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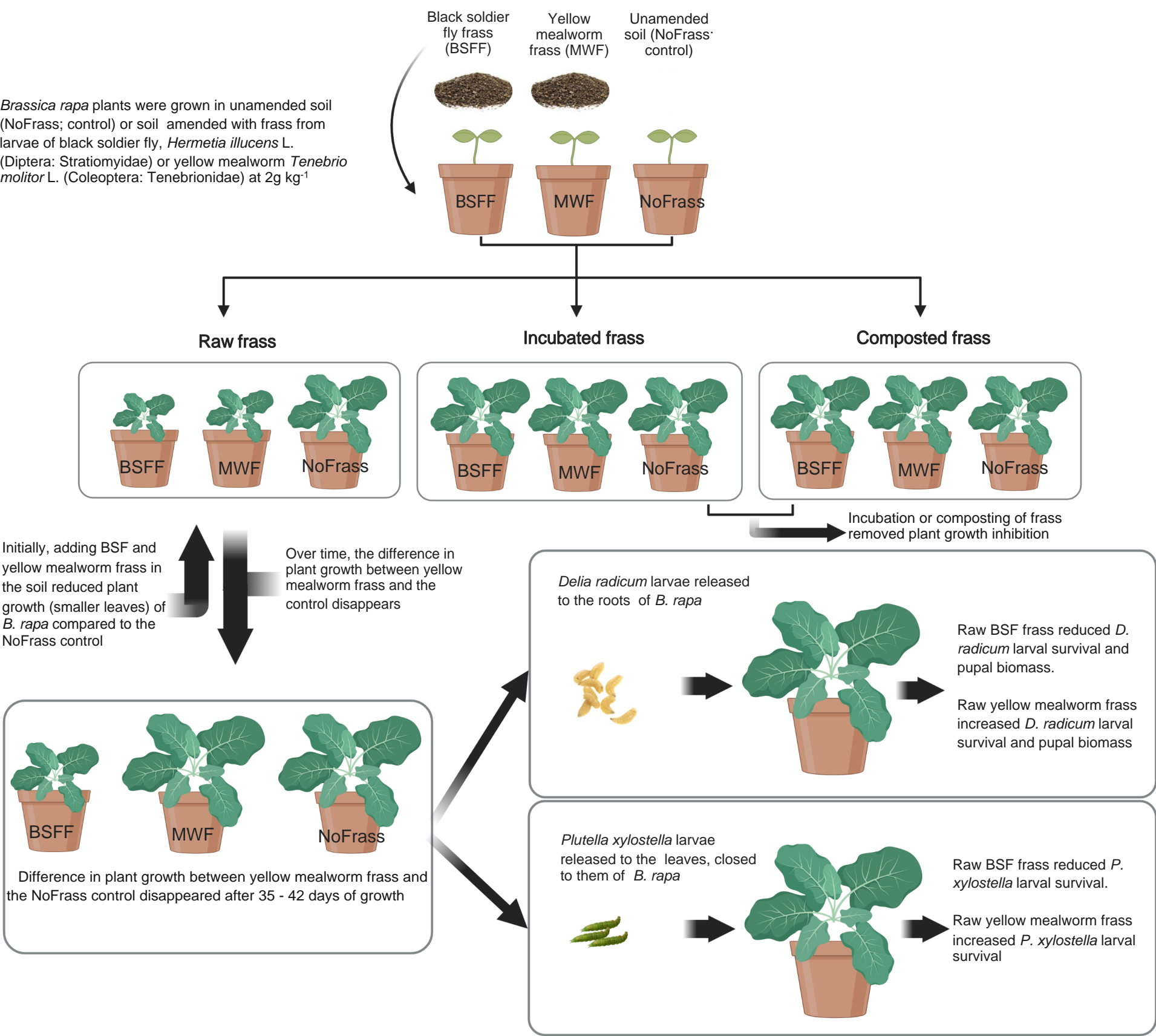
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# Effects of frass from larvae of black soldier fly (*Hermetia illucens*) and yellow mealworms (*Tenebrio molitor*) on growth and insect resistance in field mustard (*Brassica rapa*): differences between insect species and frass treatments

*Brassica rapa* plants were grown in unamended soil (NoFrass; control) or soil amended with frass from larvae of black soldier fly, *Hermetia illucens* L. (Diptera: Stratiomyidae) or yellow mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) at 2g kg<sup>-1</sup>



Original Article:

**Effects of frass from larvae of black soldier fly (*Hermetia illucens*) and yellow mealworm (*Tenebrio molitor*) on growth and insect resistance in field mustard (*Brassica rapa*): differences between insect species and frass treatments**

Shaphan Y. Chia\*, Joop J.A. van Loon and Marcel Dicke

Laboratory of Entomology, Wageningen University & Research, P.O. Box 16, 6700 AA Wageningen, the Netherlands.

Short title:

**Frass as a sustainable soil amendment, enhancing plant growth and herbivore resistance**

**\*Corresponding author:**

S.Y. Chia, e-mail: [chiashaphan2009@yahoo.com](mailto:chiashaphan2009@yahoo.com);

mobile tel.: +31620409215

**Key words:**

Insect residual streams, Organic fertiliser, Pest management, *Delia radicum*, *Plutella xylostella*, Insect herbivory

## Abstract

Frass, a byproduct of insect rearing, has become popular for its potential use in sustainable agriculture. The rapid growth of insect production results in an increased frass output. This study examined the effects of frass as soil amendment on plant growth and resistance to insect herbivory. In greenhouse experiments, *Brassica rapa* Linnaeus (Brassicales: Brassicaceae) was grown in unamended soil (NoFrass; control) or soil amended with frass (2g kg<sup>-1</sup>) from larvae of black soldier fly, *Hermetia illucens* Linnaeus (Diptera: Stratiomyidae) (BSFF) or yellow mealworm, *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) (MWF). Frass was applied as raw, incubated, or composted frass before seed germination. Plant growth and performance of root-feeding *Delia radicum* Linnaeus (Diptera: Anthomyiidae), and shoot-feeding *Plutella xylostella* Linnaeus (Lepidoptera: Plutellidae), larvae were measured. Initially, raw BSFF and MWF reduced the growth of *B. rapa* and resulted in a smaller leaf area than NoFrass. However, over time, a notable trend emerged. While the difference in leaf area between MWF and NoFrass disappeared, BSFF consistently resulted in a smaller leaf area than MWF and NoFrass. Raw BSFF reduced *D. radicum* larval survival and pupal biomass and survival of *P. xylostella* larvae. In contrast, raw MWF increased larval survival and biomass of *D. radicum* and the survival of *P. xylostella* larvae. Interestingly, incubation of frass in the soil for 16 days before seed germination removed plant growth inhibition and increased plant leaf area, especially for MWF compared to NoFrass. In addition, composting MWF increased leaf growth. Therefore, frass may be used as a sustainable and natural alternative to conventional organic fertilisers, promoting plant growth and enhancing resistance to herbivory. Our results indicate that soil amendment with raw BSFF may negatively impact herbivore performance, whereas raw MWF may enhance herbivore performance.

## Introduction

In recent years, alternative protein sources for animal feed and human food have received increasing interest because of the need to produce food for the growing human population in a sustainable manner. The use of insect-based feed, in particular, has become popular due to its high nutritional value and low environmental impact (van Huis, 2013). Among various insect species, larvae of the black soldier fly (BSF) *Hermetia illucens* Linnaeus (Diptera: Stratiomyidae) and the yellow mealworm *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) have emerged as promising candidates for animal feed and human food respectively. These insects have a high protein content, rapid growth rate, and are amenable to mass-rearing (Chia et al., 2020; Mariod, 2020; Toviho & Bársony, 2022; Zulkifli et al., 2022).

In addition to using insects as feed or food, insect products have been investigated for their potential as organic fertilisers. Insect frass, a mixture of insect excrements, leftover substrate, and exoskeletons left after moulting, is nutrient-rich and can enhance soil health and plant growth. For instance, adding frass may stimulate the growth of saprotrophic fungi, as indicated by increased extractable ergosterol contents, alongside the promotion of plant growth-promoting rhizobacteria (PGPR) in the soil microbial community (Barragán-Fonseca et al. 2022; Watson et al., 2021). This dual enhancement of beneficial soil fungi and PGPR highlights the multifaceted positive effects of frass on soil ecosystems and plant development. PGPRs are beneficial root-associated bacteria known for bolstering a host plant's defenses against diseases and insect pests (Basu et al., 2021; Berendsen et al., 2012; Gadhawe et al., 2016; Hu et al., 2018; Mahapatra et al., 2022; Pineda et al., 2010). The potential of frass to increase crop yields has been demonstrated (Barragán-Fonseca et al., 2022; Dzepe et al., 2022; Houben et al., 2021; Houben et al., 2020; Lopes et al., 2022; Poveda, 2021).

Incorporating frass into the soil can enhance a plant's natural defence against insect herbivores (Barragán-Fonseca et al., 2022; Poveda, 2021). Bacilli in particular, are renowned for boosting a plant's resistance to insect infestation and are commonly found among PGPR in agricultural soils (Gadhav et al., 2016; Pangesti et al., 2013).

When frass is introduced into the soil, it serves as a valuable nutrient and energy source for both plants and beneficial soil microorganisms. During microbial decomposition, inorganic nitrogen (N) is released from soil amendments. Furthermore, chitinolytic microbes, which are prevalent in frass, play a critical role in biologically controlling insect pests (Sharp, 2013). Consequently, amending the soil with chitin-rich residual streams, such as frass, can foster the proliferation of these beneficial microbes.

As the edible insect industry grows, so will the amount of frass produced (Chia et al., 2019; Houben et al., 2020; Poveda, 2021; Salomone et al., 2017). Following the rapid growth of the edible insect industry and the potential of frass as a viable fertiliser and its contribution to a circular economy, the European Commission has enacted legislation (Regulation (EU) 2021/1925) to regulate its production and use. Analyses of frass produced by BSF larvae (BSFF) fed various food leftovers indicate that it ranges in total N content from 0.6 to 4.8 %, in total phosphorus (P) content from 0.1 to 2.5 %, and in potassium (K) content from 0.1 to 2.1%, as well as providing trace minerals and beneficial microorganisms (Basri et al., 2022; Choi & Hassanzadeh, 2019; Poveda, 2021). Mealworm frass (MWF), on the other hand, ranges in total N content from 2.7 to 7.8%, total P from 1.0 to 1.5% and total K from 1.2 to 2.0%. It also contains calcium, magnesium, and micronutrients (Poveda et al., 2019). Moreover, BSFF and MWF contain chitin, which can enhance the abundance of soil microbiota and generate antimicrobial peptides that serve as a plant's defence barrier (Choi & Hassanzadeh, 2019; Nurfikari & de Boer, 2021; Poveda et al., 2019; Schmitt & de Vries, 2020). High concentrations of P in BSFF promote N accumulation in plants (Klammsteiner et al., 2020). This makes it an

excellent source of plant nutrients, as it can improve soil fertility, enhance plant growth and increase crop yields. By reintroducing and valorising relevant nutrients and organic matter into the soil, using frass can help close the nutrient cycle in insect farming. This strategy contributes to developing a zero-waste food production system and highlights the significance of identifying sustainable sources of organic matter for soil amendment and food production.

Field mustard, *Brassica rapa* Linnaeus, (Brassicales: Brassicaceae) is a member of the Brassicaceae family and is widely cultivated for food, oil, and feed. It has a high economic value due to its nutritional, medicinal, and bio-industrial properties (Young-Mathews, 2012). For instance, in Bangladesh, *B. rapa* serves as a major oilseed crop, contributing to approximately 70% of the total oil crop production. According to the latest available data, during the period 2017-2018, *B. rapa* was cultivated across 0.308 million hectares, resulting in a total production yield of 351,537 metric tons of oilseeds (Rahman et al., 2022). However, it is also a preferred host for various insect herbivores, including the root-feeding larvae of the cabbage root fly *Delia radicum* Linnaeus and the shoot-feeding larvae of the diamondback moth *Plutella xylostella* Linnaeus, which can cause substantial economic losses (Ahuja et al., 2010). To mitigate plant damage caused by insect herbivores, various methods have been employed, including the use of chemical pesticides. However, the overuse of pesticides has led to numerous environmental and health concerns (Nicolopoulou-Stamati et al., 2016). Therefore, effective, sustainable, and safe alternatives for managing insect herbivores are required.

To date, there is limited research on the potential of frass to enhance plant development and resistance to insect herbivory. A recent study showed that mealworm exuviae did not affect shoot and root dry biomass of *B. oleracea* (Wantulla et al., 2022). The study further recorded a reduced survival of *D. radicum* larvae in BSFF-exposed soil, but mealworm exuviae did not

affect larval survival and biomass compared to a synthetic fertiliser. However, Wantulla et al. (2022) did not investigate the effects of MWF, which is the most abundant byproduct of mealworm cultivation. They focused on a root-feeding pest, *D. radicum* and did not include shoot-feeding insect pests. Additionally, Wantulla et al. (2022) did not investigate the effects of preprocessing insect residual streams. Furthermore, evaluations of plant growth in frass-amended soil and herbivore performance on such plants have been limited to a few plant species and insect herbivores under soil treatment with frass, thus limiting the generalisation of the results. To address this knowledge gap, it is crucial to examine the impact of various types of frass and to consider other plant species and their resistance to biotic stress, such as insect herbivory. It is also important to extend investigations to multiple herbivores. Intriguingly, whether insect frass can replace traditional organic and mineral fertilisers and chemical insecticides in agricultural systems still requires further research. There is currently no single paper that addressed this question, and several studies on soil fertility have mainly focused on frass application to improve soil health and promote plant growth, with limited attention to its potential effect on insect herbivore performance (Poveda, 2021; Wantulla et al., 2022). Exploring the effects of insect frass on plant resistance to herbivores can provide insights into its use as a pest management strategy and reduce the need for chemical pesticides.

Here, we aimed to investigate the effects of frass derived from BSF and yellow mealworm larvae on growth performance of *B. rapa* and resistance of the plants to the herbivores *D. radicum* and *P. xylostella*. We hypothesised that frass, due to its high nutrient content, would enhance the growth of *B. rapa* and confer resistance to the herbivores, compared to control plants that received no frass. Additionally, we hypothesised that incubating frass in the soil or composting it will enhance its effectiveness as a soil amendment and lead to greater plant growth than non-incubated or uncomposted frass. In fact, composting is a common method of



preparing organic materials for use as soil amendments (Barthod et al., 2018; Goldan et al., 2023). The findings of this study contribute to our understanding of the potential benefits of using frass as a sustainable and environmentally friendly fertiliser in agriculture.

## Materials and Methods

### Experimental facility and greenhouse soil

We conducted greenhouse experiments to assess how frass resulting from the production of two edible insect species affected the growth of *B. rapa* plants and the survival of a belowground and an aboveground insect herbivore. The study was conducted in the greenhouse facilities at Unifarm, Wageningen University & Research, the Netherlands. The soil used in this study was collected at Unifarm's organic experimental farm Droevendaal. Various brassicaceous plant species had been grown on this soil since 2011 and black mustard, *Brassica nigra* Linnaeus (Brassicales: Brassicaceae) had recently been grown at the location selected for soil collection. Soil composition 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 (Wantulla et al., 2022).

### Raw material and soil amendments

The frass used in this study was obtained from two commercially reared edible insect species: (1) *H. illucens* provided by Bestico, Berkel en Rodenrijs, the Netherlands and (2) *T. molitor*, provided by Nijenkamp-Voederdieren, Oldenzaal, the Netherlands. Before use, frass samples were oven-dried at 60 °C for 24 h (Binder Model FED-260, Binder GmbH, Tuttlingen, Germany), pulverised using a cutting mill SM 100 (Retsch B.V., Haan, Germany), sieved (2-mm mesh size), and then stored in air-tight containers at room temperature for 78 days. We refer to the pulverised frass as “raw frass” to differentiate it from other forms of frass used in

this study i.e., “incubated frass” and “composted frass” (see details in sections below). The soil was amended with the pulverised frass by adding 2 g of frass per kg of soil previously sieved (5 mm) to remove large debris. To mix frass and soil, 20 g of frass was added to 10 kg of soil in plastic bags and mixed thoroughly by hand until there were no visible frass clumps. Soil amended with frass of the black soldier fly larvae was labelled as “BSFF”, while soil amended with frass of yellow mealworms was labelled as “MWF”. The same procedure was followed for the control (NoFrass), except that no frass was added. In two trials (Trial 1 and Trial 2), raw frass was added to the soil. Subsequently, samples of the raw frass were either incubated in the soil (Trial 3) or composted (Trial 4) before being added to the soil for plant growth. Trial 2 is a repeat of Trial 1 under similar conditions and applying similar procedures. Trial 1 started (i.e., seed germination) on January 30, 2021; Trial 2 started on March 1, 2021; Trials 3 and 4 started on March 26, 2021.

## **Insect rearing**

### *D. radicum*

The cabbage root fly, *D. radicum* is an important pest of brassicaceous vegetables. This insect species was reared by the insect rearing team of the Laboratory of Entomology, Wageningen University & Research. The larvae of this colony were fed on rutabaga *B. napus* L. (Brassicales: Brassicaceae) until pupation. Adults were kept in gauze cages and fed on a mixture of sugar, milk powder, yeast and honey. Water was provided in cotton wool. The insect colony was maintained in a climate cabinet ( $22 \pm 1$  °C, 50-70 % RH). For experiments, we obtained young larvae (< 24 h since hatching).

### *P. xylostella*

The diamondback moth (DBM), *P. xylostella*, is one of the most destructive insect herbivores of cruciferous plants worldwide (Wei et al., 2013). Neonate larvae of DBM were supplied by the insect rearing team of the Laboratory of Entomology, Wageningen University & Research, where they were fed on Brussels sprouts plants (*B. oleracea* variety *gemmifera* cultivar Cyrus) in greenhouse conditions ( $22 \pm 3$  °C, 50-70 % RH).

### **Field mustard (*B. rapa*) seeds and germination**

Field mustard, *B. rapa* is an annual or biennial herb (Ilyas et al., 2022). *B. rapa* originated from a natural population in the Netherlands and were kindly provided by Erik Poelman (Laboratory of Entomology, Wageningen University & Research). Before sowing, the seeds were stratified by maintaining them on moist filter paper at 4 °C for 7 days to break seed dormancy. Seeds were germinated using unamended soil in the greenhouse ( $22 \pm 3$  °C,  $60 \pm 2$  % RH). In this study, seeds germinated in unamended soil (NoFrass) had a high germination rate (> 90%), whereas those sown directly into the frass-amended soil had a slightly lower germination rate, but there were no significant differences among the trials ( $\chi^2 = 2.97$ ;  $df = 3$ ;  $p = 0.3961$ ; Table S1).

### **Plant growth performance in soil amended with raw frass**

At the emergence of the first true leaf (7-day-old plants), seedlings were transplanted individually into amended and unamended soil in 1 L plastic pots placed individually in round saucers (16 cm wide, 1.8 cm deep). Plants were randomly assigned to the two soil amendments (BSFF and MWF) in 30 replicate pots placed on a table in a greenhouse compartment. During the first two weeks **after** germination, plants were watered twice per week, from the third week onwards three times per week by filling the saucer until the topsoil became moist. Weeds in experimental pots were manually removed. This experiment was repeated after four weeks

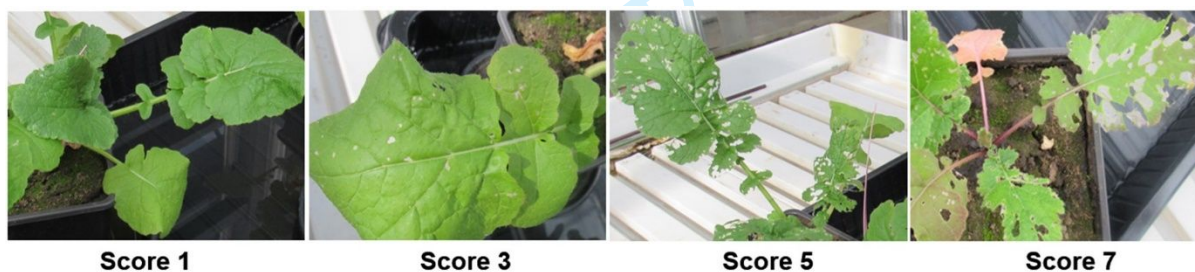
following the same procedure. At 21 days after seed germination, plant growth measurements included a leaf count to record the number of leaves per plant and the width (cm) of the second most mature true leaf (leaf formed after seedling transplant) measured at the broadest point of the leaf. The same measurements were repeated at 28, 35 and 42 days since germination. Every week, the next mature true leaf was measured until the onset of plant bolting (development of flowering stems). From this point onwards, plants were monitored daily and the number of days until the first flower emerged was recorded as the time until flowering.

### **Assessment of plant resistance to insect herbivory**

The resistance of raw-frass-exposed *B. rapa* plants to two insect herbivores, *D. radicum* and *P. xylostella*, was assessed by recording leaf damage, larval survival, and pupal biomass. When plants were four weeks old, ten larvae (< 24 h old) of *D. radicum* were released at about 0.5 cm into the soil close to the stem of each potted plant. Their survival was assessed when the larvae fed on roots of frass-exposed *B. rapa* plants. Ten plants per treatment and control (BSFF, MWF and NoFrass) were inoculated. After 21 days, all plants were uprooted, and roots were rinsed to remove adhering soil. The roots were then examined for larvae that remained, and all the soil was washed away using a Fenwick Can (Metaalgaas Twente, Hengelo, the Netherlands) and a sieve with a 0.5 mm aperture (Wantulla et al., 2022). All pupae and larvae retrieved per plant were recorded. Wet pupal weight was recorded using an Ohaus Adventurer Pro AV213 balance with an accuracy of 0.001 g. To assess the effect of soil amendment on pupal development, all pupae retrieved from roots of plants exposed to the soil treatments were placed in a Petri dish at  $22 \pm 1$  °C, 50-70 % RH. The number of adult flies that emerged and the time (days) taken to emerge were recorded daily until all pupae had either emerged as flies or appeared dead. This experiment was repeated four weeks later, following the same procedure.

To assess the effect of raw-frass-exposed plants on the survival of *P. xylostella* larvae, ten second-instar larvae were inoculated on one fully expanded leaf of each replicate *B. rapa* plant. **Ten plants per treatment and control (BSFF, MWF and NoFrass) were inoculated.** Inoculated plants were immediately enclosed in transparent mesh bags to contain the larvae and prevent their escape. The mesh bags were monitored daily to record the pupation of the larvae. The experiment was terminated when all larvae had either pupated or appeared dead. Ten replicate plants per treatment (BSFF, MWF or NoFrass) were used in this experiment. This experiment was repeated once more following the same procedure.

The extent of leaf damage by the larvae of *P. xylostella* on raw-frass-exposed *B. rapa* plants was assessed visually on a 1-to-7 scoring scale (Fig. 1). A score of 1 means no visible damage to the plant, and a score of 7 means extensive damage to the plants (Robin et al., 2017). The average values from ten plants were calculated for each soil amendment.



**Figure 1.** Visual representation of feeding scores used to assess the extent of leaf damage in greenhouse-grown *B. rapa* plants by larvae of *P. xylostella*. The score ranged from ‘1’ to ‘7’ with ‘1’ being scored for leaves with no damage symptoms and ‘7’ being scored for heavily damaged leaves. Intermediate values on the scale represent different levels of damage (Robin et al., 2017).

## 264 **Incubation and composting of raw frass: effects on plant growth performance**

### 265 Incubation of 'raw frass' in the soil

266 Incubation was achieved by mixing 2 g of raw frass per kg of soil. The amended soil was placed  
267 in 0.5 L plastic pots in saucers (14 cm wide, 1.5 cm deep). The soil mixture in pots was  
268 moistened by filling the saucers with water twice a week. This incubation of frass was  
269 maintained for 16 days under greenhouse conditions. The same procedure was followed for the  
270 unamended soil (control) except that no frass was added. Stratified seeds of *B. rapa* were sown  
271 directly into the soil. Three seeds were sown in each pot and seven days after germination,  
272 seedling numbers were reduced to maintain only one seedling per pot. When plants were 14  
273 days old, measurements of the leaf width (cm) and the number of leaves per plant were taken  
274 as described for raw frass. Six replicate plants per treatment were used in this study and  
275 measurements were repeated on the same plants at 21, 28 and 35 days since germination. Plants  
276 were further monitored, and the first flowering date was recorded to calculate the time from  
277 germination until flowering.

278

### 279 Composting of 'raw frass'

280 Fifty grams each of **BSF** larval frass and yellow mealworm frass were placed in plastic boxes  
281 (17.5 x 12.5 x 6.5 cm). The pulverised raw frass samples were moisturised with 100 mL of  
282 water, and the frass in the containers was covered with a perforated aluminium foil to allow  
283 ventilation but also to reduce evaporation and maintain a high temperature inside the box  
284 relative to the external environment. Frass inside the box was aerated by stirring it vigorously  
285 after every five days using a spatula. The composting lasted for 38 days. Composting of frass  
286 was terminated by removing the aluminium foil cover and allowing the compost to air-dry for  
287 18 days. Then, the composted frass was pulverised and added to the soil at 2 g kg<sup>-1</sup> of soil.  
288 As described above, three stratified seeds were sown in each pot and seven days after

germination, seedling numbers were reduced to maintain only one seedling per pot. Percent seed germination in amended and unamended soil was recorded. Twelve replicate plants per soil treatment were used in this study, and the number of leaves and leaf width per plant were measured at 14, 21, 28 and 35 days since germination. Plants were further monitored, and the time from germination until emergence of the first flower was recorded.

### Data processing and statistical analysis

All analyses were performed using the R environment for statistical computing (version 4.2.2) (R Core Team, 2022). A linear regression model estimated leaf area ( $\text{area} = 0.88735 * (\text{leaf width})^2 + 0.93503 * \text{leaf width}$ ) from linear measurements (leaf width) (Tartaglia et al., 2016). The normality of data was verified by visualisation using boxplots and QQ plots and subjected to the Shapiro-Wilk test. The homogeneity of variance was checked using Levene's test. Data on leaf area and the number of leaves were analysed with a generalised linear model (GLM) using the 'glm' function. For each trial, soil amendment (treatment) was included in the model as a predictor variable. Larval survival data were analysed with a Poisson-based model. Pupal biomass and leaf damage score data were analysed with a generalised linear model (GLM) using the 'glm' function. To determine the effect of soil amendments on the proportion eclosion of *D. radicum*, data on the proportion of adult flies that emerged were analysed with a Chi-square test of equality of proportions (Adedia et al., 2020). For fly emergence time of *D. radicum*, and time until flowering of *B. rapa* plants, data were analysed with the Poisson regression model using the 'glm' function, estimated by the maximum likelihood to capture the relationship between the number of days taken for flies to emerge from pupae, and for the first flower to emerge (Zeileis et al., 2008). The 'Anova' function of the 'car' package was used to generate the model output for the main effects with Chi-square ( $\chi^2$ ) values, degrees of freedom (df) and *p*-values using the Wald Chi-square test (Fox et al., 2012). Akaike's Information



Criterion (AIC) was used to estimate the degree of fit of statistical models with the lowest AIC values considered as best in estimating the model prediction error. The mean effects of treatment were considered significant at  $p < 0.05$ . The '*emmeans*' function was used to perform pairwise comparisons among soil treatments with  $p$ -values adjusted according to the Tukey method for comparing estimates when a significant effect of soil treatment was detected in the larval survival and pupal weight (Lenth & Lenth, 2018). In the leaf area and number of leaves, the '*glht*' function was used to perform pairwise comparisons with  $p$ -values adjusted according to the '*holm*' method for multiple comparisons adjustment. After conducting a Generalized Linear Model (GLM) to evaluate the differences among treatment groups for time until flowering and leaf area in composted frass treatments, post hoc comparisons were performed using the Fisher's Least Significant Difference (LSD) post hoc test. The GLM analysis revealed significant differences among the treatment groups. However, no significant differences were detected when applying the Tukey post hoc test for multiple comparisons. Considering this, the LSD post hoc test was chosen as an alternative method to investigate pairwise differences between treatments, as it does not assume equal variances and does not require homogeneous sample sizes. The LSD test allows for direct pairwise comparisons, and it was used to identify any significant differences that the Tukey test may have missed. Following a significant Chi-square test of equality of proportions, the Marascuilo procedure for multiple comparisons was used to determine significance of differences (Wagh & Razvi, 2016).

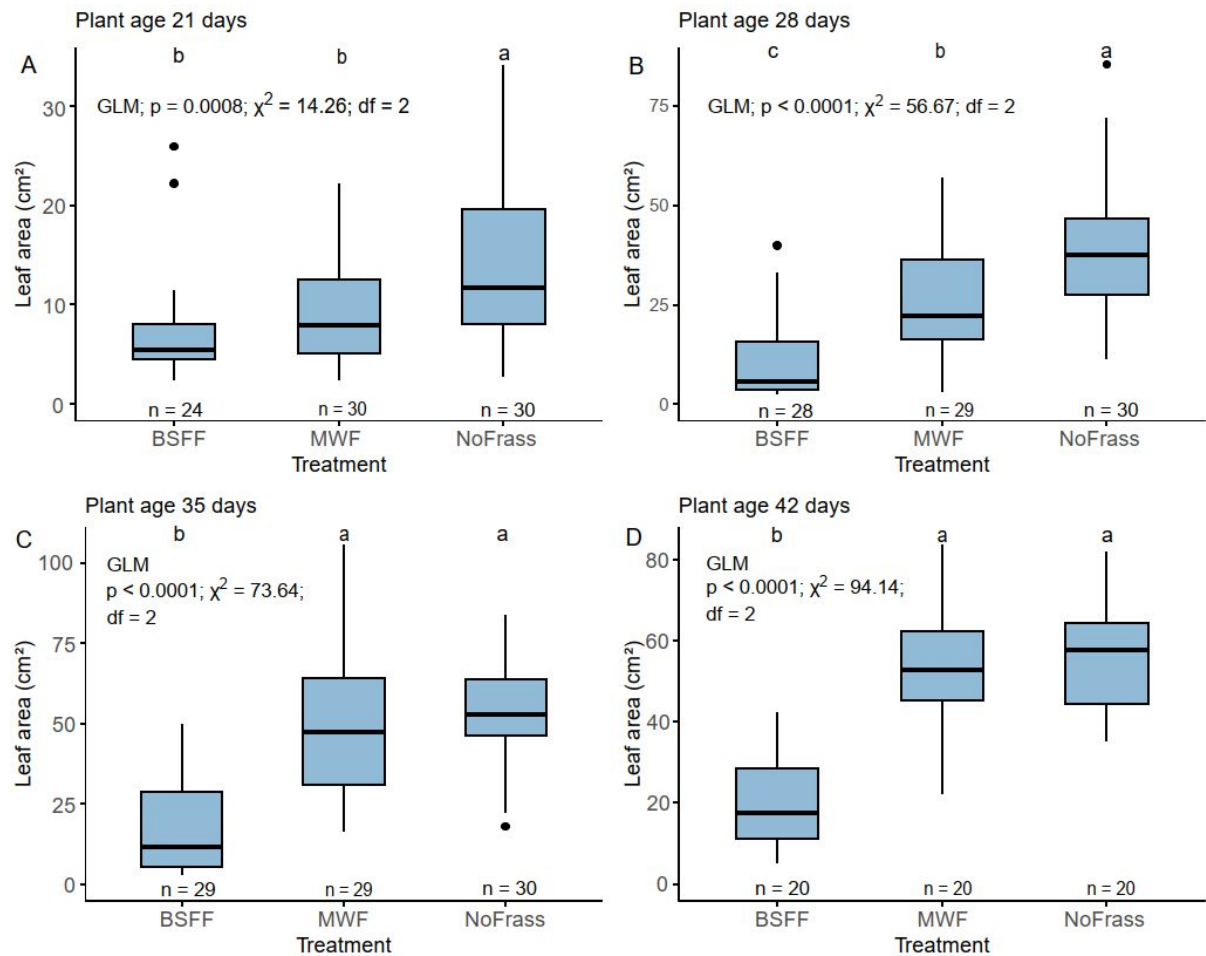
## Results

### Effects of raw frass on the growth and development of *B. rapa* plants

Amending soil with either raw BSFF or raw MWF affected the growth of *B. rapa* plants. Initially, both frass types, BSFF and MWF resulted in a significantly smaller leaf area than the control (NoFrass) (Figure 2). However, over time, an interesting trend emerged. While the



difference in leaf area between the MWF-treated group and NoFrass disappeared, BSFF consistently resulted in a smaller leaf area compared to both MWF and NoFrass (Figure 2). When this experiment was repeated under similar conditions, BSFF consistently resulted in a smaller leaf area than MWF and NoFrass (Figure S1).



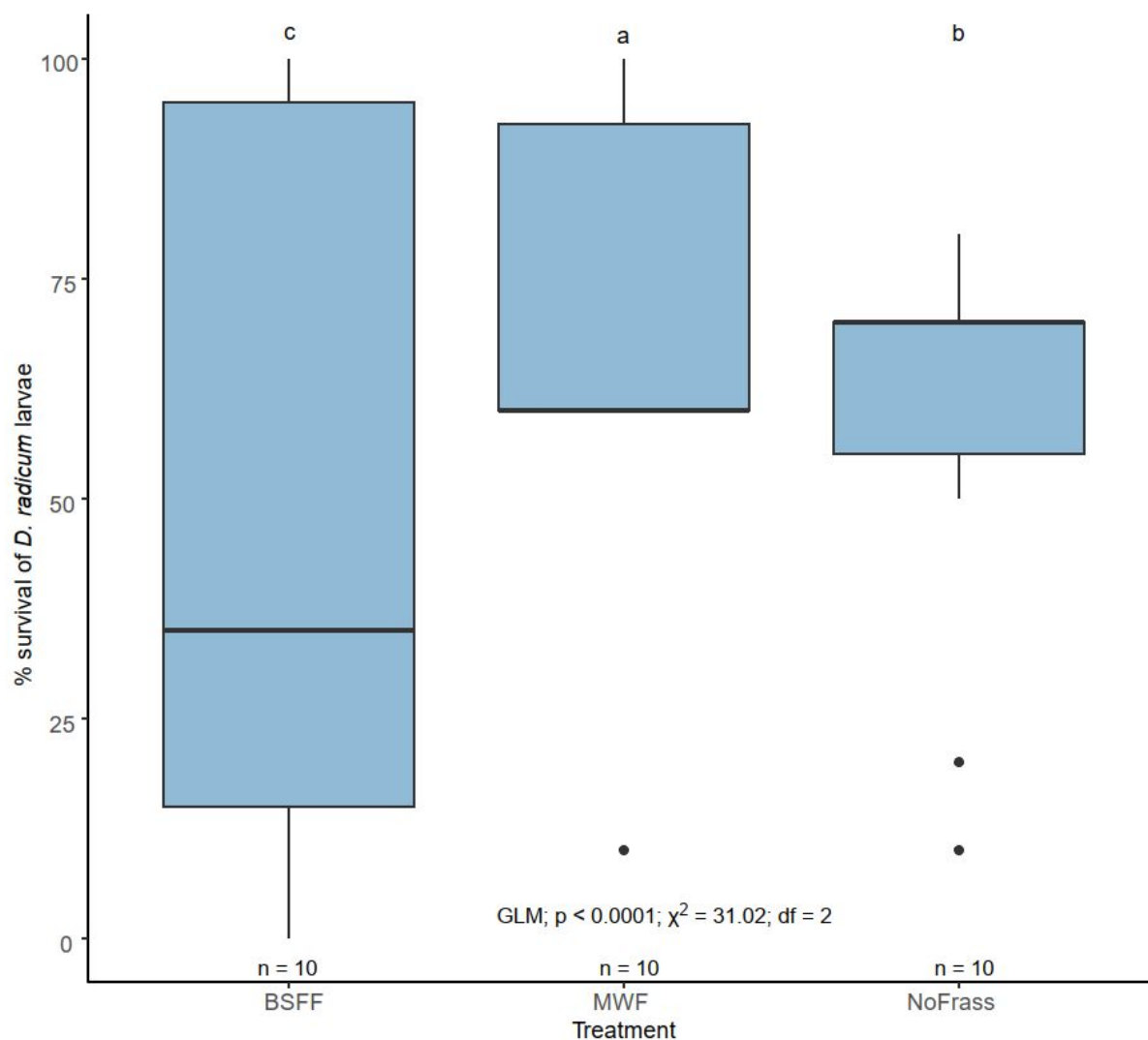
**Figure 2.** Leaf area (cm²) of *B. rapa* plants grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF) recorded in Trial 1 at ages 21 days (A), 28 days (B), 35 days (C) and 42 days (D). The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants for leaf area measurements. Boxes with different letters differ significantly (Tukey's post-hoc test,  $p < 0.05$ ).

The addition of raw BSFF or raw MWF to the soil initially resulted in a significantly smaller number of leaves per *B. rapa* plant when compared to the unamended control (Figure S2). However, over time, the difference in number of leaves between the MWF and NoFrass disappeared, while BSFF continued to exhibit a smaller number of leaves compared to both MWF and NoFrass (Figure S2). Repeating the experiment under comparable conditions gave similar results, with no significant difference between MWF and NoFrass, and BSFF displaying a consistently smaller number of leaves (Figure S3).

Amending soil with raw BSFF or raw MWF resulted in significant differences in the time until flowering of *B. rapa* plants (Figure S4). The application of raw BSFF resulted in a longer time until flowering than MWF and NoFrass (Figure S4A). There was no significant effect of soil treatment on time until flowering when this experiment was repeated under similar conditions (Figure S4B).

#### **Effect of raw frass on the survival of *Delia radicum* larvae**

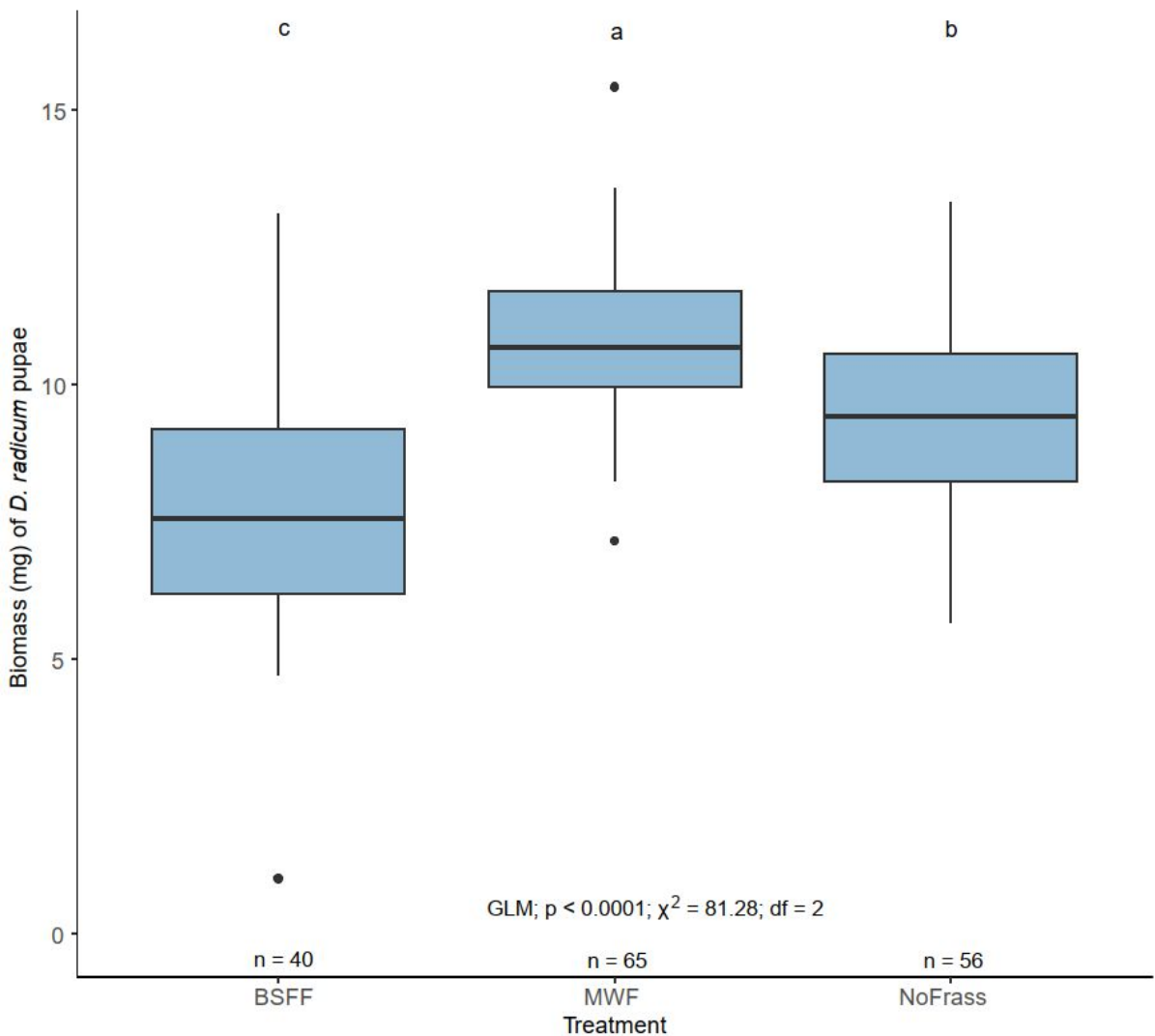
Frass treatments affected the number of *D. radicum* larvae that survived after a 21-day root infestation of *B. rapa* (Figure 3). Soil amendment with BSFF resulted in the lowest median survival rate (35%) of *D. radicum* larvae, followed by MWF (60%) and NoFrass (70%). (Figure 3). Similar results were recorded when the experiment was repeated under comparable conditions (Figure S5).



**Figure 3.** Survival of *D. radicum* larvae on roots of *B. rapa* plants grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF) recorded in Trial 1. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants that had each been infested with ten larvae. Boxes with different letters differ significantly (Tukey's post-hoc test,  $p < 0.05$ ).

**Effect of raw frass on the biomass of *Delia radicum* pupae**

Biomass of *D. radicum* pupae retrieved from the roots of *B. rapa* plants was influenced by soil treatment (Figure 4). Treatment with raw BSFF resulted in the lowest pupal biomass, whereas MWF resulted in the highest biomass (Figure 4). When the experiment was repeated under comparable conditions, the differences were **not statistically** significant ( $\chi^2 = 5.12$ ,  $df = 2$ ,  $p < 0.0773$ ; Figure S6).



**Figure 4.** Biomass (mg) of *D. radicum* pupae retrieved after a 21-day root infestation of *B. rapa* grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF) recorded in Trial 1. The box represents the interquartile range (IQR), with the bottom and top edges

corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with a generalised linear model (GLM).  $n$  is the number of pupae sampled per treatment. Boxes with different letters differ significantly (Tukey's post-hoc test,  $p < 0.05$ ).

#### **Effect of raw frass on the emergence of *D. radicum* adult flies**

The proportion of adult *D. radicum* flies that emerged from pupae was significantly affected by soil treatment (Figure S7A). The application of BSFF resulted in a substantially lower proportion of flies that emerged than the application of MWF, but the effect was not significantly different from the NoFrass treatment (Figure S7A). The proportion of flies that emerged from plants exposed to MWF was similar to that from plants in the NoFrass group (Figure S7A). Although a similar emergence pattern was recorded when this experiment was repeated under similar conditions, the proportion of flies that emerged did not differ significantly among soil treatments (Figure S7B). The time it took adult flies to eclose did not differ significantly among soil treatments (Figure S7C), and similar results were recorded when the experiment was repeated under similar conditions (Figure S7D).

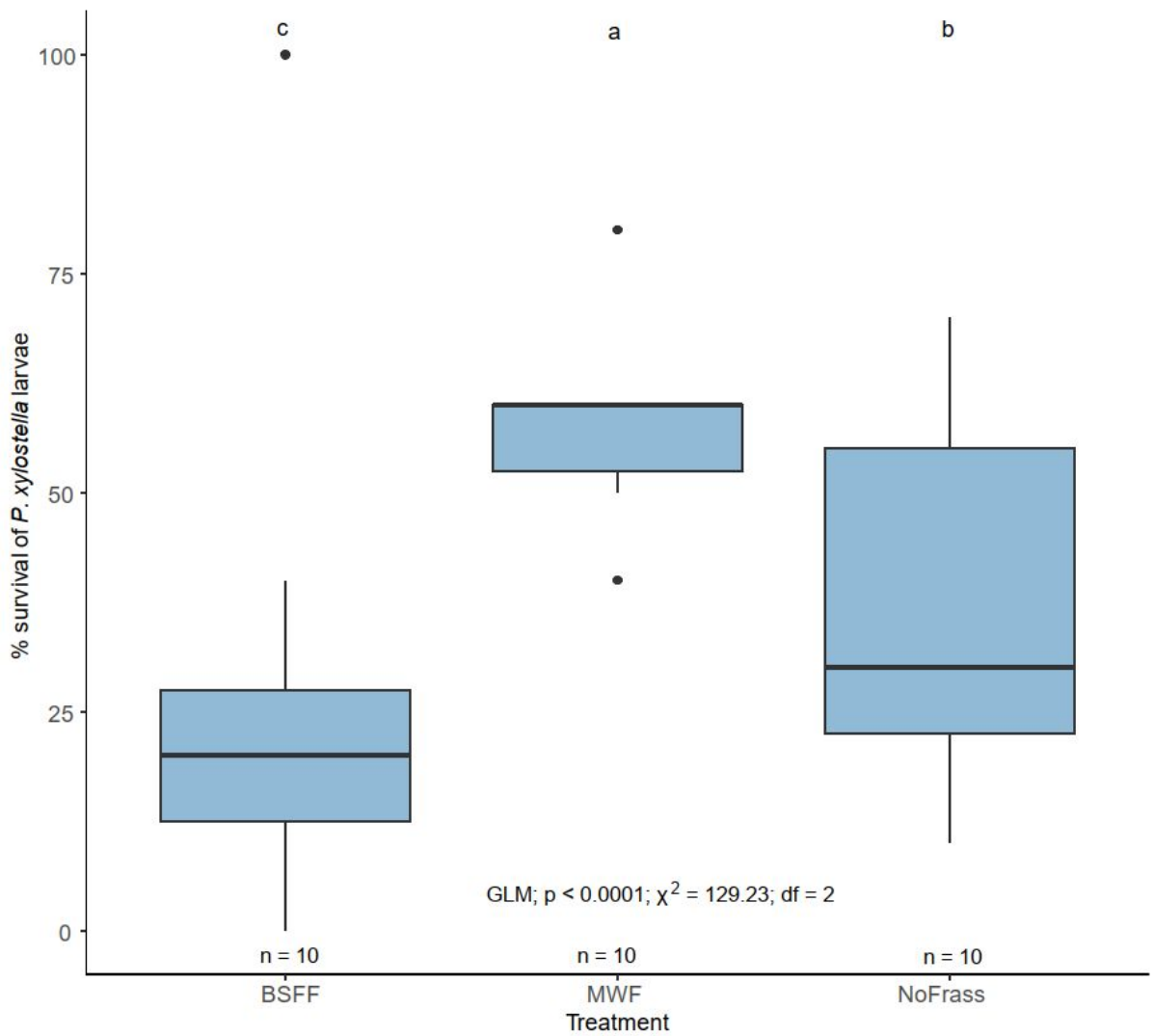
#### **Effect of raw frass on feeding damage by *P. xylostella* larvae on *Brassica rapa* plants**

Soil amendment with raw frass did not affect larval feeding damage by *P. xylostella* caterpillars on the leaves of *B. rapa* plants in either of the two trials (Figure S8).

#### **Effect of raw frass on the survival of *P. xylostella* larvae on *Brassica rapa* plants**

The number of *P. xylostella* larvae that survived on *B. rapa* plants differed significantly among treatments (Figure 5). Amending soil with BSFF resulted in the lowest mean larval survival, whereas MWF resulted in the highest larval survival (pupae retrieved) (Figure 5). **There was**

no significant difference in mean larval survival when this experiment was repeated under similar conditions ( $\chi^2 = 5.84$ ,  $df = 2$ ,  $p < 0.0540$ ; Figure S9).



**Figure 5.** Survival of *P. xylostella* larvae on *B. rapa* grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF) recorded in Trial 1. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed by generalised linear models (GLM).  $n$  is the number of replicate

plants that had each been infested with ten larvae. Boxes with different letters differ significantly (Tukey's post-hoc test,  $p < 0.05$ ).

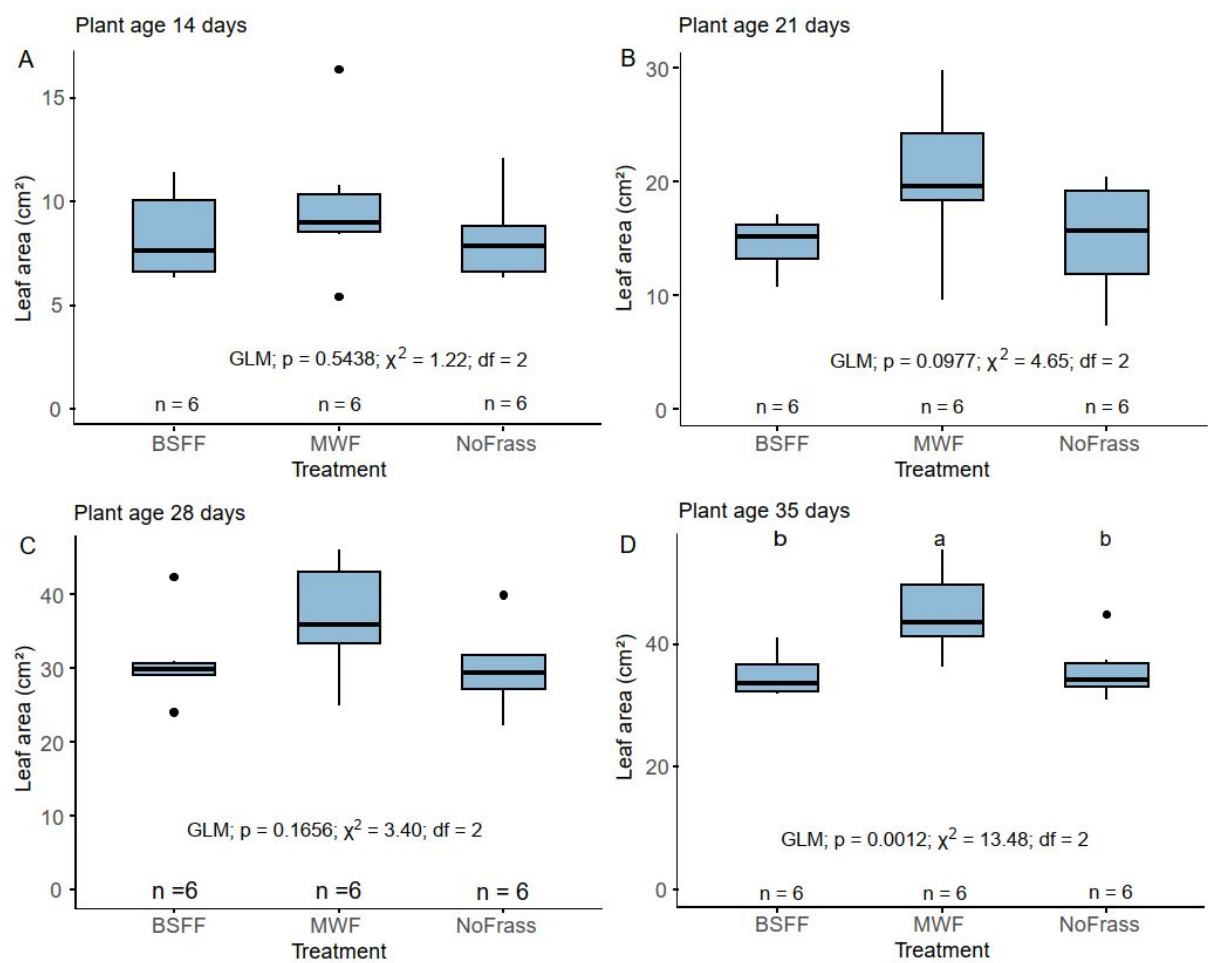
#### **Effects of incubated and composted frass on the growth of *B. rapa* plants**

Black soldier fly frass or MWF that had been incubated in the soil for 16 days had no significant effect on the growth of *B. rapa* plants from germination to 28 days but affected growth by day 35 (Figure 6). Compared to the control (NoFrass) and BSFF, incubating MWF in the soil resulted in the highest mean leaf area by day 35 (Figure 6). Plants exposed to incubated BSFF had a similar leaf area as plants exposed to the NoFrass control.

When BSFF or MWF was composted before being added to the soil, this affected leaf area at days 14 and 35 (Figure 7), but not at days 21 and 28 (Figure 7). Amending the soil with composted BSFF resulted in the lowest leaf area, significantly different from plants grown in soil amended with composted MWF and NoFrass at day 35 (Figure 7).

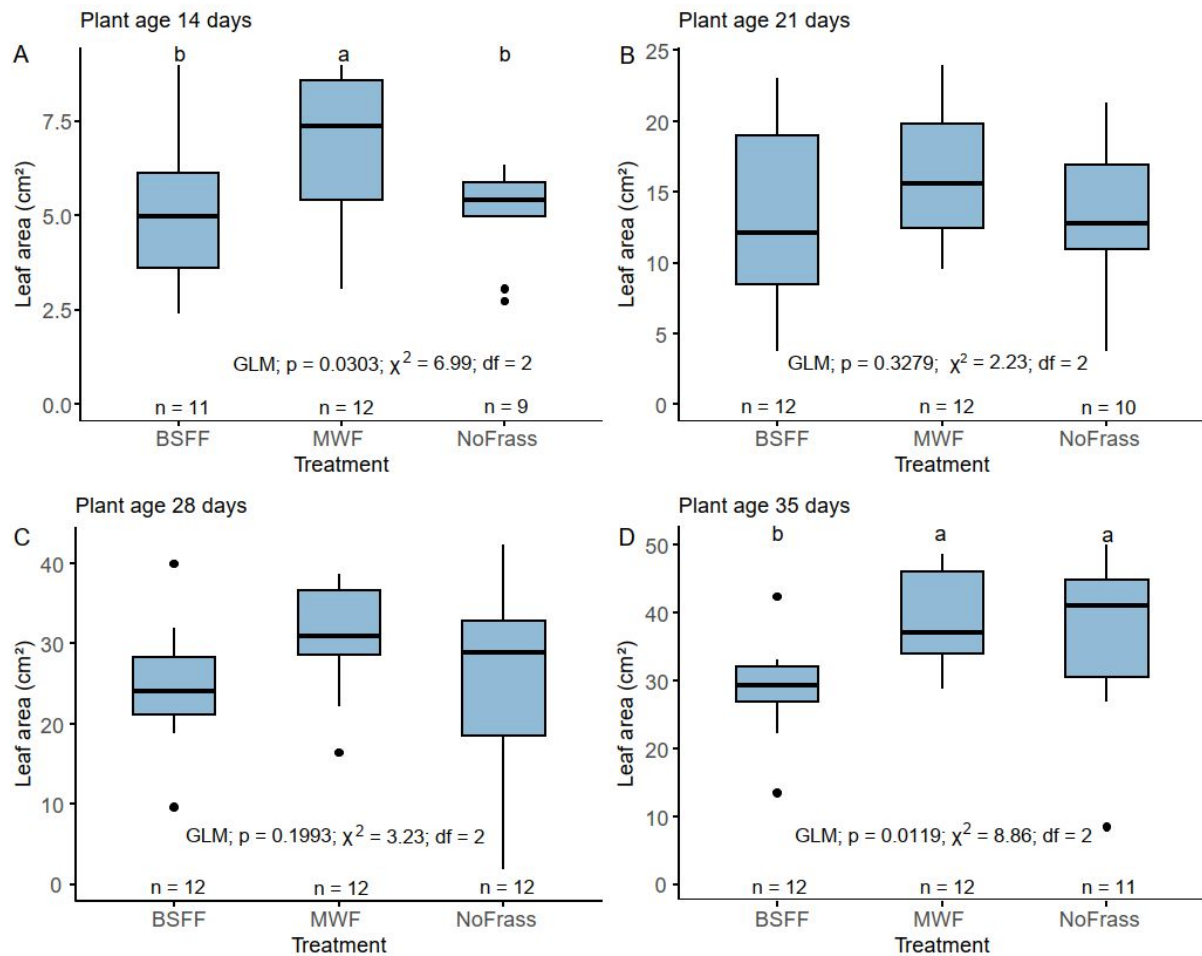
The number of leaves per *B. rapa* plant was not affected by incubated BSFF or MWF at any of the time points (Figure S10). However, composted MWF significantly increased the number of leaves per plant at days 14 and 21 compared to the NoFrass (Figure S11), but not at days 28 and 35 (Figure S11).

The time until the start of flowering was not significantly affected by the incubation of frass in the soil when compared to the NoFrass control (Figure S12A). Similarly, the time until flowering of *B. rapa* plants was not significantly affected by adding either composted BSFF or MWF to the soil (Figure S12B).



**Figure 6.** Leaf area (cm<sup>2</sup>) of *B. rapa* plants grown in unamended soil (NoFrass; control) or soil amended with BSF frass (BSFF) or yellow mealworm frass (MWF) after incubating. Leaf measurements were taken at plant ages 14 days (A), 21 days (B), 28 days (C) and 35 days (D). Incubation involved frass mixed with soil in 0.5 L plastic pots and moistened, and seeds were only sown after sixteen days under greenhouse conditions. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants for leaf area measurements. Boxes with different letters differ significantly (Tukey's post hoc test,  $p < 0.05$ ).





**Figure 7.** Leaf area (cm<sup>2</sup>) of *B. rapa* plants grown in unamended soil (NoFrass; control) or soil amended with BSF frass (BSFF) or yellow mealworm frass (MWF) after composting. Leaf measurements were taken at plant ages 14 days (A), 21 days (B), 28 days (C) and 35 days (D). Frass samples were composted for 38 days in plastic and air-dried for 18 days. The resulting compost was pulverised and added to the soil. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants for leaf area measurements. Boxes with different letters differ significantly (Fisher's Least Significant Difference post hoc test,  $p < 0.05$ ).

## Discussion

This study investigated the effect of soil amendment with raw, incubated, and composted frass of black soldier fly (BSFF) and yellow mealworm (MWF) on the growth of *B. rapa* plants. In addition, feeding damage inflicted by diamondback moth (*P. xylostella*) larvae, their survival and development and survival, growth and adult eclosion of the cabbage root fly (*D. radicum*) were quantified. Our results show that while both raw BSFF and MWF frass initially resulted in smaller leaf area and fewer leaves, the negative effect of raw MWF disappeared over time, whereas raw BSFF consistently resulted in a smaller leaf area and fewer leaves compared to both MWF and the NoFrass control. Raw BSFF resulted in longer time until flowering compared to MWF and the NoFrass control. Soil amendment with BSFF resulted in a significantly lower survival and biomass of *D. radicum* larvae and pupae respectively, while amendment with MWF frass resulted in a considerably higher *D. radicum* larval survival and biomass than on NoFrass control plants. Interestingly, soil amendment with BSFF resulted in a lower survival of *P. xylostella* larvae compared to the control and MWF. Larval feeding damage on the leaves of *B. rapa* was not significantly affected by frass treatments. Interestingly, when frass was incubated in the soil or composted before being added to the soil, it promoted the growth of *B. rapa*. Notably, the growth inhibition that was previously observed for raw BSFF and MWF had been eliminated by pre-treating the frass.

The plant growth inhibition by raw frass use in our study is consistent with previous studies. For example, maize plant growth trials showed that soil amendment with BSFF resulted in stunted growth, fewer plant leaves, smaller leaf area, and lower N use efficiencies (Alattar et al., 2016; Gärttling et al., 2020). Recently, research on *B. oleracea* grown in soil amended with BSFF revealed a decrease in dry shoot biomass compared to a synthetic fertiliser (Wantulla et al., 2022). The effects of frass vary with plant species, insect species, and with time. Applying

MWF did not increase biomass and nutrient uptake in barley plants. However, when frass was applied with a synthetic N-P-K fertiliser, biomass and nutrient uptake increased (Houben et al., 2020). Moreover, combining BSFF with synthetic fertilisers improved rice plant growth (Reswita et al., 2022; Zim et al., 2022). Lettuce plants grew better in soil amended with BSFF than in soil fertilised with urea or left unamended (Dzepe et al., 2022). Compared to unamended sandy soil, zucchini plants grown in BSFF- and MWF-treated soil were considerably taller, had a bigger leaf area, and had bigger dry leaf weights (Zim et al., 2022).

A probable explanation for the negative effect of raw frass on plant growth in the current study is that the frass used might have contained compounds that interfere with plant growth. Frass quality depends on the larval substrate as well as postharvest processing. Soil amendment with frass in this study might have altered the physical properties of the soil and obstructed root growth. Excess frass in the soil can lead to soil compaction or waterlogging, limiting the availability of oxygen and essential nutrients to plant roots (Liu et al., 2019). Alternatively, frass salinity may have caused inhibitory effects on plant growth. For example, high salt content can disrupt the balance of ions and nutrients in the soil, impairing plant growth (Zhang et al., 2012). It should be noted that the quality of the raw frass used in this study may have been impacted by the extended heat treatment (24 h at 60 °C) compared to the shorter duration of 1 h at 70 °C required by the EU Commission regulation EU 2021/1925, and confirmed by Van Looveren et al.'s (2021) study, which assessed the effects of this heat treatment on BSFF and found that a heat treatment at 70 °C for 1 h successfully eliminated detectable amounts of foodborne pathogens (*Salmonella*, *Clostridium perfringens*, and Enterobacteriaceae). Hence, this heat treatment appears suitable for ensuring the microbiological safety of insect frass as a soil amendment (Van Looveren et al., 2021). The application of raw BSFF resulted in a longer time until flowering compared to MWF and the NoFrass control. However, when the

experiment was repeated under similar conditions, no significant effect of soil treatment on time until flowering was observed. Overall, these findings suggest that using raw BSFF or raw MWF as soil amendments may negatively affect the growth and flowering of *B. rapa* plants, particularly in leaf production. The negative effects observed of raw BSFF or MWF on leaf production of *B. rapa* could be due to a combination of factors related to nutrient composition, pH, soil structure, toxicity, microbial activity and, salinity. Further research and detailed analysis would be needed to pinpoint the exact mechanisms responsible for these observations. However, the effect on time until flowering seems to be more variable. The disparities between the effects of raw frass in the current study and the positive results reported in previous studies illustrate the difficulty in generalising the effect of frass as an organic fertiliser on plant growth performance.

Pests of cruciferous plants, especially brassicas, include *D. radicum* and *P. xylostella*. *D. radicum* larvae feed on plant roots, but *P. xylostella* larvae feed on the leaves, resulting in severe reductions in plant growth and yield (Ahuja et al., 2010). In our study, amending soil with raw BSFF significantly decreased the survival of *D. radicum* larvae and *P. xylostella* larvae. Similarly, soil amendment with raw BSFF resulted in the lowest *D. radicum* pupal biomass, while MWF produced the highest. These findings suggest that the frass application negatively affected *D. radicum* larvae in the soil and *P. xylostella* larvae feeding on the leaves of *B. rapa*. However, it is important to note that the effectiveness of BSFF to control pests may vary depending on the specific properties of both the frass and the soil type used (Wantulla et al., 2023). While the activation of plant defensive responses following frass treatments has been attributed to the presence of eliciting molecules or microorganisms (Poveda, 2021), the particular mechanisms responsible for the lower herbivore performance in soil amendments with raw BSFF in the current study remain to be elucidated to assess their potential to contribute

to pest management in agriculture. Our findings indicate that soil amendment with raw frass did not significantly impact the damage caused by larvae of *P. xylostella* feeding on the leaves of *B. rapa*. It remains to be investigated what the effects are on natural enemies of *P. xylostella*. Intriguingly, amending soil with raw MWF resulted in higher herbivore performance than raw BSFF amendment. We hypothesised that adding frass to the soil would reduce herbivore performance by inducing plant defences against herbivorous insect pests (Barragán-Fonseca et al., 2022; Ray et al., 2015). However, it appears that adding MWF to the soil favoured the survival and biomass accumulation in root-feeding *D. radicum* larvae and provided better and readily available plant nutrition for leaf-feeding *P. xylostella* larvae. For instance, a pot experiment indicated high mineralisation of MWF, particularly at higher application rates (Houben et al., 2021). Moreover, the addition of MWF may have altered the soil microbial community, potentially favouring the growth of microorganisms beneficial to the cabbage root fly larvae (Wantulla et al., 2023). It is also possible that the MWF used in our study had a different chemical and/or microbial composition than other sources of insect frass that have been shown to induce plant defences (Poveda et al., 2019).

Different insect species produce different types and amounts of defensive compounds, so the composition of frass can vary depending on the insect species used (Ray et al., 2016). A greenhouse experiment to measure frass-induced plant defences of maize, rice, cabbage, and tomato plants showed that caterpillar-frass-induced plant defences are specific to each host-herbivore system and can induce herbivore or pathogen defence responses in the host plant depending on the composition of the frass deposited, the plant organ where it is deposited, and the insect species (Poveda, 2021; Ray et al., 2016). However, herbivore performance on maize plants was enhanced due to cues that suppressed herbivore defences (Ray et al., 2015). Overall, our findings indicate that soil amendment with raw BSFF has a detrimental effect on herbivore

performance, while using raw MWF may have a protective effect. The mechanisms that underpin these results and the factors that may have promoted herbivore performance in soil amended with raw MWF need further study. The results of our study align with certain prior reports while contradicting others, as anticipated, because of the differences in the frass origins and quality employed in this study and those reported previously. This discrepancy highlights the need for additional research to broaden our understanding of the potential of frass application for soil enhancement and plant growth promotion.

A fascinating finding from the present study is that the process of incubating and composting raw frass alleviated the inhibition of plant growth. Incubating MWF in the soil before sowing *B. rapa* seeds resulted in a larger plant leaf area than the NoFrass control. Furthermore, composted MWF significantly increased the number of leaves per plant. Frass contains N, P, K, micronutrients, and beneficial microbes. Adding frass to the soil makes these nutrients readily available to the plants, which in turn may improve plant growth (Gärtling & Schulz, 2022; Gebremikael et al., 2022; Houben et al., 2020; Poveda, 2021; Poveda et al., 2019). Organic fertilisers, including animal manure and compost, have been associated with enhanced soil fertility and plant growth (Bashir et al., 2021; Rayne & Aula, 2020), aligning with our findings. Interestingly, the incubation of frass in the soil did not significantly affect the time until flowering of *B. rapa*. This suggests that the effects of frass on plant growth and development may be more pronounced during the vegetative stage of growth than during the reproductive phase.

A limitation of the current study is that while we tested the effects of raw frass on both plant growth and herbivore performance, we tested the effects of incubated and composted frass only on plant growth. This means that we do not completely understand the effects of these different

types of frass on herbivore performance. In future studies, it will be important to include measurements of herbivore performance when testing the effects of different types of frass on plant growth.

This study has shown that BSFF and MWF have potential alternative sources of organic fertiliser for sustainable agriculture. However, using raw BSFF may also have implications for insect herbivore control, as it decreases the performance of *D. radicum* and *P. xylostella* larvae. In contrast, using raw MWF increases these pests' survival. Additionally, the effect of incubating and composting frass on plant growth performance highlights the importance of properly handling and treating frass to maximise its potential benefits. This study indicates that incubating frass in the soil may be more effective before sowing seeds. These findings suggest that an integrated approach, combining the use of frass as a sustainable fertiliser with pest management strategies, may lead to sustainable agricultural practices. Future studies should compare the effects of raw frass, incubated and composted frass on insect herbivores and the mechanisms of action to understand their potential for sustainable herbivore control.

### **Author Contribution**

The authors collectively developed the research question and experiments, SYC executed the experiments, analysed the data and wrote the manuscript with input from JJA<sub>v</sub>L and MD.

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634 this study.

635

#### 636 **Data availability statement**

637 The data that supports the findings of this study are available from the corresponding author  
638 upon reasonable request.

639

#### 640 **Declaration of conflict of interest**

641 The authors declare that they do not have a conflict of interest.

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**Supplementary material**

**Effects of frass from larvae of black soldier fly (*Hermetia illucens*) and yellow mealworm (*Tenebrio molitor*) on growth and insect resistance in field mustard (*Brassica rapa*): differences between insect species and frass treatments.**

Shaphan Y. Chia, Joop J.A. van Loon and Marcel Dicke

Laboratory of Entomology, Wageningen University & Research, P.O. Box 16, 6700 AA Wageningen, the Netherlands.



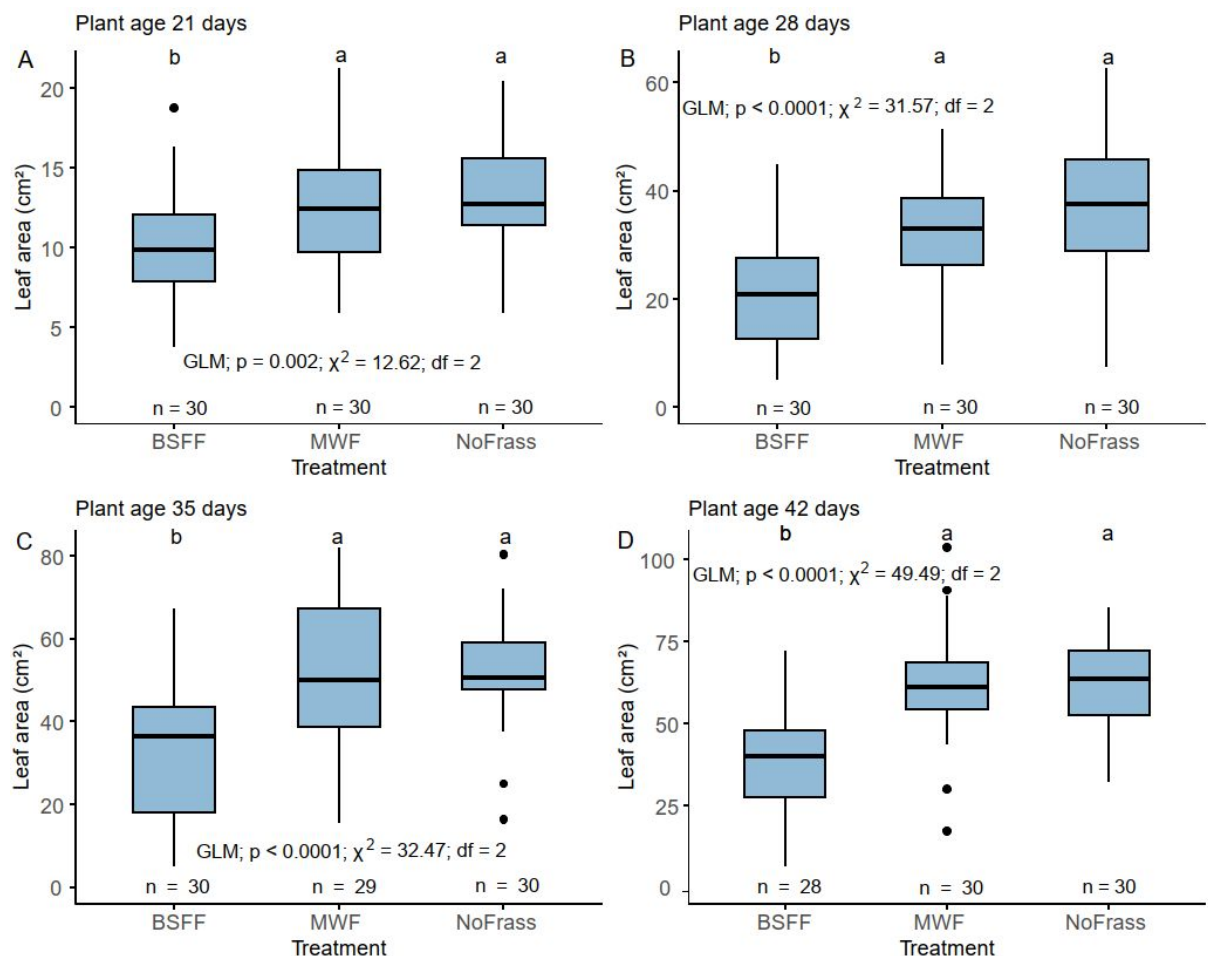
846 **Table S1.** Summary of *Brassica rapa* seed germination for the four trials.

| Trial | Seeds<br>sown | Seeds<br>germinated | * % Seed<br>germination | Time<br>(days) | Seed<br>treatment | Germination method              |
|-------|---------------|---------------------|-------------------------|----------------|-------------------|---------------------------------|
| 1     | 120           | 114                 | 95.0                    | 1-3            | stratified        | germinated in unamended soil    |
| 2     | 160           | 146                 | 91.3                    | 1-3            | stratified        | germinated in unamended soil    |
| 3     | 36            | 32                  | 88.9                    | 1-3            | stratified        | Sown directly into amended soil |
| 4     | 51            | 45                  | 88.2                    | 1-3            | stratified        | Sown directly into amended soil |

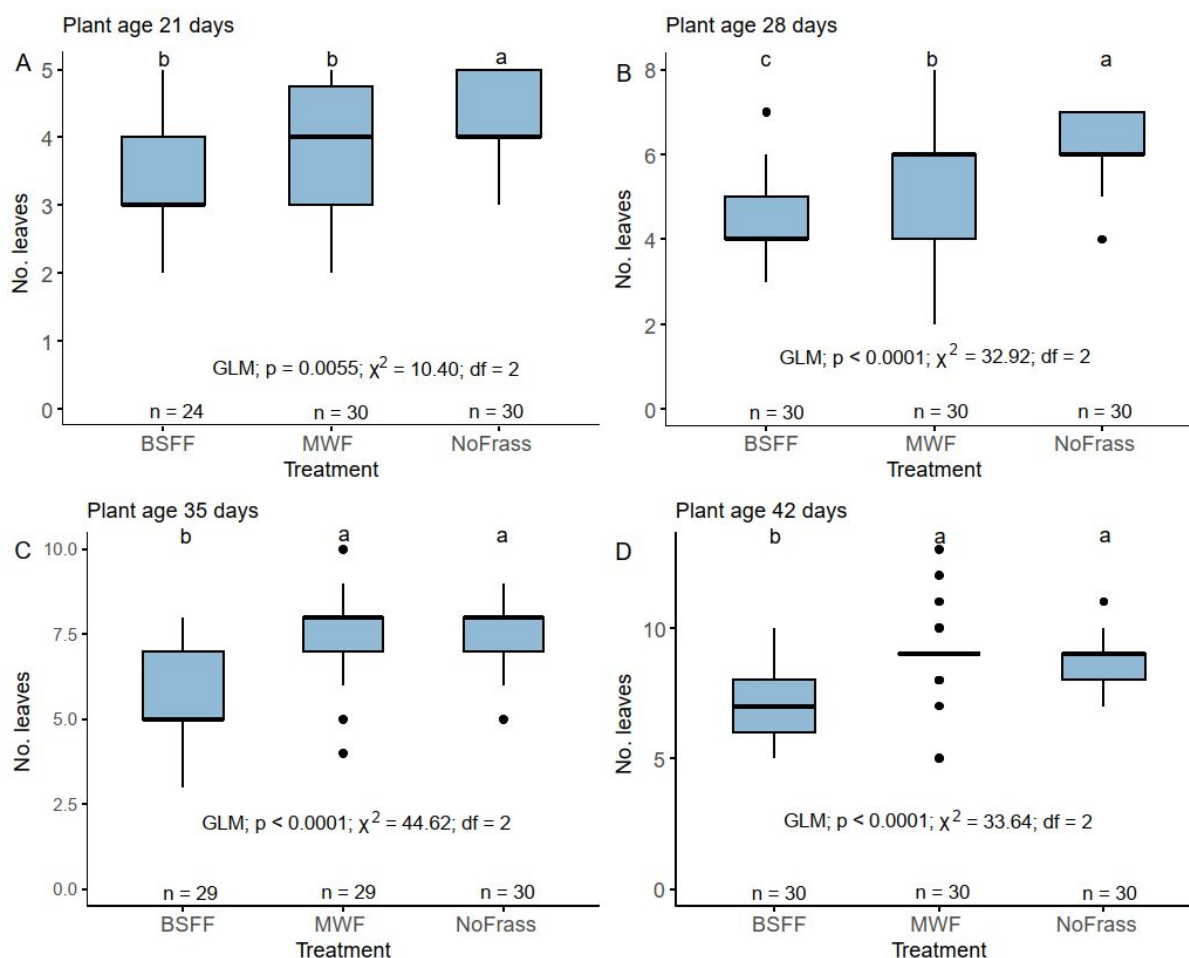
847 Seeds were stratified by maintaining them in moist filter papers in Petri dishes at 4 °C for seven days. In trials 1  
 848 and 2, seedlings were transplanted into raw frass (no incubation or composting) soil after germination; Trial 3:  
 849 frass incubated in the soil before seeds were sown; Trial 4: frass samples were composted, air-dried, and pulverised  
 850 before being added to the soil. Data were analysed using the Chi-squared test. (\*) There was no significant  
 851 difference in the proportion of germinated seeds ( $p < 0.05$ ).

852

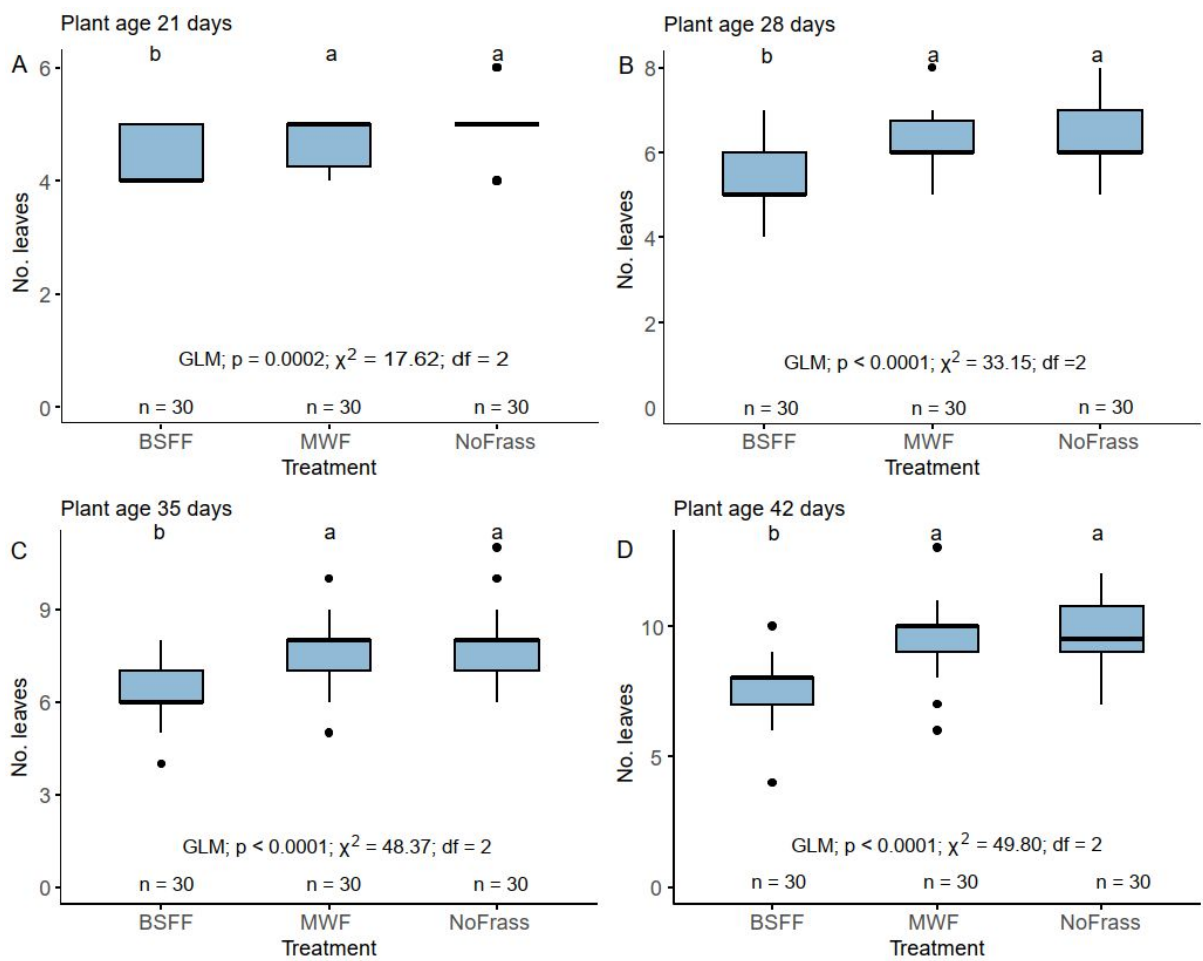
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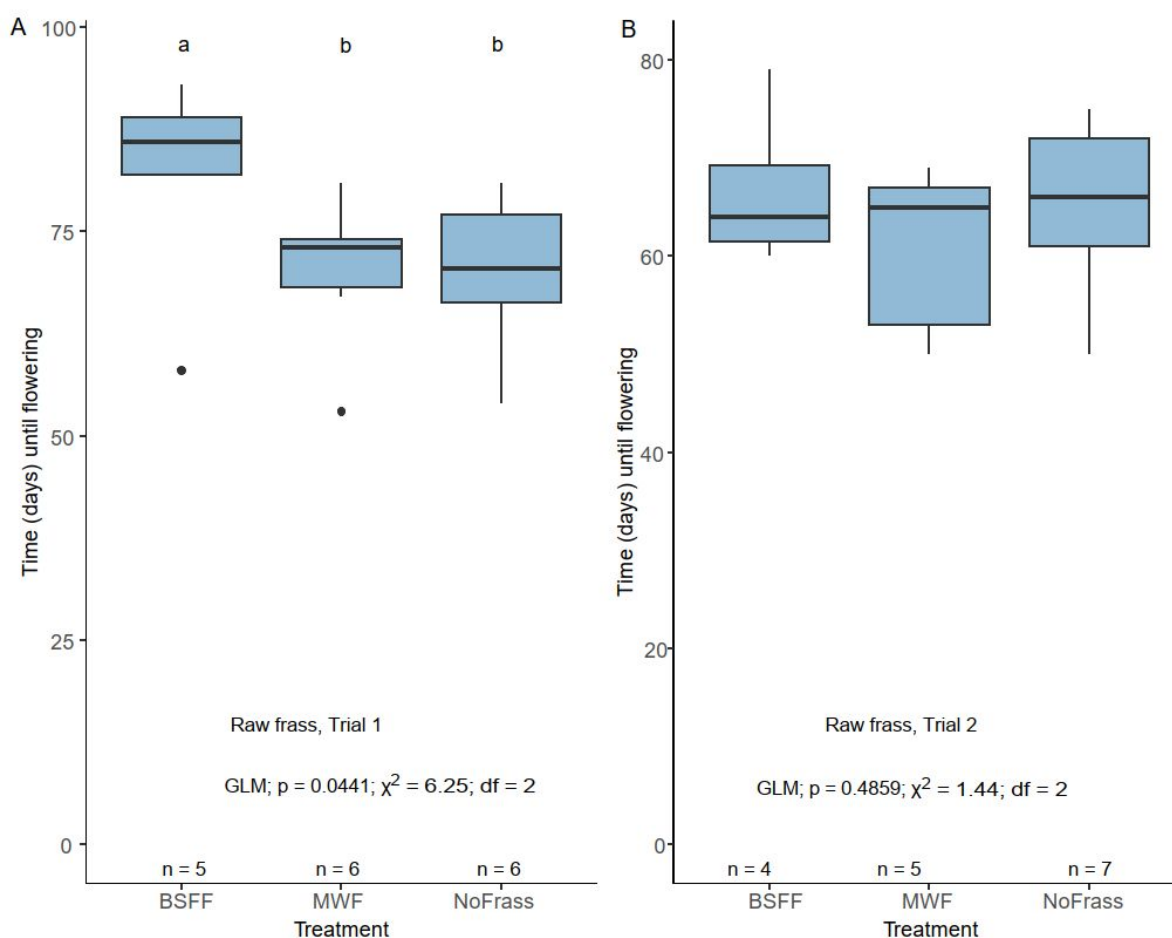
**Figure S1.** Leaf area (cm<sup>2</sup>) of *B. rapa* plants grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF), or raw yellow mealworm frass (MWF) recorded in Trial 2 at ages 21 days (A), 28 days (B), 35 days (C) and 42 days (D). The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants for leaf area measurements. Boxes with different letters differ significantly (Tukey's post hoc test,  $p < 0.05$ ).



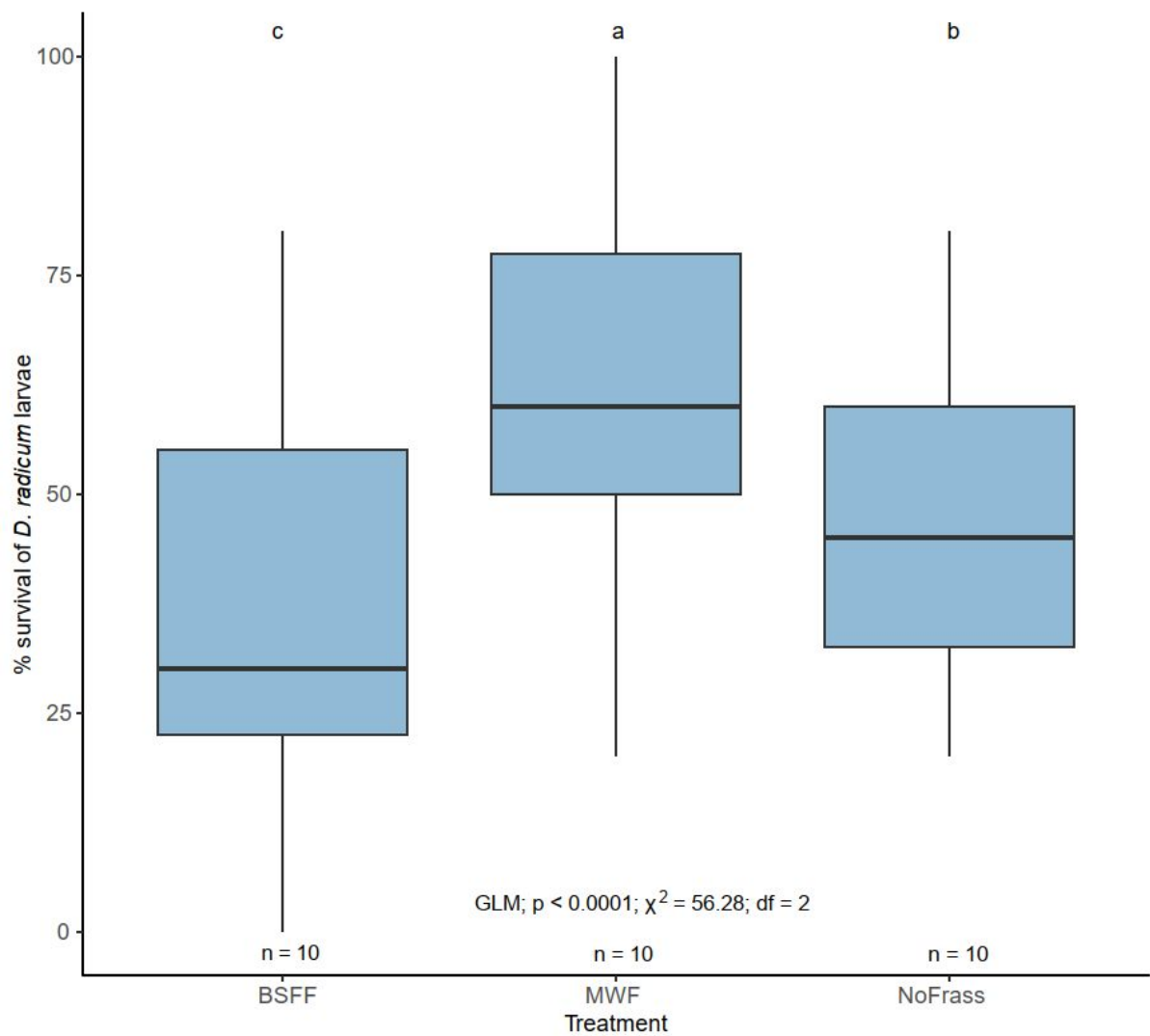
**Figure S2.** Number of leaves of *B. rapa* plants grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF) recorded in Trial 1 at ages 21 days (A), 28 days (B), 35 days (C) and 42 days (D). The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed by generalised linear models (GLM). n is the number of replicate plants for leaf counts. Boxes with different letters differ significantly (Tukey's post hoc test,  $p < 0.05$ ).



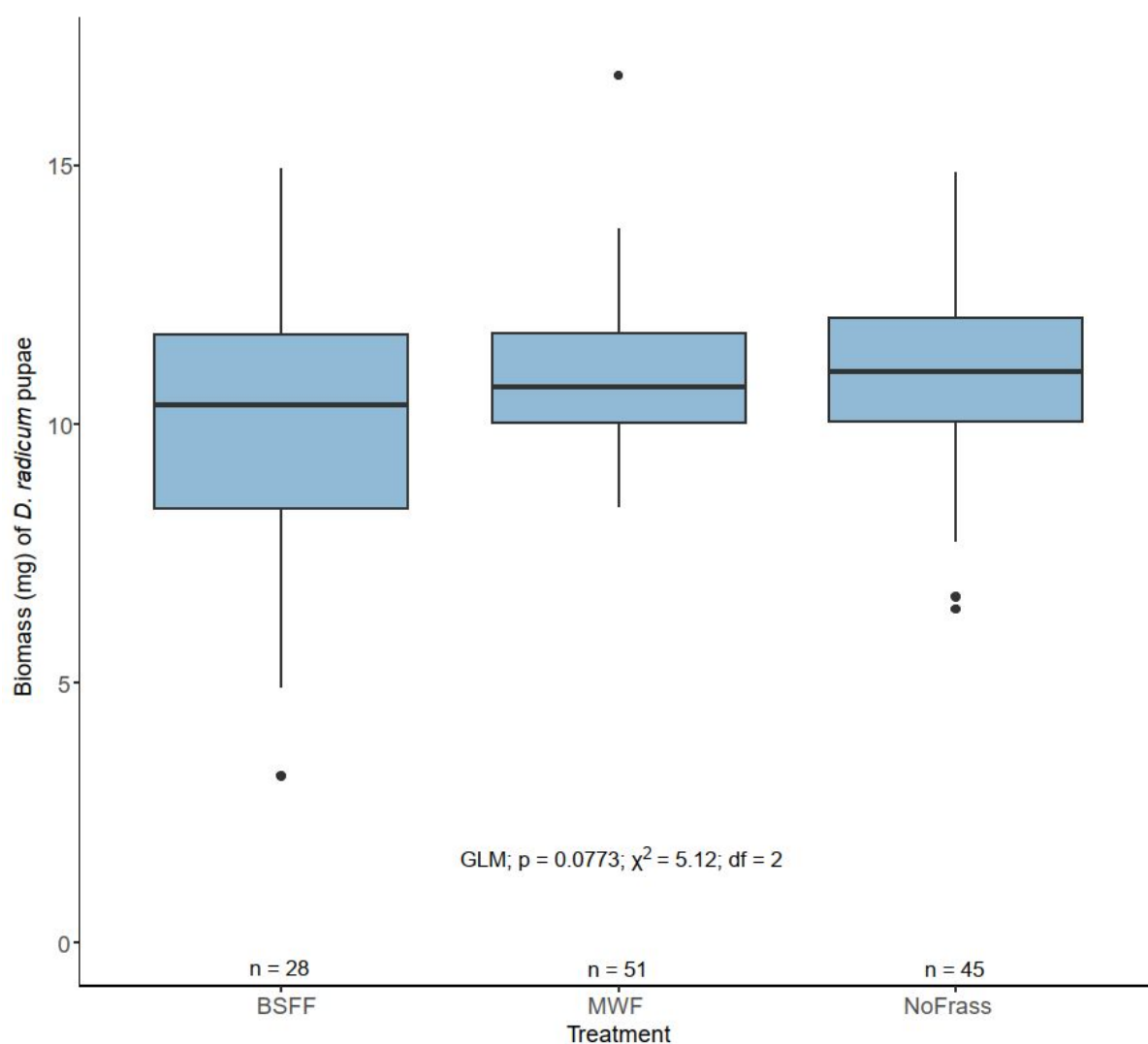
**Figure S3.** Number of leaves of *B. rapa* plants grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF), or raw yellow mealworm frass (MWF) recorded in Trial 2 at ages 21 days (A), 28 days (B), 35 days (C) and 42 days (D). The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed by generalised linear models (GLM). n is the number of replicate plants for leaf counts. Boxes with different letters differ significantly (Tukey's post hoc test,  $p < 0.05$ ).



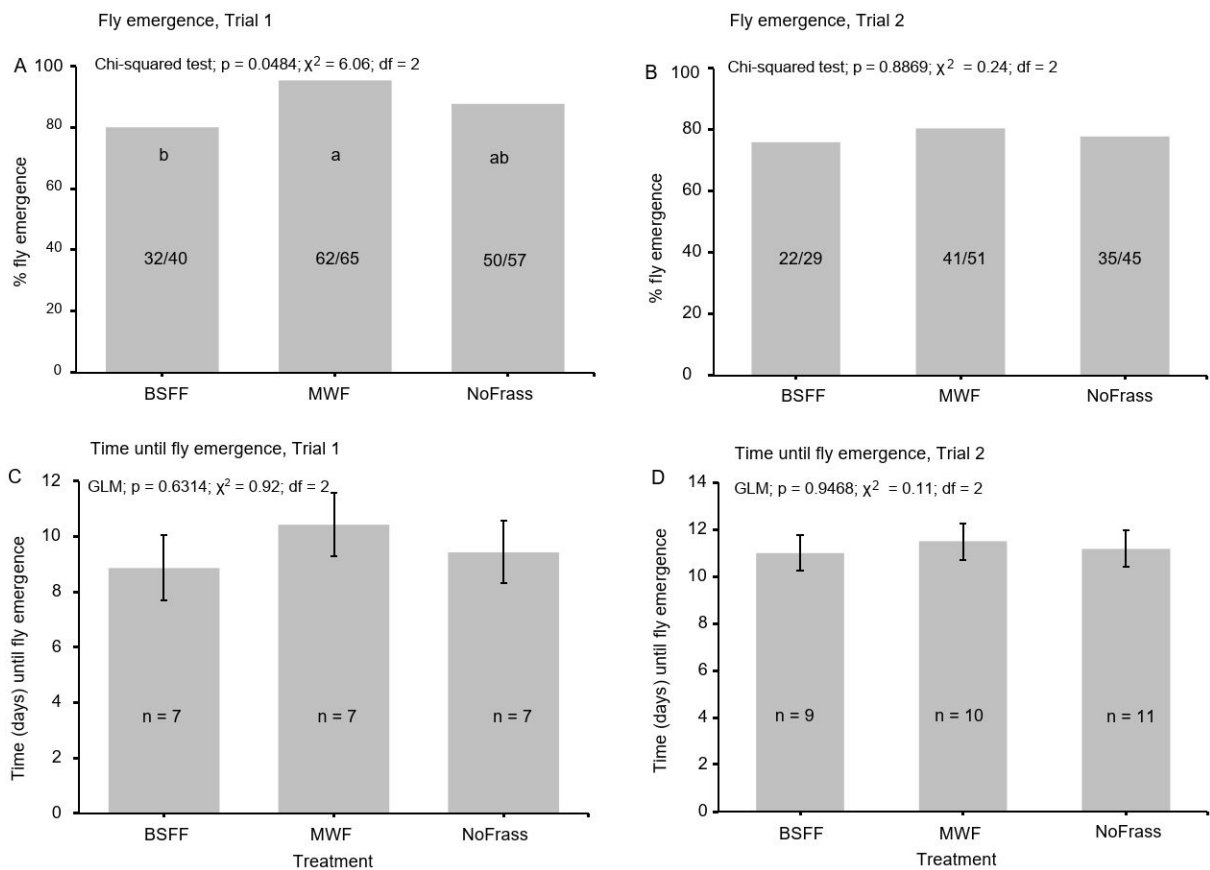
**Figure S4.** Time (days) until flowering of *B. rapa* grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF) in two trials. A = trial 1 and B = trial 2. B is a repetition of A under similar conditions. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants on which time until flowering was recorded. Boxes with different letters differ significantly (Fisher's Least Significant Difference post hoc test,  $p < 0.05$ ).



**Figure S5.** Survival of *D. radicum* larvae on roots of *B. rapa* plants grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF). The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. Data were analysed by generalised linear models (GLM).  $n$  is the number of replicate plants that had each been infested with 10 larvae. Boxes with different letters differ significantly (Tukey's post hoc test,  $p < 0.05$ ).

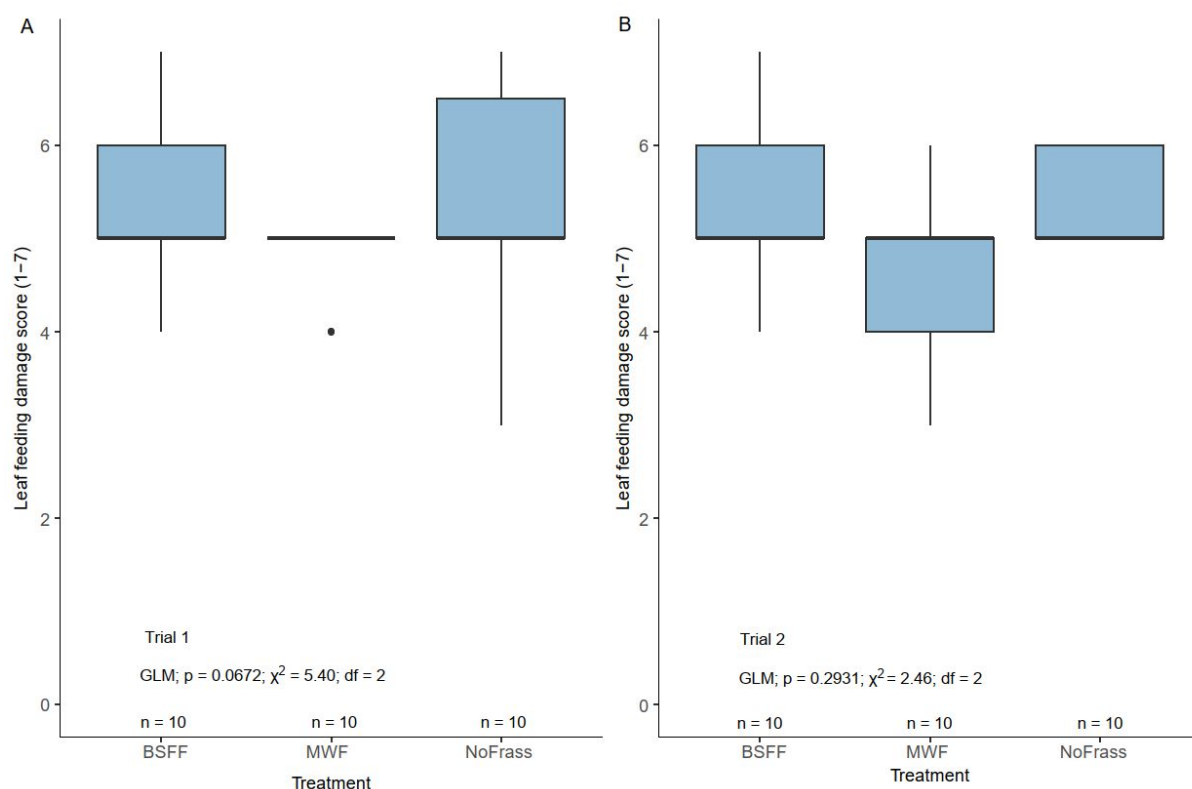


**Figure S6.** Biomass (mg) of *D. radicum* pupae retrieved after a 21-day root infestation of *B. rapa* grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF). The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with generalised linear models (GLM). n is the number of pupae weighed per treatment.

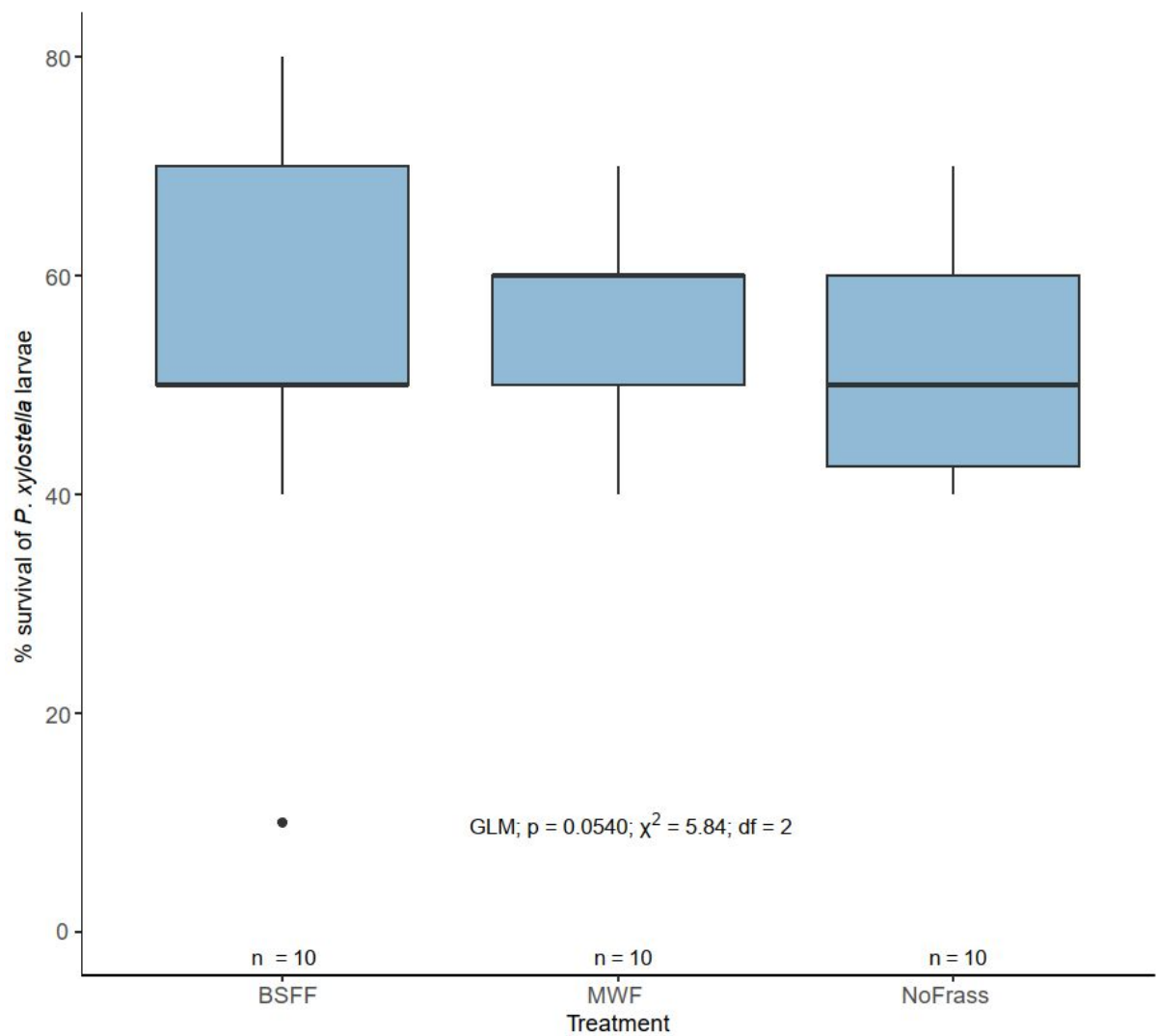


**Figure S7.** Emergence of *D. radicum* adult flies after pupae were retrieved from the roots of *B. rapa* grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF). A = proportion (%) of flies that emerged during the first trial (trial 1), B = proportion (%) of flies that emerged during the second trial (trial 2), C = time (mean  $\pm$  S.E) until fly emergence during trial 1, and D = time (mean  $\pm$  S.E) until fly emergence during trial 2. Data on the proportion of flies that emerged were analysed with the chi-squared test equality of proportions. The fractions (32/40, 62/65, 50/57, 22/29, 41/51 and 35/45) on the graph show the proportion of flies that emerged (numerator) out of the number of pupae (denominator). Data on time until fly emergence were analysed with a generalised linear model (GLM). n is the number of recorded instances of fly emergence. Error bars represent standard errors of the average time until emergence. Bars with different letters are significantly different following the Marascuilo procedure as a post hoc test (the absolute pairwise difference between proportions is statistically significant if its value exceeds the critical range value). Graphs without error bars represent single measurements (proportions).

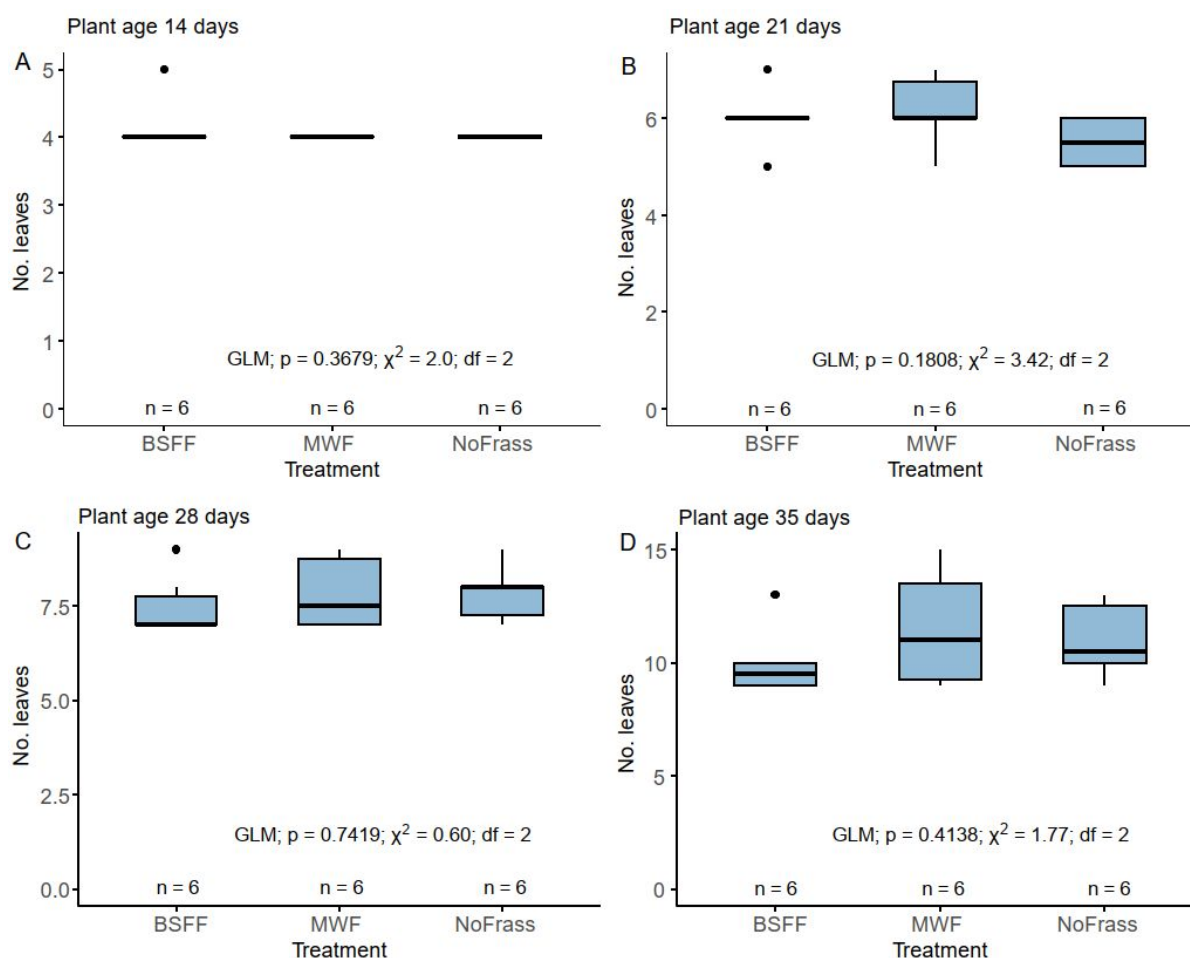




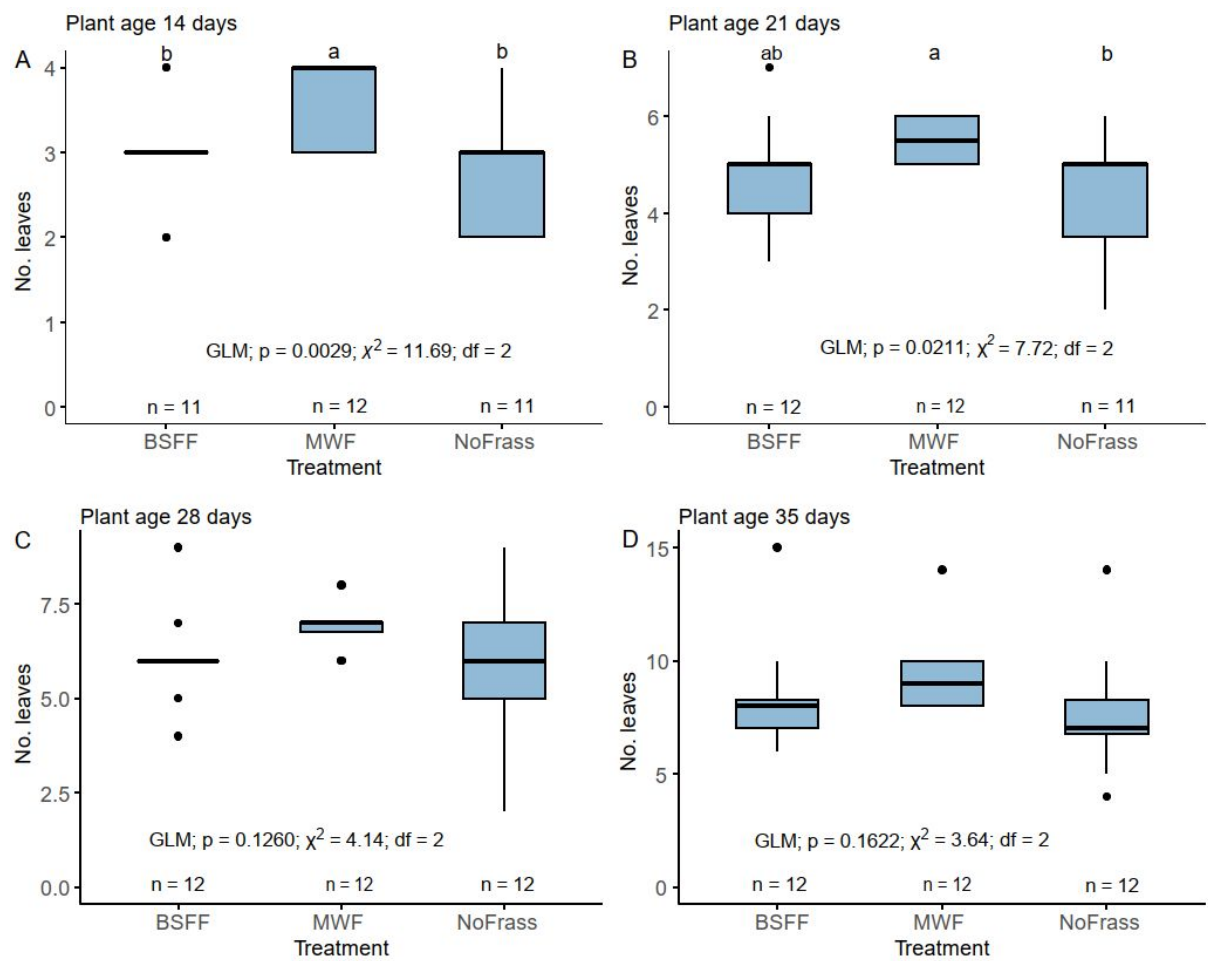
**Figure S8.** Feeding damage (scores) by larvae of *P. xylostella* on *B. rapa* grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF) in two trials. A = Trial 1 and B = Trial 2. B is a repetition of A under similar conditions. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dot beyond the whiskers represents an outlier. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants for leaf damage assessment.



**Figure S9.** Survival of *P. xylostella* larvae on *B. rapa* grown in unamended soil (NoFrass; control) or soil amended with raw BSF frass (BSFF) or raw yellow mealworm frass (MWF) in Trial 2. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dot beyond the whisker represents an outlier. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants that were each infested with ten larvae.

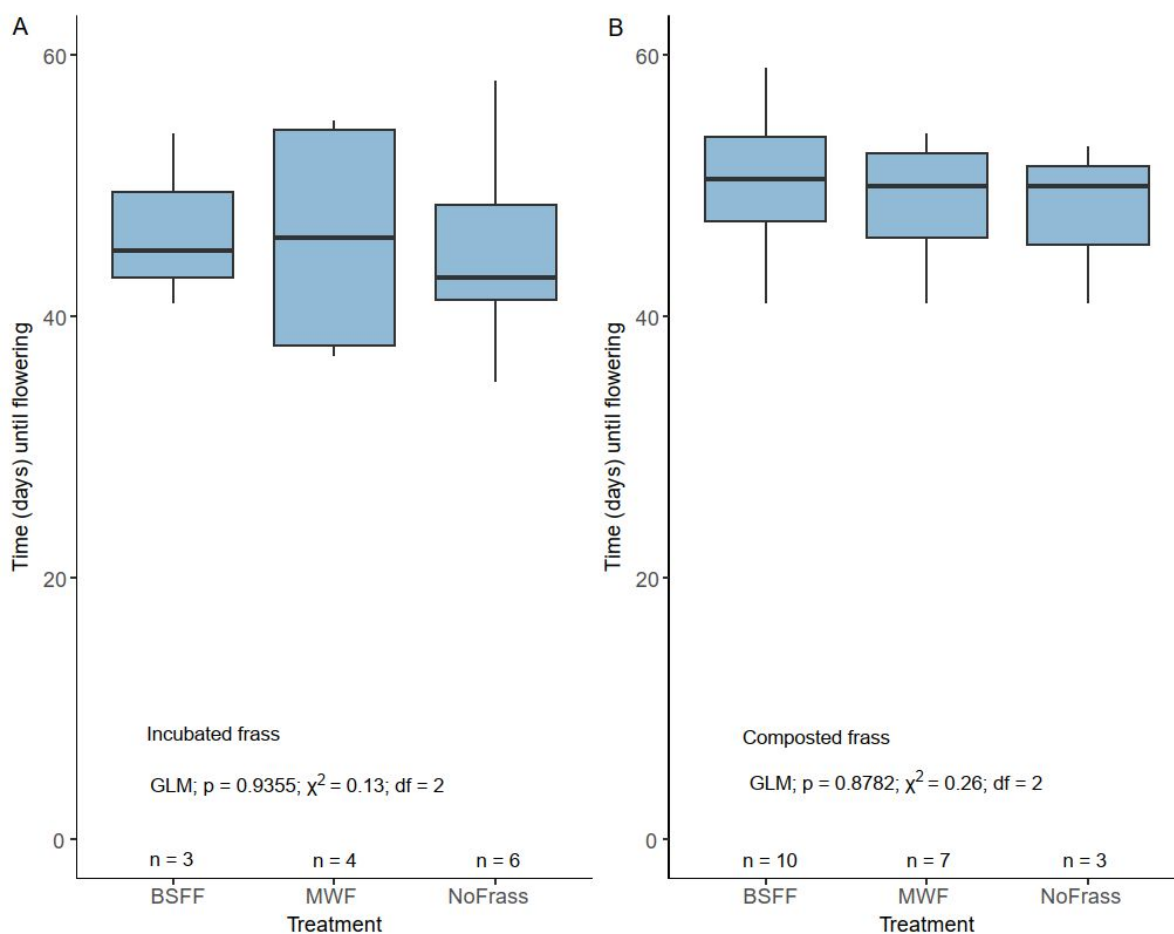


**Figure S10.** Number of leaves of *B. rapa* grown in unamended soil (NoFrass; control) or soil amended with BSF frass (BSFF) or yellow mealworm frass (MWF) after incubating. Leaves were counted at plant ages 14 days (A), 21 days (B), 28 days (C) and 35 days (D). Incubation involved frass mixed with soil in 0.5 L plastic pots and moistened, and seeds were only sown after sixteen days under greenhouse conditions. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants for leaf counts.



**Figure S11.** Number of leaves of *B. rapa* grown in unamended soil (NoFrass; control) or soil amended with BSF frass (BSFF) or yellow mealworm frass (MWF) after composting. Leaves were counted at plant ages 14 days (A), 21 days (B), 28 days (C) and 35 days (D). Frass samples were composted for 38 days in plastic boxes and air-dried. The resulting compost was pulverised and added to the soil. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. The dots beyond the whiskers represent outliers. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants for leaf counts.

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**Figure S12.** Time until flowering (days) of *B. rapa* grown soil (NoFrass; control) or soil amended with BSF frass (BSFF) or yellow mealworm frass (MWF). A = frass was incubated in the soil for 16 days before seeds were sown and B = frass was composted for 38 days in plastic boxes and air-dried. The resulting compost was pulverised and added to the soil. The box represents the interquartile range (IQR), with the bottom and top edges corresponding to the first quartile (Q1, 25%) and third quartile (Q3, 75%), respectively. The line within the box represents the median, while the whiskers extend to 1.5 times the IQR, encompassing the minimum (Q1-1.5IQR) and maximum (Q3+1.5IQR) values. Data were analysed with a generalised linear model (GLM). n is the number of replicate plants observed for time until flowering.

To: The Editor-in-Chief,

Date: 25 September 2023

Entomologia Experimentalis et Applicata

Subject: Revised manuscript submission

Dear Professor Beukeboom, dear Leo

We are pleased to submit the revised version of our manuscript titled "Effects of frass from black soldier fly (*Hermetia illucens*) larvae and yellow mealworms (*Tenebrio molitor*) on growth and resistance to insect herbivores of field mustard (*Brassica rapa*): differences between insect species and frass treatments, following a decision of "minor revision".

We appreciate the insightful evaluation provided by the reviewers, and we are encouraged by their positive feedback on our work, acknowledging our strong grasp of the research topic and the uniqueness of our idea. We have carefully addressed the reviewers' comments to improve the manuscript's readability and align it with scientific writing and journal standards.

Below, please find our detailed responses to the reviewers' comments and additional comments by the Editor.

We hope that with this revision the manuscript meets the publication standard in Entomologia Experimentalis et Applicata.

Thank you for your consideration!

Sincerely,

Shaphan Chia, Joop van Loon and Marcel Dicke

## Response to Reviewer 1 comments

### GENERAL ASPECTS:

#### Reviewer comment:

- Both the Introduction and the Discussion manifest an excellent domain of the research topic.
- A very interesting, novel and original document. Congratulations for the great work done.

#### Author response:

Thank you very much for your kind words and positive feedback. We greatly appreciate your assessment of our work.

### KEYWORDS:

#### Reviewer comment:

- Reduce the number of keywords by half. Now it is excessive.

#### Author response:

We have reduced the number of keywords as advised.

### ABSTRACT:

#### Reviewer comment:

- Very well explained and complete.

#### Author response:

Thank you

### INTRODUCTION:

#### Reviewer comment:

- L61: why are only bacteria considered and not also fungi?

#### Author response:

We have added information about fungal stimulation through frass application

#### Reviewer comment:

- L64-73: Rewriting information. It's confusing right now.

#### Author response:

We have simplified the text by breaking down complex sentences and providing a clear flow of information. The revised text emphasizes the relationship between frass, soil, beneficial microorganisms, and plant resistance to insect herbivores.

#### Reviewer comment:

- L74-75: order cites conologically.

#### Author response:

The current format adheres to alphabetical ordering of authors' names, which is consistent with the manuscript's style throughout.

#### Reviewer comment:

- L94-96: add updated production data according to FAOSTAT.

#### Author response:

We have updated the production data for field mustard in the paragraph, using the latest available information.

#### Reviewer comment:

- L98-99: repeated citation.

#### Author response:

We have removed the repeated citation on lines 98-99 as suggested.

### MATERIALS AND METHODS:

#### Reviewer comment:

- L139, 149, 150, 170, 171, 179, 180, 186, 187, 188 y 244: The full scientific name has already been written above. Reduce.

**Author response:**

We have abbreviated the scientific names consistently throughout the manuscript to reduce redundancy and align with best practices in scientific writing.

**Reviewer comment:**

- L142-143: Is the chemical analysis of the soil known? The nutritional one would be very interesting and could be added to the paper as supplementary material.

**Author response:**

We appreciate your observation and suggestion. We have cited a previous study for the soil's physical and chemical properties in our revised manuscript.

**RESULTS:**

**Reviewer comment:**

- L332, 366, 384, 393, 406, 411, 418, 427 and inside the figures: The full scientific name has already been written above. Reduce.

**Author response:**

We have removed the redundancy by omitting the full scientific names in the specified locations, as they were previously mentioned above.

**Reviewer comment:**

- Throughout the section, it is not considered necessary to put so much information from the statistical analysis in the text. However, it is very positive to put information of mean data between different treatments, which help the fluent understanding of the text and the study.

**Author response:**

We have reduced the statistical information in the text and, emphasized data visualization through figures. This should help streamline the text and enhance the reader's understanding of the study.

**Reviewer comment:**

- L468-469: spacing problem.

**Author response:**

Thank you for your observation. We have corrected the spacing problem.

**DISCUSSION:**

**Reviewer comment:**

- L538-539: The full scientific name has already been written above. Reduce.

**Author response:**

We have abbreviated the full scientific name for conciseness and clarity.

**Reviewer comment:**

- L526-527: scientific names in italics.

**Author response:**

Done. Revised text (L526-527): *Clostridium perfringens*



## Response to Reviewer 2 comments

**Reviewer comment:**

Despite the amount of work that has been done already in the case of insect frass, the overall idea is unique and the methodological approach very interesting.

The ms can be accepted for publication, but there are some parts that need to be revised.

**Author response:**

Thank you for your positive feedback and enthusiasm about our manuscript. We have carefully reviewed your comments and have made the necessary revisions to address the identified issues.

**Reviewer comment:**

97-8 and elsewhere. Use authorities in full in the first time that a scientific name appears on the ms.

**Author response:**

We have now used authorities in full for all scientific names throughout the manuscript at the first instance a scientific name was used.

**Reviewer comment:**

109. There are additional data gaps that are not addressed by Wantulla et al. (2022), which can be illustrated here.

**Author response:**

We acknowledge the presence of additional knowledge gaps beyond the scope of Wantulla et al. (2022) that we have identified and addressed in our study. These gaps are further elucidated in the manuscript.

**Reviewer comment:**

133. Expand this general scope with more text.

**Author response:**

We appreciate the suggestion for expansion, but we would like to request clarification regarding the specific section or content the reviewer is referring to. Please provide additional details so that we can address this comment effectively.

**Reviewer comment:**

Not clear how many replicates and subreplicates were used, clarify.

**Author response:**

It is not clear what section the reviewer refers to. Yet, in general, we used ten plants per treatment and control to assess the effect of raw-frass-exposed plants on the performance of *Plutella xylostella* larvae. This is mentioned in the Methods section. Number of replicates are indicated in the figures as well.

**Reviewer comment:**

524. This is the standard procedure of the authorization of frass, regardless of the overall effects of the thermal treatments. This can be further clarified.

**Author response:**

Our samples underwent extended oven drying, potentially impacting the population of beneficial microbes, which differs from the standard procedure for authorizing frass.

**Reviewer comment:**

531-3. Why- perhaps due to their increased conductance?

**Author response:**

We appreciate the reviewer's question. We have expanded the discussion to address the potential reasons behind the negative effects observed in *B. rapa* when exposed to raw BSFF or MWF. These effects are likely due to a combination of factors, including the salinity of the frass, which we have discussed in the revised manuscript.

**Reviewer comment:**

552. Merge with the next paragraph.

**Author response:**

We have merged lines 552-554 with the following paragraph as suggested.

**Reviewer comment:**

607. No need to have a separate conclusion section, use this text as the closing paragraph in the discussion.

**Author response:**

As recommended, we have eliminated the heading 'Conclusion' and integrated the text as the closing paragraph in the discussion section.

## Response to additional comments by the Editor

**Reviewer comment:**

I will add a few comments to those of the reviewers:

**Author response:**

Thank you for your additional comments on our manuscript. We appreciate your feedback and have addressed these new comments along with the ones provided by the two reviewers in our revised manuscript.

**Reviewer comment:**

Line 44, use of the word "protective" here creates ambiguity Protective might imply to some readers that plants were protected from herbivores under this treatment, which was not the case.

**Author response:**

We appreciate your observation. We have now revised the sentence to ensure that readers do not misinterpret the intended meaning of our statement. Specifically, we have eliminated the word "protective" to accurately convey that the herbivores exhibited enhanced performance under this treatment.

**Reviewer comment:**

Line 65, move the definition of PGPR to line 61, after first mention? I think this would make the paragraph flow better.

**Author response:**

Thank you for your suggestion. We have moved the definition of PGPR to enhance the flow of the paragraph.

**Reviewer comment:**

Line 122, delete comma.

**Author response:**

Thank you for your suggestion. We have deleted the comma as suggested.

**Reviewer comment:**

Lines 142-143, it would be extremely helpful to know something about the soil (e.g., pH, organic content, N, P, K levels, soil type) used in the experiments.

**Author response:**

We appreciate your observation and suggestion. We have cited a previous study for the soil's physical and chemical properties in our revised manuscript.

**Reviewer comment:**

Line 202, : "after" instead of "since"; insert "and" after the comma.

**Author response:**

We have replaced the word “since” with “after”, as suggested.

**Reviewer comment:**

Line 211, delete comma.

**Author response:**

We have deleted the comma as suggested.

**Reviewer comment:**

Figure 2, in the third and fourth panels, the letters designating the Tukey results appear to be in error – shouldn't BSFF be accompanied by a “b” and both “NoFrass” and “MWF” have “a”s?.

**Author response:**

You are right. We have corrected this error, ensuring that BSFF is now accompanied by a “b,” while both “NoFrass” and “MWF” have “a”s. Thank you for pointing this out. The conclusion remains unchanged.

**Reviewer comment:**

Line 382, were patterns similar in second experiment.

**Author response:**

Yes, the patterns were similar. In the revised sentence, we clarified that there were no significant differences among treatments.

**Reviewer comment:**

Line 416, were patterns similar in the two experiments?

**Author response:**

Yes, the patterns were similar. In the revised sentence, we have now clarified that there were no significant differences among treatments.

**Reviewer comment:**

Lines 514-515 – again, any information about the base soil used in the experiments would be helpful – also, 2 g frass per kg of soil does not seem to be an inordinate amount to me – how feasible is it that the frass led to soil compaction or waterlogging?

**Author response:**

We acknowledge that 2 g frass per kg of soil may not seem excessive. While we agree that it is unlikely to cause soil compaction or waterlogging, we cannot rule out these possibilities, as they are mentioned in the literature, although we did not measure them specifically in our study.

**Reviewer comment:**

Line 525, when what was “introduced to the frass”? Unclear.

**Author response:**

We apologize for the confusion. In the revised manuscript, we have clarified the paragraph to specify that heat treatment eliminated detectable amounts of pathogens in frass.

**Reviewer comment:**

Line 533, italicize species name.

**Author response:**

We have italicised the species name as suggested.