zoals in het eerste experiment. Terwijl de toepassing in potgrond geen invloed op *D. radicum* had, verminderde ze in vruchtwisseling veldgrond het popgewicht, maar leidde alleen in brassica veldgrond tot een significante vermindering van de overleving van *D. radicum*. Onafhankelijk van de toepassing van frass was de overleving in brassica veldgrond lager dan in de andere grondtypen. Dit suggereert dat de effecten van frass van de zwarte soldatenvlieg op *D. radicum* afhankelijk zijn van inherente *D. radicum*-onderdrukkende eigenschappen van de grond waarin frass toegepast wordt. Natuurlijke onderdrukking van *D. radicum* staat mogelijkerwijze in verband met voorgaand gebruik van de grond.

In Hoofdstuk 6 worden de effecten van verschillende bacteriële isolaten op *D. radicum* gerapporteerd. De bacteriën werden uit dode *D. radicum* larven geïsoleerd die blootgesteld waren aan brassica veldgrond gemengd met frass van de zwarte soldatenvlieg. Koolraapschijven werden met de isolaten behandeld en in het laboratorium aan *D. radicum* larven aangeboden. Een als *Pseudomonas* sp. geïdentificeerd isolaat verminderde de overleving en verpopping van de larven en werd vervolgens in een kasexperiment in brassica veldgrond toegepast. De grond werd met 10^{10}, 10^{9} of 10^{8} CFU/kg gemengd en gebruikt om *B. oleracea* planten op te kweken, die na 5 weken met *D. radicum* larven geïnfecteerd werden. De poppen werden verzameld om *D. radicum* performance te evalueren. Alleen bij 10^{10} CFU/kg leidde de toepassing van het isolaat tot een significant lagere overleving van de larven. Dit duidt erop dat het nieuwe *Pseudomonas* isolaat het potentieel heeft om *D. radicum* tot meerdere weken na bodemtoepassing te bestrijden. Het isolaat is mogelijkerwijze betrokken bij de natuurlijke en frass-geïnduceerde onderdrukking van *D. radicum* in brassica veldgrond.

De bevindingen van alle eerdere hoofdstukken in aanmerking nemend, worden in Hoofdstuk 7 drie hoofdaspecten van het in dit proefschrift gepresenteerde onderzoek bediscussieerd. De belangrijkste factoren die bepalen hoe insectenreststromen verschillende bodemorganismen beïnvloeden worden besproken met een focus op bodemmicroben als potentiële intermediair van de effecten op *D. radicum*. Bijgevolg worden aanwijzingen voor de betrokkenheid van specifieke groepen van microben bij de onderdrukking van *D. radicum* beoordeeld. Ten slotte worden de implicaties van het hier gepresenteerde onderzoek voor het gebruik van frass van de zwarte soldatenvlieg voor de bestrijding van *D. radicum* bediscussieerd en worden suggesties voor toekomstige onderzoek gedaan.

Acknowledgements

There are many people without whom this thesis never would have been written. They have motivated and supported me in various ways, whether it was in my work, my private life or both.

First of all, I want to thank my promotors, Marcel and Joop. Your friendly and approachable manner has been fundamental to me feeling comfortable during our many meetings and throughout my PhD project. I think you two complement each other perfectly to make the great supervisor duo that taught me so much. Marcel, it was an honor for me to join the 'closing the loop' project as a PhD student that had not originally been accounted for. Your optimism and positivity were an important source of motivation and often reassured me when assessing my own research. Thank you for all the uplifting mails during the pandemic, for your concern and for being so considerate, especially when I was ill. Joop, if you had not pointed out this research project to me and put me in touch with different experts during my master's, I might have never done a PhD. Your eye for detail and felicity with words are inspiring and have had a considerable influence on my writing. Thank you for not hesitating to correct me during our conversations and expanding my vocabulary, both in Dutch and English.

I am grateful to have been part of an interdisciplinary and international project team together with my fellow PhD students Azkia, Els, Katherine and Kirstin. It was exciting to all work on the same 'matter' essentially, but also in fields of research that were quite different from each other. Azkia, it was great fun starting off our work packages together and I will miss attending all those courses, small excursions and conferences with you. Thank you for welcoming me to the NIOO and for introducing me to metagenomics, I would have been pretty lost without your help. Els, your ambitions in research are most impressive and I learned so much from our different collaborations. Thank you for daring me to aim higher and for always being so kind and open during the various discussions we have had. Kathe, your heartiness and seemingly limitless positive energy never fail to brighten my day, whether in the lab or anywhere else. Thank you for being such a caring friend, for always finding the right words of encouragement when needed and for your confidence in my Spanish skills. Kirstin, it was wonderful to have you on the team as the social scientist and to hear your stories about visiting farmers and about their opinions. Thank you for your enthusiasm and sharing your refreshing perspective on everyone's research.

Over the last few years, I had the pleasure to supervise several talented and determined thesis students from all over the world. Kristian, Daniela, Esther, Sarah, Zahtamal and Tamara, thank you for all the unique experiences, for struggling and being successful together and for teaching me just as much as I taught you.

It has been a privilege to spend so many years at the Laboratory of Entomology and I could not have asked for a nicer place to do my PhD research. I also want to thank all the colleagues that worked on other projects but who were so important to me, not least because of the friendly and relaxed atmosphere in the lab, during coffee breaks, parties or outings. Filippo, it is amazing how you have an informed opinion on almost anything and how it seems like no conversation with you could ever get boring. Your avid interest in a breadth of subjects is contagious and has been a source of inspiration for me. Thank you for being a great desk neighbor, molecular biology tutor and friend. Yidong, I could always count on you when it came to having good company in the lab at a late hour, finding lab utensils I desperately needed or watching anime together. Thank you for being my Shingeki no Kyojin buddy and surprising me with Chinese snacks every time we hang out. Peter, your diligence and readiness to help others are remarkable and have been an inspiring example to me. Thank you for all your advice, organizing all those fun get-togethers and your spot-on imitation of the German accent. Julia, it was during my thesis project with you that I took my first steps at Ento and I am learning from you to this day. Thank you for introducing me to the world of cabbage and beneficial bacteria and the great time we had together as Peter's paranymphs. Patrick, Janneke, Hans and Pieter, thank you for taking your time to provide and explain all sorts of materials or techniques to me during the past years. Much of my research would not have been possible without your support. Maddalena, though you have never been a member of our lab, I still remember the first time Filippo snuck you into one of our events. Thank you for all the cozy dinners and for tolerating my occasional faux pas when trying to express my appreciation for food. Alessia, thank you for telling me about the beautiful peculiarities of the Neapolitan accent and for not letting the polentoni monopolize my image of Italy. Thibault, thank you for your R tips, for sharing your enlightening statistics book with me and for countless enjoyable trips to Orion. Davy, thank you for always being right behind my screen to help me out with random questions about Dutch or whatever else would come to my mind. Parth, thank you for being there whenever I would turn around in my chair and for the interesting discussions about entomopathogens and languages. Bram, thank you for being such a cool guy and for making the best kruidnoten I have ever had. Marieke, thank you for reminiscing about bands together, inviting me to concerts and keeping my inner core kid alive. Mitchel, thank you for always being up for a nice chat and your great sense of humor that never fails to put me in a good mood. Liana and Gabe, thank you for the fun times at barbecues, movie nights or baking sessions and always being open to discuss anything related to one of my all-time favorite topics: 'Murica. I specifically want to thank the members of Team *Delia*, Kay, Julia, Peter and Shaphan, for the wonderful experience of maintaining the rearing together. Aside from keeping the fly fest going, I also had the pleasure to be a member of the Party Committee. Maite, Yidong, Kay, Kathe, Pieter, Davy, Bram, Els, Sarah and Marcela, thank you for your commitment and creativity, I had

a lot of fun organizing all those Ento events with you. And of course, also to all other Ento friends and colleagues: thank you for your company and an amazing time during the last couple of years.

Mit am wichtigsten beim Forschen und Schreiben an einer Doktorarbeit ist es wohl, sie zwischendurch auch immer wieder einmal vergessen zu können. Mein besonderer Dank gilt deshalb meinen Freunden, die mir dabei stets behilflich waren. Für all die fantastic Wochenenden und den nahezu täglichen Austausch über alle unterhaltsamen Dinge des Lebens danke ich den Leverkusenern, Jan, Christoph, Timo, Philipp, Schwingo und Konni. Auch bei den ehemaligen Vijnreste-Bewohnern, allen voran Dustin, Jangall, Jean-Marc, Julian und Caro, möchte ich mich für die unvergessliche Zeit in Wageningen und all die schönen Dinge, die wir gemeinsam erlebt haben, bedanken.

Von ganzem Herzen möchte ich mich bei meiner Familie bedanken, bei meinen lieben Eltern und bei meinem Bruder. Diese Arbeit, die in gewisser Weise auch Agrarwissenschaften und Mikrobiologie verbindet, steht sinnbildhaft dafür, wie ich in so vieler Hinsicht einen Teil von jedem von euch in mir trage. Ohne eure vielseitige und unermüdliche Unterstützung hätte ich das alles nicht geschafft.

Dear Giudi, I could not imagine the last 4 years without you. With your cheerfulness and inspiring pragmatism, you show me all those wonderful things in life that would otherwise pass me by. Thank you for giving me strength in the most difficult times, for understanding me and my quirks and for being both my partner in silliness and my voice of reason.

About the author

Written by Alfred and Max Wantulla

Max was born on 27 December 1992 essentially as a belated Christmas present for his parents. He surely would have liked to spend the first years of his life in his native region, the Bergisches Land, a rural area east of Cologne. However, his father's employer had other plans. And so Max found himself in Istanbul, Turkey, at the tender age of 6 months, where he spent more than 4 years of his early childhood. This first experience abroad possibly shaped his open personality and curiosity about the unknown.

Back in Germany, he returned to the Bergisches Land, where his family moved into a new home at the edge of a nature reserve. For the next few years, the forest with its creek, its ponds and all the creatures that inhabit it not only became his magnificent playground, but also sparked Max's interest in all the large and small wonders of nature. His father probably contributed substantially to this by covering the neighborhood with nesting boxes and bird feeders and by setting up a pond aquarium at home. Furthermore, it was a fortunate coincidence that Max had the chance to get to know the joys and troubles of a farmer early on at his uncle's dairy farm.

During his high school time, Max spent a year abroad in Reeds Spring, Missouri. This once-in-a-lifetime experience was also crucial for learning the English language and thus for his further international career.

The course for his further life and education in the Netherlands was maybe set when Max won a raffle in high school to participate in a one-week course at the Utrecht Summer School. Stimulated by this positive experience, he considered the possibility of studying abroad following high school graduation. After a careful assessment of his preferences and the available options, it became clear to him that there was probably no better place to study life sciences than Wageningen University. Consequently, Max decided to enroll in the university's Bachelor's and subsequently its Master's program in Plant Sciences. Naturally, this decision

made learning the Dutch language inevitable. A good investment, considering that Max has now been living in the Netherlands for almost 11 years, with only a few brief interruptions.

Majoring in Plant Pathology and Entomology, Max ended up doing two theses and an internship on insects and various plant beneficial or entomopathogenic microbes. After his first thesis at the Laboratory of Entomology in Wageningen, he went to Bayer in Monheim am Rhein, Germany, for his second thesis, before returning to Wageningen for his internship at Entocare. Little did he know that he was to stay in Wageningen after his graduation to start yet another research project on insects and microbes at the Laboratory of Entomology. Having experienced the difficulties of controlling pests with microbial agents, he found his PhD project on the use of novel soil amendments to improve microbial crop protection to be a perfect next step.

List of publications

Barragán-Fonseca, K. Y.*, Nurfikari, A.*, van de Zande, E. M.*, **Wantulla, M.***, van Loon, J. J. A., de Boer, W., & Dicke, M. (2022). Insect frass and exuviae to promote plant growth and health. *Trends in Plant Science, 27*(7), 646-654. doi:10.1016/j.tplants.2022.01.007 (Chapter 2 of this thesis)

Wantulla, M., van Zadelhoff, K., van Loon, J. J. A., & Dicke, M. (2022). The potential of soil amendment with insect exuviae and frass to control the cabbage root fly. *Journal of Applied Entomology, 147*(3), 181-191. doi:10.1111/jen.13097 (Chapter 3 of this thesis)

Wantulla, M., van Loon, J. J. A., & Dicke, M. (2023). Soil amendment with insect exuviae causes species-specific changes in the rhizosphere bacterial community of cabbage plants. *Applied Soil Ecology, 188*, 104854. doi:10.1016/j.apsoil.2023.104854 (Chapter 4 of this thesis)

*These authors contributed equally to this work

Submitted

Wantulla, M., Dicke, M., & van Loon, J. J. A. Effects of amending soil with black soldier fly frass on survival and growth of the cabbage root fly (*Delia radicum*) depend on soil type. (Chapter 5 of this thesis)

Education Statement of the Graduate School Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Max Wantulla
Date: 26 June 2023
Group: Laboratory of Entomology
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presentation of your project**		
The use of insect-waste streams for control of the cabbage root fly	11 January, 2019	1,5
► **Writing or rewriting a project proposal**		
Use of insect waste-streams for control of the cabbage root fly	18 October, 2018	6,0
► **Writing a review or book chapter**		
Barragán-Fonseca KY*, Nurfikari A*, van de Zande EM*, Wantulla M*, van Loon JJA, de Boer W, Dicke M. Insect frass and exuviae to promote plant growth and health. *Trends Plant Sci.* 2022 Jul;27(7):646-654. doi: 10.1016/j.tplants.2022.01.007	20 January, 2022	3,0
► **MSc courses**		
Research Methods Microbiology (MIB-30303)	18 February-13 March, 2019	3,0
Subtotal Start-Up Phase		13,5

2) Scientific Exposure	date	cp
► **EPS PhD days**		
EPS PhD days "Get2Gether", Soest (NL)	11 & 12 February, 2019	0,6
EPS PhD days "Get2Gether", Soest (NL)	10 & 11 February, 2020	0,6
► **EPS theme symposia**		
Theme 2 symposium & Willie Commelin Scholten Day "Interactions between plants and biotic agents", Utrecht (NL)	4 February, 2020	0,3
Theme 2 symposium "Interactions between plants and biotic agents", online	9 February, 2021	0,2
Theme 2 symposium "Interactions between plants and biotic agents", online	8 February, 2022	0,2
► **Lunteren Days and other national platforms**		
Entomology day, Ede (NL)	14 December, 2018	0,3
Annual meeting "Experimental Plant Sciences", Lunteren (NL)	8 & 9 April, 2019	0,6
► **Seminars (series), workshops and symposia**		
Seminar Daniel Bopp, "Genetics and evolution of sex determination systems in the common housefly"	25 October, 2018	0,1
Seminar Elisabeth Bolund, "The evolution of sexually dimorphic life histories"	21 March, 2019	0,1
Seminar Britt Koskella, "Friends, foes, and phages in the phyllosphere"	16 May, 2019	0,1
Seminar Martin Heil, "Immunity, invitation and manipulation: how plants control their interactions with microbes and insects"	15 October, 2019	0,1
Seminar Stefan Geisen, "Challenges as a PhD and postdoc in science and tips to overcome those"	20 January, 2021	0,1
Seminar Sandra Bouwhuis, "Understanding senescence and trans-generational parental age effects in a long-lived seabird"	21 January, 2021	0,1
Seminar Cara Haney and David Thoms, "How plants engage with beneficial microorganisms while at the same time restricting pathogens"	3 May, 2021	0,2
LabLinks: Good Germs - Bad Germs: Microbiomes in Humans and Plants, Ghent (BE)	5 October, 2018	0,3
Meeting KNPV working group "Soilborne Pathogens and Soil Microbiology", Lelystad (NL)	14 November, 2019	0,3
IPIFF Workshop - Closing the loop: Improving Soil Health and Fertility through Insect Frass Application, online	2 June, 2021	0,2

Plant-Insect-Interaction Workshop - Making the invisible visible, Wageningen (NL)	21 October, 2021	0,3
WUR Open Day with farmers, Wageningen (NL)	10 June, 2022	0,3
► **Seminar plus**		
► **International symposia and congresses**		
Thünen Symposium on Soil Metagenomics, Braunschweig (DE)	11-13 December, 2019	0,9
Symposium on Insect-Plant Interactions, online	25-30 July, 2021	1,5
► **Presentations**		
Poster presentation: PE&RC & WIMEK Course "Soil Ecology"	29 January, 2019	1,0
Poster presentation: PE&RC & University of Copenhagen Course "Root Ecology"	19 January, 2020	1,0
Oral presentation: First consortium meeting "Closing the Loop"	7 March, 2019	1,0
Oral presentation: Meeting KNPV working group "Soilborne Pathogens and Soil Microbiology"	15 April, 2021	1,0
Oral presentation: Last consortium meeting "Closing the Loop"	9 June, 2022	1,0
Oral presentation: WUR Open Day with farmers	10 June, 2022	1,0
► **IAB interview**		
► **Excursions**		
Subtotal Scientific Exposure		13,4

3) In-Depth Studies	date	cp
► **Advanced scientific courses & workshops**		
Training School "Detection and Identification of Microorganisms in Plants and Arthropods", Umeå (SE)	8-10 October, 2018	0,9
PE&RC & SENSE Course "Introduction to R for Statistical Analysis", Wageningen (NL)	8 & 9 November, 2018	0,6
PE&RC & WIMEK Course "Soil Ecology - The Multifunctional Potential of Soils", Lunteren (NL)	27-31 January, 2019	1,5
PE&RC & University of Copenhagen Course "Root Ecology", Ede (NL)	19-24 January, 2020	1,5
► **Journal club**		
► **Individual research training**		
Subtotal In-Depth Studies		4,5

4) Personal Development	date	cp
► **General skill training courses**		
EPS Introduction Course, Wageningen (NL)	4 October, 2018	0,3
WGS Course "Project and Time Management", Wageningen (NL)	September & October, 2018	1,5
WGS PhD Competence Assessment, Wageningen (NL)	November, 2018	0,3
Workshop eLabjournal, Wageningen (NL)	7 February, 2019	0,1
WGS Workshop "Last Stretch of the PhD Programme", online	26 February, 2021	0,0
WGS Workshop "Writing propositions for your PhD", online	26 February, 2021	0,0
WGS Course "Supervising BSc and MSc thesis students", online	12 & 13 April, 2021	0,6
WGS Course "Searching and Organising Literature", online	15 & 16 June, 2021	0,6
► **Organisation of meetings, PhD courses or outreach activities**		
► **Membership of EPS PhD Council**		
Subtotal Personal Development		3,4

TOTAL NUMBER OF CREDIT POINTS*	**34,8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

The research described in this thesis was conducted at the Laboratory of Entomology of Wageningen University & Research (WUR) and financially supported by the Dutch Research Council, NWO (grant number ALWGK.2016.010).

Cover design and illustrations by Giuditta M. Beretta

Thesis layout by Yidong Wang

Printed by Proefschriftmaken.nl, De Bilt, the Netherlands