Biocatalysis and Agricultural Biotechnology 53 (2023) 102862

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Frass from yellow mealworm (*Tenebrio molitor*) as plant fertilizer and defense priming agent

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ARTICLE INFO

Handling Editor: Dr. Ching Hou

Keywords:
Tomato
Sunflower
Arabidopsis thaliana
Callose
Botrytis cinerea
Insect frass

ABSTRACT

World population growth requires the development of a sustainable agriculture that allows feeding all the inhabitants of the planet, while reducing the use of agrochemicals. Currently, the insect farming industry for food and feed production is developing exponentially throughout the world; also producing insect frass with a potential utilization within agriculture and greenhouse industry. In the case of the yellow mealworm (*Tenebrio molitor*), few studies have been developed so far on the use of frass as a fertilizer, and there are none on its ability to activate plant defenses. By applying 2% sterilized mealworm frass to tomato we found a significant increase in its growth, demonstrating that the microbiota might not play a key role in its fertilizing capacity. In addition, the application of frass to sunflowers under different situations of nutritional deficit allowed us to determine what specific nutrients this fertilizer may be providing to the plant, finding a possible deficiency in the supply of N, but with sufficient amounts of P, K, Ca, and S. With respect to the induction of defenses, mealworm insect frass did not induce local root defenses in a root callose deposition assay in *Arabidopsis thaliana* under our experimental conditions. However, it activated systemic defenses in *Arabidopsis thaliana* by inducing defense genes in the absence of pathogen, further enhanced by infection with the necrotrophic fungus *Botrytis cinerea*. Therefore, mealworm frass could be a good fertilizer resource and plant defense inducer to support development of sustainable agriculture.

1. Introduction

The world population is predicted to increase to an estimated 8.1 billion people by 2025 and 9.6 billion by 2050. In order to feed this population, it has been estimated that agricultural productivity should increase 70% by 2050 (Tripathi et al., 2019). Achieving this agricultural production goal requires major changes in the global agricultural system. Primarily, by basing agriculture on a sustainable system, and actively addressing the causes and consequences of climate change (Calicioglu et al., 2019). Currently, global agriculture is mainly based on the use of chemical fertilizers and pesticides (Calabi-Floody et al., 2018; Sharma et al., 2020). Both

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https://doi.org/10.1016/j.bcab.2023.102862
Received 10 December 2022; Received in revised form 22 July 2023; Accepted 19 September 2023
Available online 20 September 2023

strategies pose serious environmental and health hazards due to the severe pollution and toxicity problems they cause (Srivastav, 2020). In the case of chemical fertilizers, polluting chemicals and greenhouse gases are released in their manufacture, and also cause eutrophication of waters, or degradation of soils (Kumar et al., 2019). Chemical pesticides directly affect human health, such as immunotoxicity, respiratory disorders, reproductive impacts, hormone disruption, carcinogenicity, etc. (Rana et al., 2019). Therefore, new strategies should be developed to achieve a more sustainable agriculture.

Organic fertilizers, such as manure or compost, can be a good alternative to chemical fertilizers. They provide nutrients to plants in smaller quantities, but act over a long period of time, unlike chemical fertilizers, which are water-soluble and immediately available to the plants. Organic fertilizers present important advantages, such as soil improvement (microbiological, physico-chemical, and biochemical), organic matter supply, increase of available nutrients in the soil, or less environmental damage (Shaji et al., 2021).

As an alternative to chemical pesticides, the use of elicitors of defensive responses in crops have been proposed (Meena et al., 2022; Poveda and Díez-Méndez, 2022). When a plant is attacked by a pathogen or herbivore, its cell receptors recognize chemical components of its enemies, called pathogen-associated molecular patterns (PAMPs) and herbivore-associated molecular patterns (HAMPs). They can also recognize specific plant components released by the attack, so-called damage-associated molecular patterns (DAMPs). When the plant recognizes these molecular patterns, it activates its defenses in a specific way, through the so-called pattern-triggered immunity (PTI) (Meena et al., 2022; Poveda and Díez-Méndez, 2022). In addition to a local defensive response, PTI leads to the activation of a systemic defensive response involving the defense-related hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Poveda, 2020). Currently, different elicitors of natural origin, mainly secondary metabolites (β-aminobutyric acid, allicin, naringin or terpenes) and carbohydrate polymers (laminarin, carrageenans, chitin or chitosan), are in use in agriculture (Jamiołkowska, 2020; Zheng et al., 2020; Meena et al., 2022).

As a response to the abovementioned challenges with population growth and the increased need for sustainable food sources, insects have been proposed as part of the solution. Insects present a sustainable and healthy alternative to conventional meat products, while their ability to eat food waste can be utilized for recycling organic side streams (Van Huis, 2013). This has led to a rapid growth of the global insect farming industry in recent years. An abundant by-product of this industry is the insect feces, known as frass.

Recently, the use of insect frass as an agricultural resource has been gaining more and more interest (Poveda, 2021; Barragán-Fonseca et al., 2022). Insect frass can be used as a fertilizer, and as a phytofortifier, as it might be able to improve tolerance to abiotic stresses and resistance against biotic stresses in the plant. This is due to a direct contribution of nutrients to the soil and plants, together with biomolecules of interest (such as chitin), and beneficial microorganisms for crops (plant growth promoters, antagonists) (Poveda, 2021). Frass usually contains significant amount of insect exoskeleton fragments, which in turn contain the well-known plant and soil stimulant chitin (Chavez and Uchanski, 2021; Barragán-Fonseca et al., 2022). Chitin also exists in the peritrophic membrane in the insect gut, which is excreted together with the feces (Fescemeyer et al., 2013). Chitin can stimulate the plant immune system, but only when present in shorter oligomers (6-8mer) (Li et al., 2020). The chitin found in the insect exoskeleton must likely be degraded (e.g. by soil microorganisms) before it can have an immune stimulating effect. Addition of mealworm exuviae to soil has been shown to stimulate growth of chitinolytic bacteria, leading to degradation of chitin, and plant growth promoting rhizobacteria (PGPR) (Bai, 2015). Barragán-Fonseca et al. proposed that insect frass and exuviae can benefit plant resistance in various ways, such as stimulation of beneficial microorganisms leading to induced systemic resistance (ISR), or chitin induced immune responses (Barragán-Fonseca et al., 2022).

Tenebrio molitor L. (Coleoptera: Tenebrionidae), or yellow mealworm, is one of the largest stored-product beetles in agricultural products, mainly in grains, flour and bran (Rumbos et al., 2020). Due to its cycle and nutritional content, *T. molitor* has been proposed as one of the most appropriate insect species for the development of large insect farming industries (Arévalo et al., 2022). Frass is an important by-product of insect production for feed and food. Specifically, to produce 100 g of mealworm-biomass, 200–300 g of frass-biomass are produced (He et al., 2021). One proposed use for this by-product is as an agricultural resource (Poveda, 2021).

So far, not much work has been done on the application of mealworm frass on plants. As a fertilizer, it has been shown that it provides nutrients such as N, P and K and promotes the growth of chard (Poveda et al., 2019), barley (Houben et al., 2020), ryegrass (Houben et al., 2021), and zucchini (Zim et al., 2022). It has also been proposed that plant growth promotion could be related to a stimulation of soil microbiological activity (Houben et al., 2020). Plant-beneficial microorganisms present in mealworm frass are also involved in increased tolerance to salinity, drought and flooding in bean plants (Poveda et al., 2019). However, the use of mealworm frass as a plant resistance activator against biotic stresses has to our knowledge not yet been explored.

This work studied the use of mealworm frass in agriculture with benefits for crops. The first objective was to analyze its use as a fertilizer for various crops, under standard conditions and nutrient deficiency. Finally, it was intended to analyze the use of mealworm frass as a resource of plant defense elicitors, an aspect unexplored until now.

2. Materials and methods

2.1. Obtaining mealworm frass

Mealworm frass was provided by Invertapro (Voss, Norway), a company dedicated to the *T. molitor* insect farming. The insects were fed with wheat bran and different food wastes, and maintained at an ambient humidity of 70% and temperature of 25–27 °C. The frass was collected mechanically by sieving, eight weeks after hatching of the *T. molitor* larvae. The nutritional analysis of mealworm frass is reflected in Table S1, presenting a NPK content of 2.6–1.8–2.8.

2.2. Biological materials

In the fertilization trial, tomato (*Solanum lycopersicum*) plants of the Moneymaker variety were used. The nutrient deficiency fertilization trial was conducted with sunflower (*Helianthus annuus*) plants of the Giganteus variety. Immune stimuli trials were conducted with the *Arabidopsis thaliana* accession Columbia-0 (Col-0). Tomato and sunflower seeds were purchased from Plantasjen (Oslo, Norway) or LOG Frø (Oslo), respectively, while *A. thaliana* seeds were purchased from Lehle seeds (Texas, USA) and propagated in the lab.

The pathogen used in the leaf infection assays was the necrotrophic fungus *Botrytis cinerea* isolate CECT2100, provided by Dr. Imre E. Somssich (Max Planck Institute for Plant Breeding Research, Cologne, Germany) (Birkenbihl et al., 2012). The fungus was routinely grown on potato dextrose agar (PDA) plates at 22 °C. The spores were maintained in 0.8% NaCl at −80 °C.

2.3. Application as fertilizer in tomato

Tomato is a model plant in plant growth studies with different fertilization (Stikić et al., 2015). Tomato seeds (Moneymaker variety) were germinated in seedbeds with peat-based substrate (Tjerbo, Rakkestad, Norway), covered with plastic (to maintain humidity), and located in the greenhouse at Ringve Botanical Garden (Trondheim, Norway). After 5 weeks, individual plants were transplanted into 2 L pots with a 4:1 peat:perlite mixture. At that time, the different fertilization treatments were applied (6 plants per treatment).

- C - Control: no additional fertilization.
- MF - Mealworm Frass: application of 2% (v/v) mealworm frass.
- SMF – Sterilized Mealworm Frass: application of 2% (v/v) autoclaved (at 121 °C for 20 min) mealworm frass; to identify the possible effect of the microorganisms present.
- OF – Organic Fertilizer: application of 1% (v/v) commercial organic fertilizer (NaturGjødsel from Hageland, Norway), based on pelletized chicken manure. As its NPK content (6–4.5-5) is twice that of mealworm frass, half the amount was applied.

The peat-based substrate used as base soil already had significant amounts of nutrients. Tomato plants were maintained in a greenhouse under artificial lights (12-h photoperiod, 145 μmol $m^{-2}s^{-1}$, 40–65% humidity, 20 °C), randomizing the distribution of pots. Irrigation was performed several days per week, as needed, always with the same amount of water per plant. After 6 weeks, the plants were harvested, taking measurements of fresh shoot weight and shoot height.

2.4. Nutrient deficiency assay with sunflower

Due to its economic importance and ecophysiological adaptability, sunflower represents an interesting model plant for nutrient deficiency studies (Dimitrijevic and Horn, 2018). The substrate for sunflower cultivation was acid-washed sand (Alfa Aesar/ThermoFisher, Kandel, Germany) (2:3) in 0.2 L pots. The different fertilizer treatments were applied to 2-week-old seedlings (8 plants per treatment). Plants were grown in a greenhouse under artificial light (18-h photoperiod, 200 μmol m^{-2} s^{-1}, 21 °C), randomizing the distribution of pots.

A modified Hoagland solution (Hoagland, 1920) was used as fertilizer, from which each of the nutrients (N, P, K, Ca, or S) was removed to create nutrient deficiency situations. Therefore, 2-week-old seedlings were irrigated with 6 different nutrient solutions, at the rate of 15 ml/plant/day for 5 weeks, then 40 ml/plant/day for another 3 weeks, with 8 plants per treatment. In plants that were fertilized with mealworm frass, it was applied at a rate of 5% (v/v). See Table S2 for total amount of nutrients that were added through each nutrient source. Plants were harvested at 10 weeks-old, quantifying shoot and root dry weights (dried at 70 °C for 3 days).

2.5. Root callose deposition analysis

To analyze the possible activation of local root defenses by mealworm frass, the model plant *A. thaliana*, widely used for this type of analysis (Poveda, 2022), and the deposition of callose as a plant defense response were used.

First, an elicitation solution was prepared with mealworm frass. A mixture of sowing soil (Norgro, Hamar, Norway) (64%), perlite (Norgro, Hamar, Norway) (16%), compost (12%) and sand (Hageland, Kristiansand, Norway) (8%) (v/v) was used as soil. This soil was mixed with either mealworm frass at the rate of 2% (v/v), or with the organic fertilizer to an equivalent NPK content. After two weeks of incubation with one *A. thaliana* planted in the soil (for enhanced frass breakdown through plant-microbiota interactions), 15 g of soil was added to 10 ml of double-distilled water and filtered through 0.2 μm pore size filters.

A. thaliana seeds were surface sterilized using chlorine fumes, following the methodology described by Clough and Bent (1998). Subsequently, seeds were deposited individually on a 24-well plate (Sigma-Aldrich, Missouri, USA) with 1 ml of MS nutrient medium, as described by Millet et al. (2010). Seedlings were grown for 8 days in a growth chamber at 16-h photoperiod at a light intensity of 125–140 μmol $m^{-2}s^{-1}$, and a temperature of 22 °C. Subsequently, the liquid culture was replaced by 1 ml of: elicitor solution with soil and frass, soil filtrate, or soil filtrate supplemented with 250 μg/ml chitin solution. Each treatment was applied to 12 seedlings. The plate was placed back into the growth room for 2 days.

To perform the callose staining, the elicitor solutions were replaced with 1 ml of 3:1 ethanol/acetic acid fixing solution and put on shaking at 90 rpm for 24 h. The fixative was changed 3 times during this time to ensure complete clearing of the tissues. The seedlings were then rehydrated in 70% ethanol for 4 h, in 50% ethanol for 2 h, and finally in double distilled water overnight. The water was then replaced with 1 ml 10% NaOH per well for 90 min at 37 °C. After 2 washes with double distilled water, roots were incubated in 1 ml 0.01% aniline blue (Sigma-Aldrich, Missouri, USA) in 150 mM K_2HPO_4, covered with aluminum foil, and put on shaker at

90 rpm for 2 h. Whole seedlings were mounted on slides in 50% glycerol and roots were observed with a Nikon Eclipse E800 epi-fluorescence microscope under UV light.

2.6. Expression analysis of defense genes in A. thaliana

A. thaliana is a model plants in plant-pathogen interaction studies (Poveda, 2022), with a large number of studies developed on systemic resistance against the pathogen *B. cinerea* (Poveda et al., 2020). *A. thaliana* seeds were germinated in seedbeds with the substrate mixture described in the previous section (soil, perlite, compost, and sand) in a Vötsch VB 1514 culture chamber (12-h photoperiod, at 75–90 μmol $m^{-2}s^{-1}$, relative humidity of 40% and temperature of 22 °C). After 3 weeks, the seedlings were transplanted into 80 ml pots with the same culture substrate, fertilized either with mealworm frass at 2% (v/v), or with organic fertilizer to an equivalent NPK content. The plants were kept in the culture chamber for another 2 weeks.

Inoculation of *A. thaliana* with the pathogen *B. cinerea* was performed by depositing 2 μl drops at 10^6 spores/ml in filtered-sterile Vogelbuffer (Birkenbihl et al., 2012), on each side of the midrib of two fully grown leaves per plant, 12 plants per treatment. Vogelbuffer was used as mock treatment and 2 μl droplets were placed similarly. Plants were randomly distributed in separate covered trays, sealed with cling film (resulting in 100% relative air humidity) before being returned to the growth chamber. After 8, 24 and 48 h, the *B. cinerea* and mock inoculated leaves from 4 plants per soil treatment were harvested and frozen in liquid nitrogen. The tissue pooled from one plant was considered as a biological replicate. Frozen plant tissue was stored at −80 °C.

For leaf RNA isolation, the Spectrum Plant Total RNA Kit (Sigma-Aldrich, Missouri, USA) was used, starting from 100 mg of leaf tissue. Plant cell disruption was performed using a TissueLyserII (Qiagen, Hilden, Germany) for 2 min at 25 Hz, and 500 μl lysis-buffer containing 10 μl/ml β-mercaptoethanol was added to each tube. Samples were then run in the TissueLyserII once more for 2 min at 25 Hz, and the supplier's instructions were followed for the rest of the RNA extraction. An on-column DNase digestion using the RNase-Free DNase Set (Qiagen, Hilden, Germany) was included to remove trace DNA. The concentration and purity of total RNA were measured with a NanoDrop One spectrophotometer. The cDNA synthesis from 1 μg total RNA was performed using the Quanti-Tect Reverse Transcription Kit (Qiagen, Hilden, Germany).

Gene expression was analyzed by RT-qPCR, using the LightCycler 480 SYBR Green I Master Kit and a LightCycler96 (Roche Life Science, Penzberg, Germany). All PCR reactions were performed in a total volume of 20 μL for 45 cycles under the following conditions: denaturation, 95 °C, 10 s; annealing, 55 °C, 10 s; extension, 72 °C, 15 s. The gene for protein phosphatase 2A subunit A2 (*PP2AA2/At3g25800*), and the gene for TAP42 interacting protein of 41 kda (*TIP41/At4g34270)* was used as reference genes. The primers used are listed in Table 1: Zinc finger of *Arabidopsis thaliana* 10 (*ZAT10/At1g27730*), the genes of the ethylene-responsive transcription factor 5 (*ERF5/At5g47230*), transcription factor WRKY33 (*WRKY33/At2g38470*), calmodulin like 37 (*CML37/At5g42380*) transcription factor WRKY75 (*WRKY75/At5g13080*), cytochrome P450, family 71, subfamily A, polypeptide 13 (*CYP71A13/At2g30770*).

2.7. Statistical analysis

GraphPad Prism software (GraphPad Software, CA, USA) was used for statistical analysis. A one-way ANOVA analysis and a Tukey-Kramer multiple comparison test were performed, except for pairwise comparisons, where the Sidák test was used.

3. Results

3.1. Fertilization of tomato with mealworm frass

Six weeks after the application of the different treatments with mealworm frass and organic fertilizer, data on growth parameters were collected from the tomato plants. The application of mealworm frass (MF and SMF) resulted in a significant increase in shoot fresh weight of plants compared to unfertilized plants (control, without fertilization) (Fig. 1a). However, no significant differences were reported with respect to plant height (Fig. 1b). The data obtained with plants fertilized with sterilized mealworm frass (SMF) showed no significant differences to unsterilized mealworm frass (MF). Fertilization with the organic fertilizer (OF) (positive control) reported no significant differences with respect to plants fertilized with frass.

3.2. Fertilization of sunflower under nutrient deficiencies

In order to determine the exact nutrient supply to the plant by mealworm frass, a specific nutrient deficiency test was conducted with sunflower plants. Two-week-old sunflower seedlings were given a nutrient solution with a missing macronutrient, once a day for

Table 1
Primers used in this work.

GENE		FORWARD PRIMER SEQUENCE	REVERSE PRIMER SEQUENCE
PP2AA2 (ref. gene)	*Phosphatase 2A subunit A2*	TGGCTCCAGTCTTGGGTAAG	ATCCGGGAACTCATCTTTCA
TIP41 (ref. gene)	*TAP42 interacting protein of 41 kda*	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
ZAT10	Zinc finger of *Arabidopsis thaliana* 10	TAGCTTCTCCGATTCCTCC	GTGGAAATCGGATCTTGATC
ERF5	*Ethylene-responsive transcription factor 5*	TCTTCGGATCATCGTCCTCTTC	GGTTTGCATACGGATTCAGAGAA
WRKY33	*Transcription factor WRKY33*	GACATTCTTGACGACGGTTACA	CGATGGTTGTGCACTTGTAGTA
CML37	*Calmodulin like 37*	CGTTTGGGATGTATGTTATGG	CAAAGCTGAGAACTCCATCG
WRKY75	*Transcription factor WRKY75*	TATGCGTTTCAAACAAGGAG	CTATAGTAACTCCTAGGGAACTTGTTG
CYP71A13	*Cytochrome P450, family 71, subfamily A, polypeptide 13*	ATGGATAGATGGGATCCGT	GAAATCCGCTTTATCGTTACTC

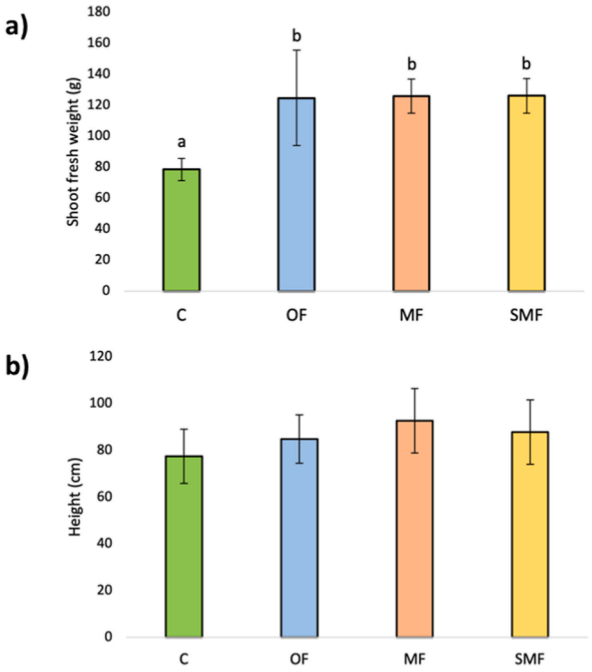

Fig. 1. Effect of mealworm frass fertilization on tomato plant growth. Monitored parameters were fresh weight (a) and height (b) of 11-week-old tomato plants after 6 weeks of the following treatments: C: not fertilized; OF: organic fertilizer 1%; MF: mealworm frass 2%; SMF: sterilized mealworm frass 2%. The bars represent the means of 6 plants, together with their standard deviation. The letters denote statistically significant differences between treatments, by Tukey-Kramer multiple comparison test ($p < 0.05$).

8 weeks. Frass was added to half the plants to see if it could provide the missing nutrient. Deficiency in any of the nutrients (N, P, K, Ca, or S) resulted in significantly lower plant growth (both shoot and roots) compared to plants fertilized with the complete nutrient solution (Fig. 2). Deficiencies in N, P and K reduced plant growth to a greater extent than deficiencies in Ca or S (Fig. 2). In all cases, the addition of mealworm frass significantly increased plant growth, except in roots under S deficiency. The addition of mealworm frass further increased the shoot biomass of plants fertilized with the complete Hoagland nutrient solution (Fig. 2).

Analyzing from the point of view of possible nutritional compensation, the addition of mealworm frass compensated for the deficiencies of all nutrients, except N, in the growth of the shoots (Fig. 2a). At the root level, the addition of mealworm frass compensated for the deficiency of all nutrients (Fig. 2b).

3.3. Deposition of callose in A. thaliana roots

To analyze the activation of local defenses in roots in response to frass, staining for callose, a polysaccharide that accumulates as a defensive response, was performed. Roots of *A. thaliana* were treated with a solution prepared from soil inoculated for 2 weeks with or without mealworm frass, or with a chitin oligomer solution as a positive control. Roots treated with the solutions prepared either from soil without frass (Fig. 3a) or from soil with mealworm frass (2%) (Fig. 3b) did not induce local root accumulation of callose. A pure frass solution did not lead to callose deposition in roots either under these conditions (data not shown). However, application of the soil solution supplemented with chitin (250 μg/ml) did induce local callose accumulation (Fig. 3c).

3.4. Gene expression analysis in A. thaliana

In order to identify possible systemic resistance pathways activated by root application of mealworm frass, a gene expression analysis was performed on *A. thaliana* leaves with and without infection with the pathogen *B. cinerea*. The expression of defense related genes was assessed over time, after inoculation with the pathogen or mock treatment. Application of frass led to higher gene expressions compared to the control in all genes tested, in many cases both in infected plants and in mock treated plants.

ZAT10, ERF5, WRKY33, and *CML37* displayed the highest gene expressions at 8 h post inoculation (hpi), which decreased over time (Fig. 4). Frass treated plants showed significantly higher expression than control in most cases, both for the mock treated plants and the infected plants. At 48 hpi, the combination of frass treatment and *Botrytis* inoculation generally displayed higher expression values compared to the other treatments (Fig. 4).

WRKY75 and *CYP71A13* did not show a significant change in expression by any treatment at 8 and 24 hpi. At 48 hpi, however, plants inoculated with *Botrytis* showed a highly induced expression of *WRKY75* and *CYP71A13*, particularly in combination with the frass treatment. The variation between replicates of this treatment was very high for both genes, which resulted in large confidence intervals and no significant differences between control and frass treatments in the infected plants at 48 hpi (Fig. 4).

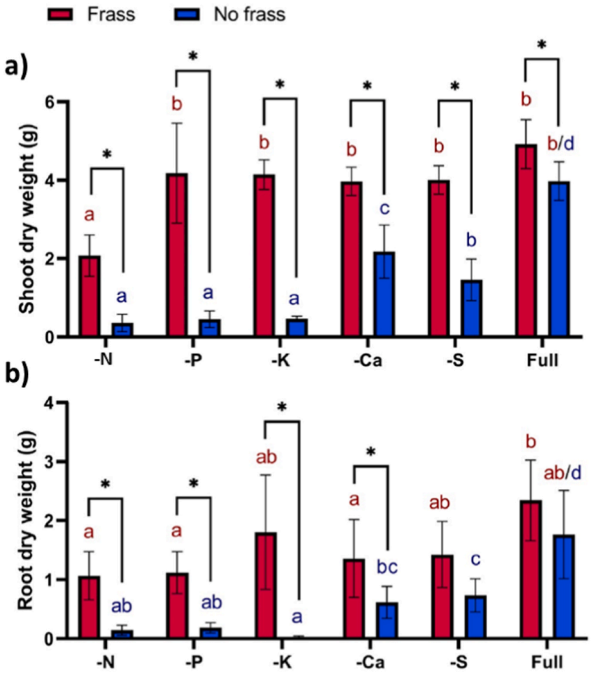

Fig. 2. Biomass production of nutrient deficient sunflowers with or without frass. Shoot (a) and root (b) dry weights of 10-week-old sunflower plants grown in the absence or presence of mealworm frass and watered for 8 weeks with nutrient solutions lacking nutrient -X (indicated below bars on the x-axis) or complete nutrient solution (Full). The bars represent the means of 8 plants, together with their standard deviation. The letters denote statistically significant differences between nutrient treatments (red letters = frass, blue letters = no frass) by Tukey-Kramer multiple comparison test ($p < 0.05$). Asterisks represent statistically significant differences between frass and no frass within each nutrient treatment by Sidák test ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

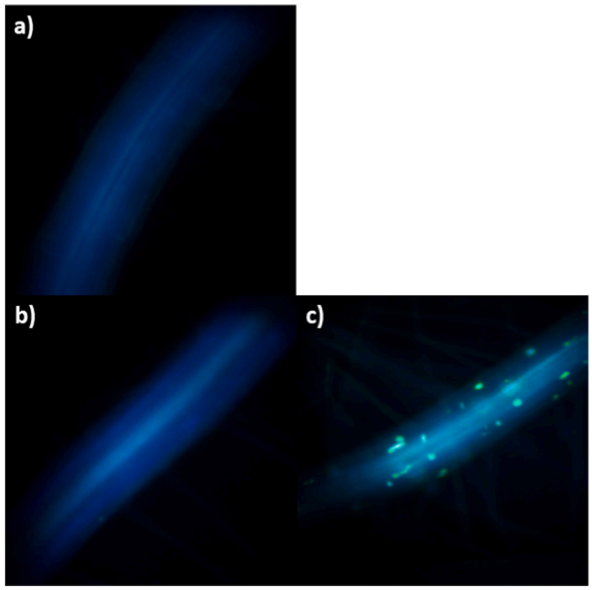

Fig. 3. Callose deposition in *A. thaliana* roots. Representative pictures of roots of 10-day-old *A. thaliana* seedlings grown in liquid medium, treated with a filtered solution from solution (a), soil containing frass (b), or pure chitin (c) for two days. Callose was stained with aniline blue and observed under UV light in a Nikon Eclipse E800 epi-fluorescence microscope (10x magnification). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

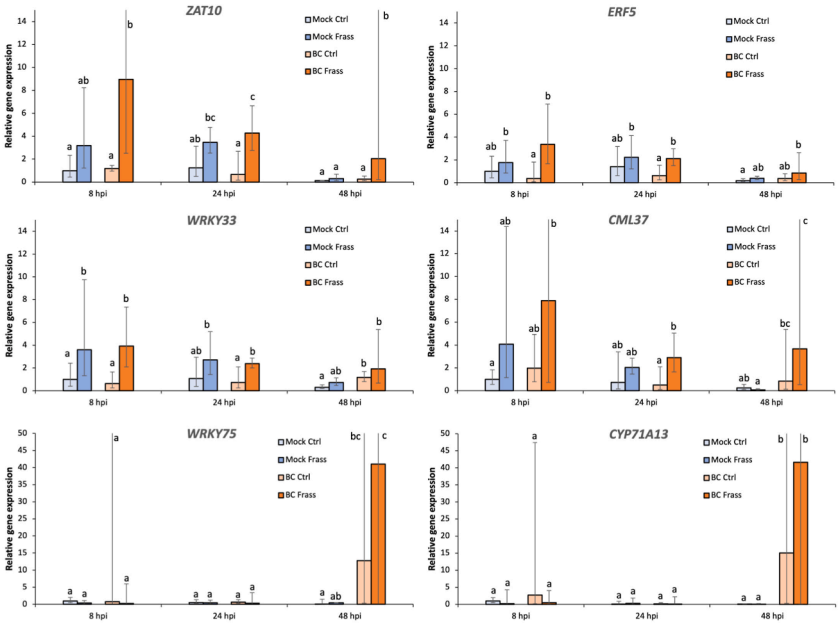

Fig. 4. Gene expression analysis of *A. thaliana* defense genes after *B. cinerea* infection. Gene expression was monitored in leaves of 5-week-old *Arabidopsis* Col-0 plants grown for 2 weeks in control soil (Ctrl) or a frass-soil mixture, inoculated with *Botrytis cinerea* spores (BC, orange bars) or Vogelbuffer (Mock, blue bars), and harvested at 8 h, 24 h or 48 h post inoculation (hpi). All values are relative to the gene expression in plants grown on Ctrl soil, mock-inoculated and subsequently incubated for 8 h (set to 1). Error bars show 95% confidence intervals (CI). Some of the upper CI are capped in the figure for presentation purposes. Different letters denote significant differences within the same time point by Tukey- Kramer multiple comparison test, $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

4.1. Fertilizing aspects of mealworm frass

Here we have reported how the root application of mealworm frass promoted the growth of tomato plants. This corroborates results from other crops, such as chard (Poveda et al., 2019), barley (Houben et al., 2020) or ryegrass (Houben et al., 2021). In particular, this plant growth promotion would be a consequence of the nutritional content of mealworm frass or the presence of active biomolecules, since sterilizing the frass to eliminate the microbiota did not reduce the beneficial effects obtained. This contrasts with a similar experiment with chard plants, where sterilization of mealworm frass resulted in lower growth compared to unsterilized frass (Poveda et al., 2019). As heat treatment of insect frass fertilizers is mandatory in EU countries (EU, 2021/1925), it is of relevance to understand how the microbiota of frass is affecting its quality as a fertilizer and soil improver.

To further investigate its fertilizer capabilities, we analyzed the specific nutrient supply of mealworm frass by means of a nutrient deficiency study in sunflower plants. Even though higher amount of nutrients was added through the nutrient solution (see Table S2), the application of mealworm frass at 5% v/v inclusion restored the growth of the sunflower plants to the level of the complete nutrient solution in almost all the treatments. The exception is nitrogen, as plants fertilized with frass had stunted growth and N deficiency symptoms. The sunflowers received approximately 2.4 times more nitrogen through the nutrient solution than through frass (Table S2), which probably explains this difference in growth. The fact that nitrogen in frass is mainly bound to organic compounds instead of existing as plant-available ammonium or nitrate could also contribute to the reduced effect. Houben et al. reported that 40% of nitrogen in mealworm frass mineralized after 20 days, while mineralization of the remaining N occurred over a long period of time (Houben et al., 2020). In our experiment, we used acid washed sand as substrate, which most certainly has a poor microbiota and is not likely to have mineralized organic N from frass at a very high rate. This result shows a potential need for combining frass with another nitrogen rich fertilizer for nitrogen demanding plants.

A combination of the complete nutrient solution and frass resulted in significantly increased growth compared to the complete nutrient solution alone, suggesting that frass is able to increase plant growth even when there are ample amounts of nutrients in the soil. This may be attributed to potential biostimulating traits of frass (Barragán-Fonseca et al., 2022).

4.2. Immune stimulating aspects of mealworm frass

Chitin in frass originates from either the insect exoskeleton, or from the peritrophic membrane of the insect's gut. In both cases, the chitin exists as long chains and is bound to other molecules such as proteins and calcium carbonate (Brandt et al., 1978; Vincent and Wegst, 2004). As chitin oligomers are known to be the plant stimulating form of chitin, it is reasonable to assume that the fragments of exoskeleton in frass must undergo a degradation process in order to produce chitin elicitor molecules. This degradation can occur in soil, and will likely require a long-lasting microbial action for its release (Nayak et al., 2020).

Therefore, it was theorized that frass in itself does not stimulate plant immunity, but frass mixed in soil with a rich microbiota should in theory generate eliciting chitin oligomers at some point. No literature was found that investigates the time it takes for insect exuviae to degrade in soil.

To test this hypothesis, an elicitor solution was prepared from a frass-soil mixture that had been incubated for two weeks, and applied to *A. thaliana* roots to monitor callose deposition.

Cellular deposition of callose is one of the main local plant defensive responses against pathogen attack and will also form during chitin recognition by plants (Wang et al., 2021). In this experiment, roots in contact with mealworm frass solution did not accumulate callose, while roots incubated with a pure chitin oligomer solution did. This indicates that the elicitor solution did not contain chitin oligomers at sufficient concentrations. The reason for this may be that the inoculation time was too short for chitin oligomers to appear in the soil, or that the microbiota in the soil was insufficient for degrading chitin to oligomers that can act as elicitors.

If degradation of chitin takes place, plants grown for several weeks in soil containing frass would be stimulated at some point. This could lead to a priming effect, where the plant is put in a state of increased alertness towards pathogen infection (Aranega-Bou et al., 2014). The use of insect frass as a possible elicitor of plant defense responses has been gaining attention lately (Ray et al., 2015, 2016a, 2016b; Poveda, 2021), although this has to our knowledge not been tested with mealworm frass yet. Therefore, plants were grown for 2 weeks in compost soil containing frass, and subsequently infected with the necrotrophic fungus *Botrytis cinerea.* Expression of genes related to the defense against necrotrophic pathogens were monitored over 48 h after inoculation (Fig. 4).

Indeed, application of frass led to an increased expression of the investigated genes compared to control treatments (Fig. 4), indicating a priming effect. Interestingly, this response was also found in mock-treated plants, especially in the earlier time points after inoculation. A possible explanation for this can be that the inoculation procedure led to an abiotic stress response, amplified in frass treated plants. Several of the analyzed genes, including *ZAT10*, *ERF5* and *WRKY33*, are linked to abiotic stress responses as well as biotic stress (Mittler et al., 2006; Pan et al., 2012; Wang et al., 2013). This can imply that frass application can prime plant defenses against abiotic stress, as previously shown by Poveda et al. (2019).

Compared to mock inoculated plants, genes in *Botrytis* inoculated plants displayed the biggest differences 48 hpi, especially in plants grown on soil with frass. *WRKY75* and *CYP71A13* were notably different in their responses compared to the other genes investigated, with very low expression values at early time points and highly induced levels at 48 hpi. It has been shown that *WRKY75* is not triggered by the abiotic stresses drought and heat (López-Galiano et al., 2018), while it has been described as a critical component in *A. thaliana* defense against necrotrophic pathogens (Chen et al., 2021). It may, therefore, be hypothesized that abiotic stress led to the early responses in the other genes investigated, while *WRKY75* only reacted to the biotic stress of the pathogen. While *CYP71A13* showed a similar change in expression as *WRKY75*, it has a role in abiotic as well as biotic stress response through their involvement in the synthesis of the phytoalexin camalexin (Xu et al., 2008). Nevertheless, this might indicate that the pathogen had not yet triggered a plant response at 8 and 24 hpi, perhaps as a result of a slow infection by the pathogen.

This proposed delayed infection corresponds quite well with previous research looking at gene responses in *A. thaliana* after *Botrytis* infection. AbuQamar et al. (2006) used 24 hpi as the first time point after inoculation where gene expression was analyzed. At this time point, *ZAT10* had ~10-fold-increase (compared to mock treatments), while *ERF5* had a ~4-fold-increase. Sham et al. (2019) used 18 hpi as the first time point analyzed, where gene expression was increased 15-fold for *WRKY33* and 83-fold for *CYP71A13*. Ferrari et al. (2007) showed that *CYP71A13* was induced 8-fold after 18 hpi, while it was induced 45-fold after 48 hpi.

The increased expression of defense genes at 48 hpi in plants treated with frass indicates a priming effect by frass against the pathogen. Both *B. cinerea* and chitin is known to trigger the JA pathway in *A. thaliana*, leading to increased resistance against necrotrophic pathogens and herbivores (Sharp, 2013; Windram et al., 2012). Several of the genes investigated in this experiment are known to be JA dependent, e.g. *WRKY75* (Chen et al., 2021), suggesting that mealworm frass acts through JA-mediated signaling pathways. The defense gene *WRKY53*, which is known to be SA dependent and a negative regulator of JA (Miao and Zentgraf, 2007), was also tested (data not shown). There were no significant differences in expression at 48 hpi, when the pathogen is thought to be detected by the plant, supporting the hypothesis that the JA pathway is activated.

Despite this activation of defense genes, *A. thaliana* plants grown in frass did not show increased resistance to *B. cinerea* compared to plants grown on control soil under these conditions, likely because of the low virulence of this *B. cinerea* strain on *A. thaliana* Col-0 (Birkenbihl et al., 2012) and the resulting low disease symptoms in both cases (data not shown). Further assays should be conducted to ascertain under which conditions the exposure to frass leads to increased disease resistance.

Our results indicate that mealworm frass contains an elicitor source of systemic plant defense responses, likely chitin, leading to priming against necrotrophic pathogens such as *B. cinerea.* As suggested by Barragán-Fonseca et al. (2022), other characteristics of frass may lead to a similar effect, such as PGPRs stimulated by frass or chitin, causing ISR (Barragán-Fonseca et al., 2022). Priming of plant defenses would be a valuable additional characteristic to the fertilization effect of frass, as it could potentially lead to a reduction in chemical pesticide use in the future, and increased crop yield for organic farmers. In addition, our results indicate a possible priming effect against abiotic stress. This trait could be very beneficial in the face of future agricultural challenges related to climate change. Further research is required in order to fully document the priming capabilities of mealworm frass.

As a conclusion, mealworm frass seems suitable as fertilizer due to its ability to promote plant growth by providing nutrients to the soil and plant. However, an additional source of plant available nitrogen may be necessary for nitrogen demanding plants. Furthermore, a potential priming effect of mealworm frass was found, likely caused by the chitin content of the frass. This could make mealworm frass useful as an alternative to pesticides, but more research is needed to ascertain whether application of frass will lead to increased plant tolerance to abiotic and biotic stress.

Authors contributions

J.I.B. designed and performed the experiments and analyzed the data. R.K. helped design and perform all of the experiments. A.M.B. helped design some of the experiments and supervised all the work, and provided the material means and facilities to carry out the experimentation. R.S. designed and helped execute one of the experiments. J.P. analyzed the data and wrote the manuscript. J.I.B., R.S., A.M.B. and R.K. contributed to the manuscript correction and critical reading. All authors have read and agreed to the published version of the manuscript.

Funding

The study was funded by internal Norwegian University of Science and Technology (NTNU) seed money and project grant RCN 250953 from the Norwegian Research Council to A.M.B. Open Access funding provided by University of Valladolid (Spain).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: We declare that J.I.B. works for the mealworm production company Invertapro, a private corporation, which also provided us the mealworm frass used in this study; however, we assure that the research is free of bias.

Data availability

No data was used for the research described in the article.

Acknowledgements

The authors want to thank Vibekke Vange and the staff at Ringve Botanical Garden for the use of their greenhouse and the help received during tomato growth trials. We also want to thank Invertapro for supplying mealworm frass, and Imre E. Somssich for generously providing the *B. cinerea* strain. Lastly, thanks to Torfinn Sparstad and Javad Nafaji at the Bones lab for valuable help with the plant immune system elicitation experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2023.102862.

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