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Research article

Towards circularity for agro-waste: Minimal soil hazards of olive pomace bioconverted frass by insect larvae as an organic fertilizer

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ABSTRACT

As global populations escalate and the demand for food and feed intensifies, the generation of agri-food waste is becoming an increasingly critical issue. Addressing this challenge is crucial for optimizing food production and advancing sustainable waste management practices. In this context, insects, including the Black Soldier Fly (BSF, *Hermetia illucens*), present opportunities for circularity through the bioconversion of organic waste. Olive pomace (OP), a by-product of the olive oil industry, is known for its phytotoxic properties due to its high phenolic content and acidic pH. Using BSF for OP bioconversion could mitigate the environmental disposal of this by-product while producing valuable resources such as protein, fats, and insect frass. Insect frass is the excrement of insects that can be used as an entomofertilizer. Building from BSF feeding on OP, this study aimed to evaluate the safety of applying the resultant frass in soil amendment applications for the first time. Here are explored the effects of olive pomace-derived frass (OP-BSF_{frass}) on soil health and plant growth by evaluating the soil model invertebrate *Enchytraeus crypticus* and phytotoxicity bioassays using the forage crop ryegrass (*Lolium perenne*) and the agricultural species broccoli (*Brassica oleracea*). Our methodologies included direct soil applications and aqueous extract tests, with a range of OP-BSF_{frass} concentrations (from 0 to 9.8% w/w) and observation periods (2 and 32d). Despite initial concerns over the phytotoxic nature of OP, our findings revealed that OP-BSF_{frass} did not adversely affect the survival of *E. crypticus* and even enhanced its reproductive success. Furthermore, while higher frass concentrations elicited some adverse effects on plant germination and growth, these were limited to levels unlikely to be used in practical applications. The outcomes of this study suggest that OP-BSF_{frass} could be safely integrated into the soil as a fertilizer, promoting a circular bio-economy by converting waste into economically and environmentally friendly products. This study underscores the potential of insects in transforming waste management paradigms and enhancing food security, particularly in regions like the Mediterranean, thus contributing to a more sustainable and resilient agricultural sector.

1. Introduction

An increasing world population is escalating food demands and waste production, resulting in global concerns and identifying the need for more sustainable production and integrative waste management practices (Kaza et al., 2018; Godfray et al., 2010). The Food and Agriculture Organization of the United Nations (FAO) has reported that 1.6 billion tons, equal to 27% of the current world agricultural production, is wasted (van Huis and Oonincx, 2017). According to the "zero hunger" and the "responsible consumption and production" goals of the United Nations Sustainable Development Goals, sustainable and innovative solutions must be implemented to reuse waste, including the development of opportunities to mitigate severe environmental and socio-economic challenges that result from waste generation (United Nations Department of Economic and Social Affairs, 2022). Insects represent an efficient tool for such beneficial reuse because these organisms can consume organic materials and transform agro-waste into added-value products (Čičková et al., 2015). Thus, reusing agricultural

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and agro-industrial coproducts to feed insects promises to support more sustainable food production and waste management while advancing toward circular economy goals (Leni et al., 2021). The high bioconversion capacity and high protein and nutrient content of insects, associated with numerous environmental benefits (e.g., less land use, less carbon dioxide footprint, little water consumption in comparison with livestock and poultry breeding), make rearing insects an emerging environmentally friendly and economically profitable approach at the global level (van Huis, 2013; da Silva and Hesselberg, 2020; Houben et al., 2020; Makkar et al., 2014).

The selection of adequate feed substrates is necessary for sustainably feeding insects. However, every country has legal frameworks for feeding insects, which can be highly restrictive, as is the case of those from the EU (European Commission, 2017; Riekkinen et al., 2022). Consequently, commodities such as wheat bran, soybean meal, and others are most commonly used to achieve optimal insect diets (Čičková et al., 2015). In contrast, using agricultural by-products may support sustainable up-scaling of insect production (van Huis and Oonincx, 2017), increasing the circularity of the entire feeding process and contributing to waste management. For example, olive oil production growth in recent years, especially in the Mediterranean region, has resulted in vast amounts of waste and by-products produced each year (Kashiwagi et al., 2020; Ruschioni et al., 2020). Managing waste streams from olive oil production, such as olive pomace (OP), represents an expensive and prolonged process (DellaGreca et al., 2002; Klisović et al., 2021) because it contains olive pulp and fruit skin (Slama et al., 2021; Rubio-Senent et al., 2012) and is known for its high phytotoxicity, mainly due to low pH and the presence of polyphenolic compounds (Adnan Khdair, 2020; Omer and Mohamed, 2012). Therefore, this material can only be discharged into the environment after treatment (Rubio-Senent et al., 2012). However, it has been defined as a possible animal feed source (European Commission, 2017), and feeding OP to black soldier fly (*Hermetia illucens*, BSF) larvae has been identified as an alternative for beneficial reuse of increasing OP production, thus providing by-products with direct environmental benefits and economic applications (Ameixa et al., 2023; Ramzy et al., 2022). Specifically, generated organic material following BSF biodigestion, or frass, which includes insect excreta, undigested food, and exuviae of the larvae, can be used as organic fertilizer and soil amendment, offering more sustainable alternatives to conventional mineral fertilizers (e.g., nitrogen–phosphorus-potassium) (Leni et al., 2021; Beesigamukama et al., 2022a; Anyega et al., 2021; Lopes et al., 2022).

The beneficial reuse of insect frass for fertilizer is growing and receiving more attention from producers, farmers, and regulatory authorities (Menino et al., 2021; Klammsteiner et al., 2020). Insect frass is not only rich in essential macro and micronutrients for plants. Still, it contains bio-stimulatory compounds that can increase soil fertility by directly providing plants with available nutrients (nitrogen, phosphorus, and potassium) and/or by stimulating soil microorganisms and nutrient cycling (Choi and Hassanzadeh, 2019; Poveda et al., 2019; Gärttling and Schulz, 2022; Chiam et al., 2021). Applying insect frass or manures in soil can alter soil processes and properties over time, including ammonium and nitrate levels (Luo et al., 2018). Beyond simple nutrient supplementation, insect frass has been observed to foster a robust soil microbiome, enhancing the diversity and abundance of beneficial bacteria and fungi and their functions in soil by influencing biogeochemical cycles and plant resistance to diseases (Houben et al., 2020; Poveda et al., 2019; Esteves et al., 2022). In addition, frass contains chitin, which can alter the microbial properties of the soil by promoting beneficial antimicrobial peptides (Basri et al., 2022). Compared to synthetic mineral fertilizers, insect frass can stimulate natural defensive responses in plants, reducing dependence on conventional pesticides (Ray et al., 2016). Beneficial reuse of frass is, in fact, more sustainable and has a significantly lower environmental impact compared to traditional fertilizers, which are often associated with greenhouse gas emissions and water pollution (Poveda, 2021). Furthermore, the chitin in insect frass can enhance the activity of chitinolytic microorganisms in the soil, decreasing pathogenic plant fungi and undesirable nematodes that act as disease vectors (Oka, 2010).

While entomo-based organic fertilizers promise to promote soil health and increase crop productivity, the environmental safety of applying these materials to soils needs to be better understood, mainly when these fertilizers are generated from insects processing phytotoxic feedstock, such as the OP from olive oil production. To our knowledge, there is no current information regarding the potential impacts of OP-based frass (OP-BSF_{frass}) on non-target soil biota. Thus, the present study examined the influences of applying OP-BSF_{frass} to soil invertebrates and plants. To simulate different application rates, we manipulated different concentrations of OP-BSF_{frass} in soil. Then we evaluated the adult survival and reproduction responses of *Enchytraeus crypticus* as a model soil invertebrate (Castro-Ferreira et al., 2012). Seed germination of two plant species, the ryegrass *Lolium perenne* (monocotyledon) and the broccoli *Brassica oleracea* (dicotyledon), using both OP-BSF_{frass} incorporation in soils and aqueous extracts from amended soils during a shorter and longer periods study. Therefore, we hypothesized that invertebrate and plant responses would not be adversely affected by OP-BSF_{frass} amendments, regardless of time.

2. Methodology

2.1. OP-BSF_{frass} production

The OP-BSF_{frass} was obtained from Ingredient Odyssey SA – EntoGreen (Santarém, Portugal), which has a BSF colony that has been running since 2015, using standard cereal-based substrates and pre-consumer byproducts of plant origin to feed the BSF larvae. For this specific study, before initiating experiments, the standard diet of larvae was replaced by a mixture of OP (56.14%), Gainesville feed (25.18% - constituted by 50% wheat bran, 30% alfalfa meal, 20% corn meal, and 18.59% water) (Hogsette, 1992). At Ingredient Odyssey SA – EntoGreen, newly hatched larvae were fed for five days in a mixture of chicken feed and water at a controlled room temperature (27 ± 1 °C, 55 ± 5% relative humidity). This ratio of OP and Gainesville feed was previously tested by the company that provided the OP-BSF_{frass} as the best ratio regarding insect's safety and good performance. Bioconversion took place in plastic boxes (60 x 40 × 12 cm) (n = 18), with approximately 15,000 larvae (6.25 larvae cm^{-2}), feeding on a diet (initial moisture content of 65%) containing OP (7.6 kg), Gainesville feed (3.4 kg) and water (2.5 kg). Subsequently, these larvae (approximately 1 mg in weight) were fed a diet containing OP as the main feed component. This process lasted 12 days, after which larvae were separated from OP-BSF_{frass} by sieving (5 mm mesh), treated at 70 °C for 1h, and subsequently analyzed and examined using soil invertebrate and plant assays.

2.2. OP-BSF_{frass} characterization

The total nitrogen and carbon contents were analyzed in an elemental analyzer (LECO® CN 628) to calculate C:N. Moisture levels were measured by drying samples at 60 °C for 24h, and organic matter was determined by combustion at 500 °C for 4 h in a muffle oven, according to Alcarde (Carlos Alcarde, 2009). OP-BSF_{frass} samples were sent to Silliker Portugal, S. A. - Mérieux NutriSciences (Vila Nova de Gaia, Portugal) for metal analysis, but the methods employed were not fully disclosed. In addition, the concentration of total phenolic compounds was assessed in both the OP and the obtained OP-BSF_{frass}, following the previously reported methodology (Singleton and Rossi, 1965). Briefly, samples were diluted in deionized water, and 5 mL of Folin-Ciocalteu reagent was added (diluted 10-fold). After adding 4.0 mL of a saturated sodium carbonate solution (75 g L^{-1}), this solution was maintained at room temperature for 1 h, and then absorbance was measured at 765 nm.

2.3. Soil amendment with BSF_{frass}

Lufa 2.2 standard soil (Speyer, Germany) was incubated after being amended with different treatment levels of OP-BSF_{frass} (0% as a negative control; 0.3%, 0.6%, 1.2%, 2.4%, 4.8% and 9.6% w/w). These concentrations were chosen based on the widely variable doses of OP-BSF_{frass} when applied in cropland, as distinct crops have different nutrient demands; therefore, several application levels were determined and used in all bioassays. Notably, although 9.6% is considered a very high concentration when applying organic fertilizers, it was chosen at a lab scale as part of the safety assessment approach employed here. All treatments were incubated with distilled water with the maximum allowed percentage of moisture in test media according to OECD guidelines, or 60% of the maximum water holding capacity (maxWHC) in soil (OECD, 2006; OECD, 2016). The incubated soil was kept at 20 ± 2 °C for 16/8h (light/dark) for two and 32 days. The first sampling time was two days after the incubation, as recommended in OECD guidelines (Esteves et al., 2022), and the second was 32 days after the incubation, a more extended incubation period that has been proposed for frass application because it affords sufficient time to observe significant mineralization of the nutrients present in the frass (Houben et al., 2020). For each amendment level, pH and electrical conductivity were measured weekly during the 32 days of the longer-term study. Also, soil moisture adjustment was performed weekly to prevent moisture loss (Seyfried and Murdock, 2001).

2.4. Invertebrate bioassay with Enchytraeus crypticus

For the *E. crypticus* experiment, we followed the OECD 220 guideline (OECD, 2016) to evaluate potential adult survival and reproduction perturbation when the soil was amended with OP-BSF_{frass} at different concentrations. Bioassays were conducted using organisms cultured at the applEE-applied Ecology and Ecotoxicology laboratory, CESAM, University of Aveiro (Portugal). Five replicates of each treatment level were used; each replicate consisted of 10 *E. crypticus* adults with visible clitellum and similar size in glass experimental units (jars) containing 30 g of the incubated soil with a maximum of 60% WHC. The experiment was performed at 20 ± 2 °C with 16:8h (light/dark) for 21 days. Moisture content was adjusted weekly, and ≈2 mg smashed oats were used as the food source per replicate. At the end of the study, approximately 100 mL of ethanol (96%) was added to each flask with several drops of Bengal rose (1% in ethanol) and mixed gently. All the glass jars were kept at 4 °C overnight for organisms to be stained entirely. Finally, soil from each replicate was washed and sieved (125 μm mesh) to separate enchytraeids from most soil particles and transferred to white trays to facilitate counting the number of survived adults and juveniles. A magnifying glass (x 2.25 power) was used for this process.

2.5. Plant bioassays with Lollium perenne and Brassica oleracea

Phytotoxicity experiments were conducted by incorporating OP-BSF_{frass} in natural soil and collecting aqueous extracts from the same soil mixture. The rationale between these two exposure methods is that aqueous extracts allow for a more controlled environment, where the experimental factor, OP-BSF_{frass}, can more uniformly contact the seeds, providing more consistent exposure across all seeds (Nunes et al., 2016, Kebrom et al., 2019). However, it is essential to note that while bioassay conditions using aqueous extracts can provide valuable information on potential phytotoxic effects, they do not fully replicate the complex interactions in soils when an organic fertilizer is applied. Therefore, complementing these approaches with more realistic assays in soil provides a more complete understanding of potential environmental impact. Subsequently, both types of media were used to determine the effects of OP-BSF_{frass} on plant germination. This evaluation was performed during two steps: initially examining the germination of the seeds in both media and then evaluating potential influences on the (early-stage) growth of the plants after germination. The second step only addressed the OP-BSF_{frass} effects using a phytotoxkit test (soil media) since experimental conditions (e.g., absence of light) for the aqueous extracts were not intended to promote plant growth after germination (Luo et al., 2018). Phytotoxkits are transparent plastic devices designed to evaluate seed germination and early plant growth under laboratory conditions. These kits allow for observing germination and plant growth outside the transparent container. In this study, Phytotoxkits were employed with soil media to facilitate precise monitoring of plant development (Figure SD 1.).

The following equations were utilized to calculate seed germination rate (SGR), germination index (GI), relative seed germination (RSG), and relative radicle growth (RRG) presented (Luo et al., 2018). RSG and RRG were calculated using the control mean of germinated seeds and radicle length for each experimental treatment level, including negative controls.

$$SGR = \frac{Number\ of\ germinated\ seeds}{Number\ of\ total\ seeds} \times 100\%$$

$$RSG = \frac{Number\ of\ germinated\ seeds\ in\ test\ media}{Number\ of\ germinated\ seeds\ in\ control} \times 100\%$$

$$RRG = \frac{Radicle\ lenght\ of\ germinated\ seeds\ in\ test\ media}{Radicle\ lenght\ of\ germinated\ seeds\ in\ control} \times 100\%$$

$$GI = RSG \times RRG \times 100\%$$

2.5.1. Aqueous extract bioassay

To evaluate seed germination in aqueous extracts, 3g of the incubated soil with OP-BSF_{frass} were mixed on an orbital shaker with deionized water (1:10 ratio (w/v)) for 3 h, in a 50 mL-Falcon tube and then centrifuged (15 min, 4000 rpm, 20 °C) and filtered through 1.2 μm paper filters (Ø 45 mm). Bioassays were performed in Petri dishes (90 mm diameter × 10 mm height), where three paper filters were added and soaked with 4 mL of the aqueous extract to hold ten seeds on top. Samples were kept at 25 °C in the dark in a climatic chamber for 72h. After the test concluded, each seed with a root length>2 mm was considered germinated (Luo et al., 2018; Kebrom et al., 2019).

2.5.2. Soil bioassays

Phytotoxicity bioassays with commercially acquired kits were designed according to the ISO-18763 guideline (ISO, 2016; van der Vliet et al., 2012), with some adaptations to examine the potential effects of OP-BSF_{frass} on seed germination and early-stage plant growth performance (fresh weight (FW) and root/shoot length three days after germination) with two different seeds, *L. perenne* (Flora Lusitana, Lda) and *B. oleracea* (Rocalba, S.A.). Phytotoxkit kits (MicroBioTestsInc., Belgium) used amended soil with OP-BSF_{frass} applied at the treatment levels and 60% of the soil WHC. These bioassays were performed in a climatic chamber (Binder KBWF 720) at 25 ± 1 °C and 16/8h (light/-dark) photoperiod, and experimental duration was based on germinated seeds in controls (Phase 1), which consisted of germination of more than 70% of the seeds in negative controls on the same day. Subsequently, plants were kept in experimental condition for three more days after germination (Phase 2). For each kit, 100 ± 5 g of incubated soil and 10 seeds of each species were added. At phase 1 of the bioassay, all plants with ≥2 mm length in the plant root were considered germinated seeds (Luo et al., 2018; Kebrom et al., 2019). Furthermore, at the end of phase 2, plants were cut into two parts (root/shoot), and then the root and shoot length (in cm) and biomass as FW (in mg) were measured.

2.6. Data analysis

Data obtained from invertebrate survival and reproduction, SGR, GI, biomass, and length in the two different assays (soil and aqueous

extract), and early plant growth stage of both plants in soil were analyzed using a Two-Way ANOVA to confirm the influence of both OP-BSF_{frass} concentrations and soil incubation period in the responses of each parameter (Sigmaplot software v.14). For each experiment at each incubation time, the responses when OP-BSF_{frass} was incorporated in soil and the negative control (no amendment) were compared using a post hoc Dunnett's method ($p \leq 0.05$). In addition, comparisons between the two incubation periods were conducted at each concentration using a post hoc Dunnett's method ($p \leq 0.05$). Before performing each Two-Way ANOVA, the Shapiro-Wilk test for the normality of data and the Brown-Forsythe equal variance test for homoscedasticity were carried out $\alpha = 0.05$). Raw data was transformed using square root if these assumptions were not met.

3. Results & discussion

BSF larvae bioconversion of organic materials has grown exponentially over the last few years (Hénault-Ethier et al., 2024). After being considered an underlooked by-product in the past, research and industry innovation have finally turned their attention to frass (regulated as soil fertilizer and placed on the EU market - EU 2021/1925 (European Parliament and Council, 2021), perceiving it as an added-value product that can not only benefit insect farmers economically but also contribute to the establishment of circular economy models when it comes to food production and waste management practices (Lopes et al., 2022; Beesigamukama et al., 2022b). Substitution of traditional soil fertilizer with BSF frass can contribute to more sustainable fertilizer production (Elissen et al., 2023). In fact, the Haber-Bosch process for ammonia production for traditional synthetic fertilizers uses over 1% of global energy and produces over 1% of global CO_2 emissions (Smith et al., 2020).

Considering the importance of reducing waste, using different feeding substrates to rear insects/for insect bioconversion is crucial but needs further exploration. For example, approval for different feed substrates, such as olive pomace, has been implemented. Using OP as a feed substrate for insects and the conversion of OP to valuable products such as protein and insect frass, which can be used as soil fertilizer, may present an alternative for organic waste management. However, a complete characterization of the obtained sub-products (e.g., insect frass) must be assessed. Olive pomace usually presents high levels of polyphenols and is considered a phytotoxic component, which should not be released into the environment, although OP contains beneficial nutrients such as minerals and organic matter (Akratos et al., 2017).

Recently, the OP was considered and studied as part of the insect feeding substrate, resulting in its incorporation in EU 2022/1104 (European Comission, 2022) amending EU 68/2013 (European Commission, 2013), leading to several studies regarding its effects only on insect quality as a protein feed source (Ameixa et al., 2023). Ruschioni et al. (2020) found the optimal balance between growth performance (including larval and pupal weights, survival rate, and development time) and nutritional properties (precisely, the protein content of 47.58% dry matter, yield of 38.5% dry matter and essential/non-essential amino acids ratio (1.16)) in mealworm larvae using a feeding substrate comprised of 25% OP and 75% wheat middling (w/w). Ramzy et al. (2022) explored using BSF larvae to turn OP into insect biomass. The high lignin content in OP delayed the larvae's development, but using 75% OP (d/w) as a substrate increased the conversion efficiency of 33% protein, lauric, and palmitoleic acid (omega-7) by 79.76% and 65.05%, respectively. With that inclusion level of OP (75%), the larvae could convert it to 22% insect biomass. A recent study by Ameixa et al. (2023) demonstrated a high potential for using an olive pomace-based diet (75% OP d/w) in BSF rearing, which can result in an adequately tailored insect meal product by increasing the protein content up to 25%. The authors revealed certain trade-offs regarding the quality of the insect meal. Still, they emphasized the positive impact of such a waste management solution for Portugal, given the current and projected environmental and health pressures from the olive oil industry (Ameixa et al., 2023). Aligned with the urge to improve olive oil industry waste management in the Mediterranean region, our work extends well beyond previous studies of impacts on insects, looking to the safety of the application of insect frass – a significant product that results from the biodigestion of feedings substrates by insects - in soils due to its capacity to increase soil health by providing nutrients and biostimulants to crops, promoting plant growth (Elissen et al., 2023). This study used representative and model soil invertebrates and plants to examine the potential effects of OP-BSF_{frass} on soil biota.

3.1. OP-BSF_{frass} characterization

Different organic feedstocks can affect the gut microbiome community of insects; thus, the resulting frass can also vary based on the food and activities of the insect gut microbiome (Innangi et al., 2017; Palma et al., 2020). Depending on the organic substrate characteristics, the produced insect frass should be analyzed carefully before soil application. Results obtained from the characterization of olive pomace-derived frass, produced through bioconversion of OP by BSF larvae, revealed alterations in various parameters compared to the original OP substrate insect feed (Table 1). Inherent to bioconversion, OP-BSF_{frass} exhibited a lower moisture content (22.5 g/100 g) than OP (78.5 g/100 g), indicating efficient dehydration during bioconversion. Moreover, the

Table 1
Characterization of olive pomace-derived frass (OP-BSF_{frass}, d/w), obtained through the bioconversion of OP (w/w) with black soldier fly larvae. LOQ – Limit of quantification. "-" data not available.

Parameters	Olive pomace	L.Q.	OP-BSF_{frass}	L.Q.
Moisture	78.5 g/100g	–	22.5 g/100g	–
Organic Matter	74%	–	81%	–
Crude Protein	2.16 g/100g	–	–	–
Total Nitrogen	0.35 g/100g	–	2.85 g/100g	–
Organic Carbon	112 g/kg	–	45.2 g/100g	–
C/N Ratio	32	–	17.5	–
Fatty Matter	3.6 g/100g	–	–	–
Crude cellulose	7.7 g/100g	–	–	–
Arsenic	<0.020 mg/kg	0.020 mg/kg	<0.500 mg/kg	0.500 mg/kg
Cadmium	<0.005 mg/kg	0.005 mg/kg	<0.0500 mg/kg	0.0500 mg/kg
Chromium	0.10 mg/kg	0.05 mg/kg	1.52 mg/kg	0.50 mg/kg
Copper	4.24 mg/kg	0.5 mg/kg	25.3 mg/kg	1 mg/kg
Mercury	<0.005 mg/kg	0.005 mg/kg	<0.00010 mg/kg	0.00010 mg/kg
Nickel	<0.50 mg/kg	0.5 mg/kg	2.0 mg/kg	1.0 mg/kg
Lead	0.011 mg/kg	0.00 mg/kg	<1.0 mg/kg	1.0 mg/kg
Zinc	4.44 mg/kg	0.05 mg/kg	104 mg/kg	3.0 mg/kg
Total Phenols	6.06 g/kg	–	2.02 g/kg	–

organic matter content was 74% in OP and 81% in OP-BSF$_{frass}$. Despite this increase, the total nitrogen content increased remarkably from 0.35 g/100 g in OP to 2.85 g/100 g in OP-BSF$_{frass}$, indicating a considerable increase in nitrogen-rich compounds. The C:N ratio decreased from 32 in OP to 17.5 in OP-BSF$_{frass}$, reflecting a shift towards a more nitrogen-rich composition, increasing the potential nitrogen availability to plants. Additionally, concentrations of essential elements such as copper and zinc increased in OP-BSF$_{frass}$ compared to OP, highlighting the possible enrichment of essential nutrients during bioconversion. Conversely, non-essential metals such as arsenic, cadmium, and lead either remained below quantifiable limits or exhibited minimal increases below the maximum allowed in EU legislation for organic fertilizer (European Parliament and Council, 2019), suggesting a negligible impact on the overall safety profile of OP-BSF$_{frass}$.

In the specific case presented here, the use of a low-value waste stream (OP) resulted in a product with reduced content of total phenolic compounds (from 6.06 to 2.02 g/kg; section 3.1, Table 1), even considering that insects were fed with a substrate with approximately 55% of OP. In terms of nitrogen and organic carbon content, this frass fulfilled the EU criteria for organic fertilizers for containing above 2 % and 15 % by mass, respectively (precisely 2.85 % of N and 45.2 % of C; Table 1) (European Parliament and Council, 2019). It is worth mentioning that, despite the metal levels in the raw OP being lower (Table 1), the final frass did not exceed either the metal contaminant required limits or the copper (300 mg kg $^{-1}$) and zinc (800 mg kg $^{-1}$) levels (the most present elements), as stated in the EU regulation on fertilizing products (European Parliament and Council, 2019).

3.2. Soil bioassay with Enchytraeus crypticus

Due to the presence of frass in the soil, soil chemistry can change (Esteves et al., 2022), mainly if frass is produced from phytotoxic feeding substrates, which could impact non-target soil-dwelling organisms such as enchytraeids. Hence, a safety evaluation is necessary to address the potential valorization of OP-frass. In the current study, an Enchytraeid reproduction bioassay met the validation criteria within the OECD 220 guideline (OECD, 2016). In the negative control treatment (no frass application), adult mortality was below 20%, the coefficient of variation in the number of juveniles was less than 50%, and more than 25 juveniles per test vessel were recorded. No statistical differences were observed in adult survival, considering the OP-BSF$_{frass}$ (Two-way ANOVA $p = 0.191$) and incubation time (Two-way ANOVA, $p = 0.062$) (Fig. 1. A, Table SD 1). However, the reproduction of *E. crypticus* was dependent on the OP-BSF$_{frass}$ (Two-way ANOVA, $p < 0.05$), incubation time (Two-way ANOVA, $p<0.05$), and the interaction between both factors (Two-way ANOVA, $p = 0.040$). A significantly increasing reproductive output was observed when the concentrations of OP-BSF$_{frass}$ on both days 2 and 32 of incubated soil compared to negative control soil (Dunnett's Method $p < 0.05$, Fig. 1. B). In addition, the number of juveniles was significantly lower at 32 days of soil incubation compared to 2 days in each respective treatment level while still being higher. This increase in the reproductive output of *E. crypticus* was also observed by Malheiro et al. (2024), with an increase in the reproduction of the same organisms with increased concentrations of frass from bioconversion of vegetables and cereals, and this increase is suggested to be related to organic matter content.

The absence of adverse effects observed in our model invertebrate species contrasts with previous reports using olive oil industry-generated waste (Hentati et al., 2016). Due to the unavailability of data related to the potential toxicity of raw OP to soil invertebrates, we compared the absence of toxicity of frass produced by the biodigestion of OP with other olive oil industry waste. In Hentati et al. (2016) study, the characteristics of the tested media (olive mill wastewater and olive mill contaminated soil) were similar in terms of polyphenol concentration (8.868 g/kg), as in the present study (total phenol 6.60 g/kg) before BSF larvae digestion. Olive mill wastewater and olive mill waste contaminated soil negatively affected the model organism *Folsomia candida* at different levels by adversely impacting neurotoxicity, oxidative stress, changes in available energy, and both survival and reproduction (Kovačević et al., 2022). In addition, Hentati et al. (2016) demonstrated that olive mill-contaminated soils induced an avoidance of *E. fetida* and *F. candida* to control soils. Further, an adverse impact on the reproduction of *E. crypticus* was observed when olive oil-contaminated soils were compared to reference soil. In the same study, the incorporation of olive mill waste in soils affected the cocoon and juvenile protection of *E. fetida* in a dose-dependent manner. The study stated that the mentioned adverse effect was due to the available polyphenolic compounds, salinity, and high amount of phosphorus in the tested media (Hentati et al., 2016).

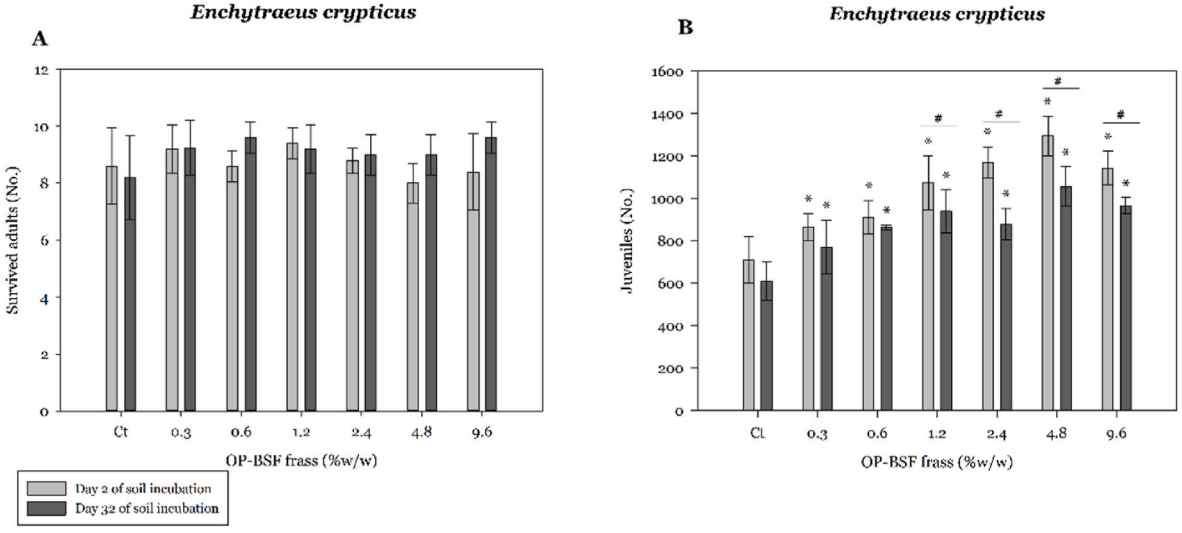

Fig. 1. *Enchytraeus crypticus* adult survival **(A)** and reproduction **(B)** after a 21d experiment with different olive pomace-derived frass (OP-BSF$_{frass}$) concentrations with 2 and 32 days of incubation. Data is presented as mean with standard deviation, and statistical analysis was performed using two-way ANOVA (OP-BSF$_{frass}$ concentrations and incubation period as factors) to determine significant differences in both endpoints. (*) significant differences in reproduction compared to the respective control (Ct) group (Dunnett's Method $p \leq 0.001$) at each sampling time. (#) represents significant differences in the same OP-BSF$_{frass}$ concentration in soil, comparing both incubation periods (Dunnett's test $p \leq 0.001$).

3.3. Plant bioassays with Lollium perenne and Brassica oleracea

We also examined the potential impacts of OP-BSF_{frass} on two relevant plant species: broccoli (*B. oleracea)* and ryegrass (*L. perenne)*. Broccoli was selected as an agricultural crop and ryegrass as a forage crop, allowing for the evaluation of different scenarios to assess whether OP-BSF_{frass} exerted adverse effects with various agricultural applications. This evaluation focused on both species' germination success and early growth stages by considering potential alterations in root length, shoot height, and overall biomass production. Through robust phytotoxicity assays, this study evaluated the possible inhibitory effects of OP-BSF_{frass}, providing an understanding of its influence on plant development and vigour.

3.3.1. Exposure to aqueous soil extracts OP-BSF_{frass}

The validity criteria of phytotoxicity assays using soil were achieved by the germination rate (SGR) > 70% in controls for phase 1 – germination period (OECD, 2006).

3.3.1.1. B. oleracea. SGR (Table 2) of *B. oleracea* was not affected by the extracts obtained from different treatment levels of OP-BSF_{frass} incorporated in soil (Two-way ANOVA, $p = 0.598$). However, it was influenced by the incubation time (Two-way ANOVA, $p < 0.05$) (Table SD 2). However, no specific differences were detected ($p > 0.05$) for SGR from the different incubation periods at the different treatment levels of OP-BSF_{frass} (Table SD 3). In addition, GI of *B. oleracea* (Table 2) was not dependent on the aqueous extract made from different treatments of OP-BSF_{frass} in soil (Two-way ANOVA, $p = 0.169$) but was influenced by the incubation time (Two-way ANOVA, $p < 0.05$). However, similar to our observations for SGR, no significant differences were identified ($p > 0.05$) for GI from the different incubation periods at the different concentrations of OP-BSF_{frass} amendment (Table SD 4).

3.3.1.2. L. perenne. Similar to observations for *B. oleracea*, SGR (Table 2) of *L. perenne* was not affected by both extracts performed with OP-BSF_{frass} incorporated in soil (Two-way ANOVA, $p = 0.677$) and with OP-BSF_{frass} incubation time (Two-way ANOVA, $p > 0.05$). However, a significant effect was observed between the two experimental factors of extracts and time (Two-way ANOVA, $p < 0.05$) (Table SD 5). In addition, GI (Table 2) was significantly affected by both OP-BSF_{frass} concentration in soil (Two-way ANOVA, $p < 0.05$) and incubation time (Two-way ANOVA, $p < 0.05$). In this case, after 32 days of incubation, aqueous extracts performed with OP-BSF_{frass} at treatment levels above 0.3% statistically increased the GI of plants compared to the aqueous extracts of non-amended soil (Dunnett's Method $p < 0.050$), which was not observed in soil incubated for only two days (Dunnett's Method $p > 0.050$). Further, when we compared each aqueous extract performed with OP-BSF_{frass} with 2 and 32 days of incubation, we observed that GI in all the aqueous extracts performed with OP-BSF_{frass} (except for the 0 % - Ct) were statistically different between the two incubation periods tested (Dunnett's Method $p < 0.05$), increasing with the time of incubation (Table SD 6).

3.3.2. Exposure in soil incorporated with OP-BSF_{frass}–Phytotoxkit bioassays

Similar to our results presented above, the validity criteria of phytotoxicity assays using soil were also achieved by the germination rate (SGR) > 70% in controls for phase 1 – germination period (OECD, 2006).

3.3.2.1. Brassica oleracea. SGR of *B. oleracea* (Table 3) was dependent on the OP-BSF_{frass} (Two-way ANOVA, $p < 0.05$) but not from the incubation period (Two-way ANOVA, $p = 0.167$). However, both factors interacted (Two-way ANOVA, $p < 0.05$). The presence of OP-BSF_{frass} in soils led to a statistical increase in SGR of seeds germinated in 2.4 and 4.8% OP-BSF_{frass} in 2-days incubated soils compared to the control (Dunnett's Method $p < 0.050$), along with a decrease in SGR when germinated in 9.6% OP-BSF_{frass} incubated by 32 days, compared with the respective control (Dunnett's Method $p < 0.050$) (Table SD 7).

OP-BSF_{frass} incorporated in soils (Two-way ANOVA, $p < 0.05$) and incubation period (Two-way ANOVA, $p < 0.05$) affected the GI of *B. oleracea,* and both factors interacted (Two-way ANOVA, $p < 0.05$) (Table 3). After 2 days of incubation, GI was significantly increased when OP-BSF_{frass} was applied at 2.4 and 4.8% in soils compared to the non-amended soils (Dunnett's Method $p < 0.050$). In addition, both 2.4 and 4.8% OP-BSF_{frass} application rates statistically differed between both incubation periods, being lower in the 32 days of the incubation period (Dunnett's Method $p < 0.050$), however at the same levels of the

Table 2
Seed germination rate (SGR) and germination index (GI) of *Lolium perenne* and *Brassica oleracea* exposed to aqueous extracts from olive pomace-derived frass (OP-BSF_{frass}) incorporated in soils at different application rates with the respective standard deviation (SD). (*) significant differences in comparison to negative controls (ct) (Dunnett's test $p \leq 0.05$) at each incubation period. (#) significant differences at each treatment level between the two incubation periods (Dunnett's test $p \leq 0.05$).

***Brassica oleracea* - aqueous extract**								
	Day 2				**Day 32**			
Treatments (%w/w)	**SGR (%)**	**SD**	**GI (%)**	**SD**	**SGR (%)**	**SD**	**GI (%)**	**SD**
0 (ct)	96.6	5.7	100	15.6	96.6	5.7	100	10.1
0.3	86.6	15.2	101	39.7	86.6	15.2	91.2	14.6
0.6	100	0	121	14.3	86.6	15.2	104.4	39.5
1.2	96.6	5.7	117	15.3	80	20	90.3	17.7
2.4	90	10	95	18.2	80	20	89.4	23.4
4.8	96.6	5.7	142	24.7	90	10	105.1	21.1
9.6	96.6	5.7	141	25.1	86	5.7	102.6	28.1
***Lolium perenne* - aqueous extract**								
	Day 2				**Day 32**			
Treatments (%w/w)	**SGR (%)**	**SD**	**GI (%)**	**SD**	**SGR (%)**	**SD**	**GI (%)**	**SD**
0 (ct)	90	10	99.4	4	93.3	5.7	99.5	14.8
0.3	76.6	5.7	96.4$^{\#}$	7.8	96.6	5.7	145$^{\#}$	31.2
0.6	90	10	112.2$^{\#}$	6.8	90	0	164 $^{*\#}$	7.1
1.2	86.6	11.5	115.7$^{\#}$	32.9	96.6	5.7	187 $^{*\#}$	1.4
2.4	90	10	116.5$^{\#}$	16.3	93.3	5.7	156 $^{*\#}$	30.7
4.8	90	0	119$^{\#}$	14.4	83.3	5.7	177 $^{*\#}$	17.4
9.6	96.6	5.7	133.7$^{\#}$	13.7	86.6	5.7	189 $^{*\#}$	49.1

Table 3
Phytotoxicity bioassay using *Brassica oleracea* exposed to soil amended with olive pomace-derived frass (OP-BSFfrass) in increased concentrations in phytotoxkits. The seed germination rate (SGR) (Number of germinated seeds/number of total seeds), germination index (GI), and standard deviation (SD) of each treatment have been presented. (*) significant differences in comparison with the respective control (ct) group (Dunnett's test $p \leq 0.05$) at each incubation period. (#) significant differences at each concentration between the two incubation periods (Dunnett's test $p \leq 0.05$).

***Brassica oleracea* in Phytotoxkit test**								
	Day 2				**Day 32**			
Treatments (%w/w)	**SGR (%)**	**SD**	**GI (%)**	**SD**	**SGR (%)**	**SD**	**GI (%)**	**SD**
0 (ct)	80$^{\#}$	±10	98	±5.5	100$^{\#}$	±0	100	±15.7
0.3	86.6	±5.7	110.8	±9.9	96.6	±5.7	115.2	±10.6
1.2	90	±0	112.8	±31.2	100	±0	113.2	±11.7
2.4	96.6 *	±10	157.5 *$^{\#}$	±26.4	100	±0	115.03$^{\#}$	±3.0
4.8	100 *	±5.7	172.1 *$^{\#}$	±17.6	96.6	±5.7	111.5$^{\#}$	±14.1
9.6	93.3$^{\#}$	±5.7	84.9	±5.0	70 *$^{\#}$	±20	72.4	±24.1

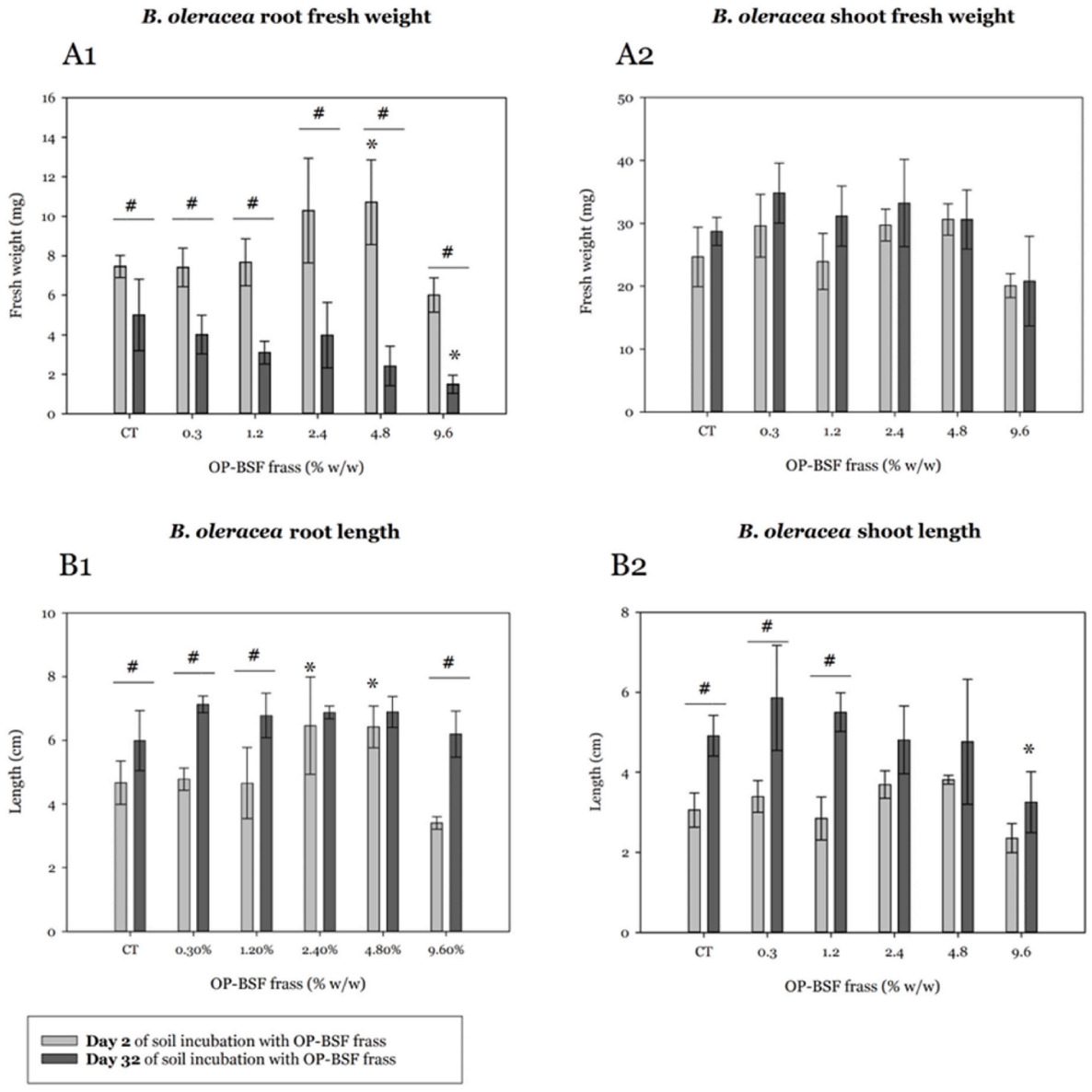

Fig. 2. *Brassica oleracea* root (A1) and shoot (A2) fresh weight and root (B1) and shoot (B2) length of plants exposed after 3 days of germination to soil amended with increased concentrations of olive pomace-derived frass ($OP\text{-}BSF_{frass}$). Two-way ANOVA analysis was conducted based on $OP\text{-}BSF_{frass}$ concentrations and incubation period. (*) significant differences in comparison with the respective control (CT) group (Dunnett's Method $p \leq 0.001$). (#) represents significant differences in the incubation period (Dunnett's test $p \leq 0.001$).

respective control (Table SD 8).

Root FW was dependent on the OP-BSF_{frass} (Two-way ANOVA, $p < 0.05$) and incubation time (Two-way ANOVA, $p=<0.001$). However, two days of incubation of OP-BSF_{frass} in the soil at 4.8% increased the root FW of *B. oleracea* compared with the control (Dunnett's test $P < 0.050$, Fig. 2 – A1). When *B. oleracea* grew with soil amended with OP-BSF_{frass}, root FW was significantly reduced compared to the control soil at the higher OP-BSF_{frass} application rate for Day 32 of incubation in soils (Dunnett's test $P < 0.050$, Fig. 2 – A1) (Table SD 9). Root FW was always significantly lighter in plants that grew in soils incubated for 32d, compared to those incubated by 2d (Dunnett's test $P < 0.050$, Fig. 2 – A1), even in control treatments.

Regarding shoot FW, despite the effects revealed by the Two-way ANOVA results induced by OP-BSF_{frass} (Two-way ANOVA, $p < 0.05$) and incubation time (Two-way ANOVA, $p < 0.05$), post-hoc tests did not detect effects on shoot FW during the 2nd phase (3 days after germination) at both incubation periods (Dunnett's test $p < 0.05$, Fig. 2 – A2) (Table SD 10). For root length, both OP-BSF_{frass} (Two-way ANOVA, $p < 0.05$) and incubation time (Two-way ANOVA, $p < 0.05$) statistically affected the root length of plants, with a statistically significant interaction of both factors (Two-way ANOVA, $p = 0.05$).

Three days after germination, OP-BSF_{frass} at 2.4 and 4.8% in soils induced a statistical increase in root length (cm) in *B. oleracea* on Day 2 of soil incubation with OP-BSF_{frass} in comparison with the respective control (Dunnett's test $P < 0.050$, Fig. 2 – B1) (Table SD 11). The root length of *B. oleracea* was significantly shorter in 2 days of incubation compared to 32 days of incubation in all the concentrations except for 2.4% and 4.8% of OP-BSF_{frass} (Dunnett's test $p < 0.050$). Shoot length was affected by both OP-BSF_{frass} (Two-way ANOVA, $p < 0.05$) and incubation time (Two-way ANOVA, $p < 0.05$). The highest OP-BSF_{frass} application rate in soils induced a statistical reduction in the *B. oleracea* shoot length on day 32 of soil incubation with OP-BSF_{frass} compared with the respective control (Dunnett's test $P < 0.050$, Fig. 2 – B2) and the shoot length was statistically different between both sampling times at Ct, 0.3 and 1.2% (Dunnett's test $P < 0.050$, Table SD 12)

3.3.2.2. Lollium perenne. SGR of *L. perenne* (Table 4) was statistically affected by both OP-BSF_{frass} (Two-way ANOVA, $p < 0.001$) and incubation period (Two-way ANOVA, $p < 0.05$). Specifically, after 2 days of incubation, the highest concentration, 9.6% of OP-BSF_{frass} statistically reduced the SGR of *L. perenne* (Dunnett's Method $p < 0.050$), with no effects on the 32d of incubation (Dunnett's Method $p > 0.050$), compared with the respective controls. Differences between the two incubation periods were observed at 1.2, 4.8, and 9.6% of OP-BSF_{frass} (Dunnett's Method $p < 0.050$), with an increase in SGR with the incubation time (Table SD 13).

GI of *L. perenne* was also significantly affected by the presence of OP-BSF_{frass} in soils (Two-way ANOVA, $p < 0.05$) but not by the incubation period (Two-way ANOVA, $p = 0.163$); there was a significant interaction between factors (Two-way ANOVA, $p < 0.05$). A significant reduction in the GI of plants that grew at 9.6% of OP-BSF_{frass} during 2 days of the incubation period was observed when compared with the respective control (Dunnett's Method $P < 0.050$) (Table SD 14). In addition, a statistical increase in GI was observed at 9.6% of OP-BSF_{frass} in soils, with the time of incubation (Dunnett's Method $P < 0.050$).

The early-stage growth of *L. perenne* demonstrated that OP-BSF_{frass} did not affect root FW in the Two-way ANOVA results (Two-way ANOVA, $p = 0.053$), even though incubation time had an impact on the root weight (Two-way ANOVA, $p < 0.05$). An interaction between these two factors was observed (Two-way ANOVA, $p < 0.05$), with a statistical increase at 1.2% of OP-BSF_{frass} incubated for 2 days compared with the respective control (Dunnett's test $P < 0.050$, Fig. 3 – A1). In addition, at Ct, 0.3 and 9.6% of OP-BSF_{frass} in soils, results indicate a statistical increase when plants grew in 32d incubated soil, compared with the ones that grew in 2d incubated soil (Dunnett's test $P < 0.050$, Fig. 3 -A1) (Table SD 15).

Shoot FW depended on the OP-BSF_{frass} in soils (Two-way ANOVA, $p < 0.05$) but not from incubation time (Two-way ANOVA, $p = 0.094$), although an interaction was observed (Two-way ANOVA, $p < 0.05$). This led to an increase of shoot FW in plants that grew in OP-BSF_{frass} at 1.2% in soils incubated for 2 days, compared to the control (Dunnett's test $P < 0.050$, Fig. 3 -A2) and a decrease in the same parameter when plants grew in soils incubated for 32d when compared with soils incubated by 2 days at concentrations of 1.2 and 4.8% of OP-BSF_{frass} (Dunnett's test $P < 0.050$, Fig. 3 -A2) (Table SD 16).

L. perenne root length was not affected by the presence of OP-BSF_{frass} (Two-way ANOVA, $p = 0.609$) but was altered by the incubation period (Two-way ANOVA, $p < 0.05$). At the concentrations of 0.3, 1.2, 2.4 and 4.8% of OP-BSF_{frass} in soils incubated for 32 days, a reduction of *L. perenne* root length was observed, compared with plants that grew in 2 days incubated soil (Dunnett's test $P < 0.050$, Fig. 3 -B1). In addition, root length was higher when plants grew in soils incubated for 2 days at 1.2% of OP-BSF_{frass}, compared with the control (Dunnett's test $P < 0.050$, Fig. 3 -B1) (Table SD 17).

L. perenne shoot length followed the same responses, with the incubation period (Two-way ANOVA, $p < 0.05$) imposing a statistical effect on the shoot length but not by the OP-BSF_{frass} (Two-way ANOVA, $p = 0.098$). Also, the same 0.3, 1.2, 2.4 and 4.8% of OP-BSF_{frass} in soils demonstrated the same decrease pattern regarding plants from 2 days of the incubation period when compared to 32 days of incubation (Dunnett's test $p < 0.050$, Fig. 3 -B2) (Table SD 18).

In general, the results obtained from the seed germination studies revealed that despite statistically significant reductions in plant growth when high concentrations of OP-BSF_{frass} were applied in the soil (non-relevant for soil application), a general lack of phytotoxicity was found, with frequent significant stimulation of germination parameters at lower concentrations. Early plant growth parameters in soils revealed no adverse effects of OP-BSF_{frass} in both root and shoot FW and length for both tested plants. The highest treatment levels used in the present study are relevant for revealing potentially toxic responses and their

Table 4
Phytotoxicity bioassay using *Lolium perenne* exposed to soil amended with olive pomace-derived frass (OP-BSF_{frass}) in increased concentrations in phytotoxkits. The seed germination rate (SGR) (Number of germinated seeds/number of total seeds), germination index (GI), and standard deviation (SD) of each treatment have been presented. (*) significant differences in comparison with the respective control (ct) group (Dunnett's test $p \leq 0.05$) at each incubation period. (#) significant differences at each concentration between the two incubation periods (Dunnett's test $p \leq 0.05$).

***Lolium perenne* in Phytotoxkit test**								
	Day 2				**Day 32**			
Treatments (%w/w)	**SGR (%)**	**SD**	**GI (%)**	**SD**	**SGR (%)**	**SD**	**GI (%)**	**SD**
0 (ct)	80	±20	99.3	±24.05	80	±17.3	97.8	±9.4
0.3	76.6	±5.7	113.9	±6.29	90	±10	114.6	±22.3
1.2	66.6$^{\#}$	±11.5	106	±26.4	90$^{\#}$	±10	96.3	±4.0
2.4	83.3	±5.7	122.8	±3.2	93.3	±5.7	116.8	±20.2
4.8	70$^{\#}$	±10	98.7	±18	93.3$^{\#}$	±5.7	105.4	±11.3
9.6	26.6 $^{*\#}$	±5.7	35.4 $^{*\#}$	±12	70$^{\#}$	±20	98.9$^{\#}$	±40.7

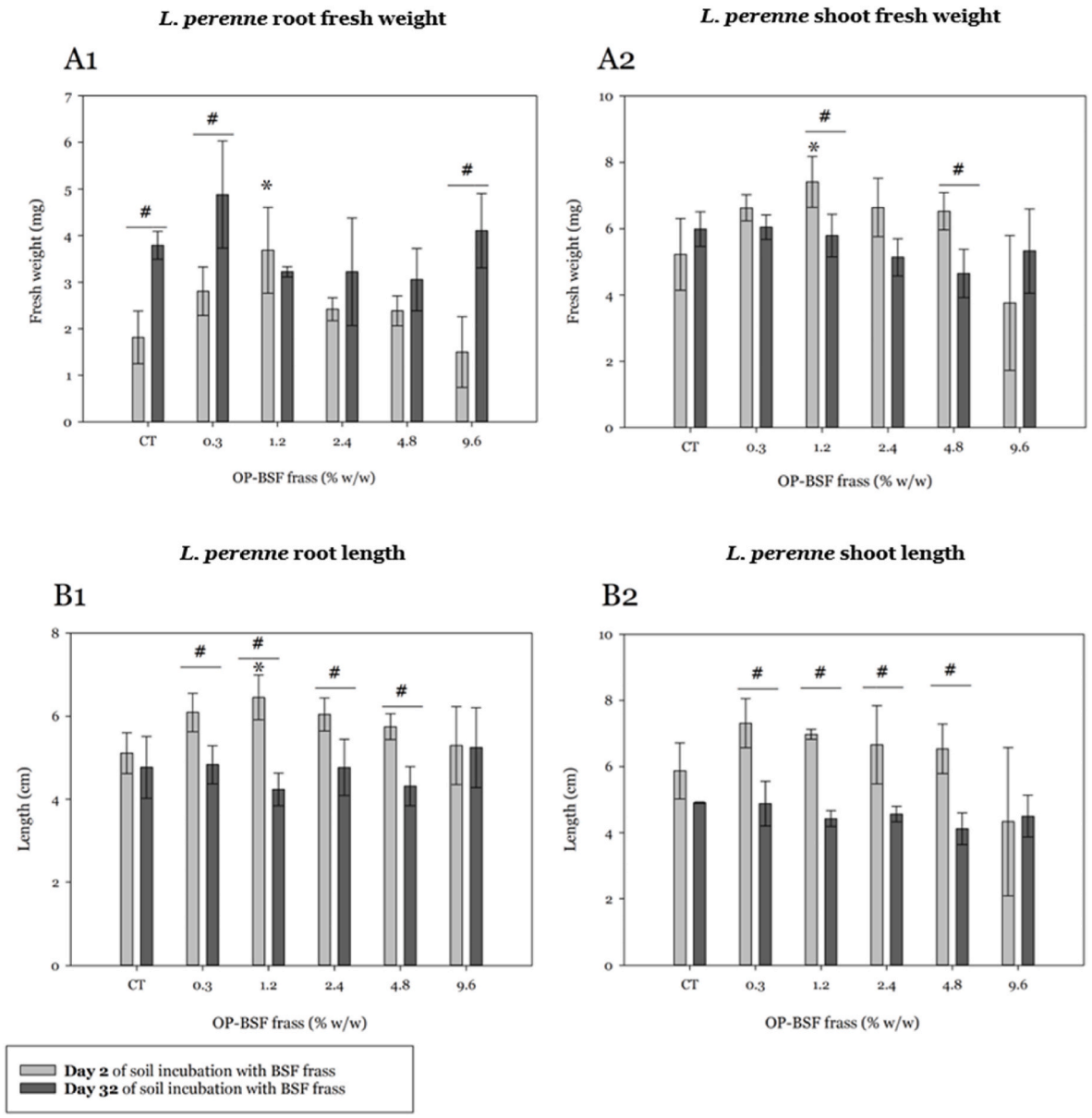

Fig. 3. *Lolium perenne* root (A1) and shoot (A2) fresh weight (FW), root (B1), and shoot (B2) length in plants exposed after three days of germination to soil amended with increased concentrations of olive pomace-derived frass (OP-BSF_{frass}). Two-way ANOVA analysis was conducted based on OP-BSF_{frass} concentrations and incubation period. (*) significant differences in comparison with the respective control (CT) group (Dunnett's Method $p \leq 0.001$). (#) represents significant differences between both incubation periods (Dunnett's Method $p \leq 0.001$).

mechanistic effects. However, one can argue that these higher application rates (above 2.4 % w/w, equivalent to ~48 t/ha, considering an incorporated depth of 15 cm and a soil bulk density of 1.33 g/cm^3 for the reference soil) would not be considered environmentally relevant nor cost-effective for farmers. Our results significantly contribute to the understanding and validating the potential uses of this specific type of OP-BSF_{frass} as an entomofertilizer and its effect on the soil environment. This is crucial for the upscaling of using insects as circularity tools, transforming low-value waste (OP) into valuable products (OP-BSF_{frass}) with significant environmental, social, and economic benefits (Beesigamukama et al., 2022b). For example, for *B. oleracea*, a general decrease in the germination parameters with the increase in incubation time was observed, leading to an increase in the early plant growth stages parameters with the increase in incubation time. *L. perenne* presents the opposite pattern, with an apparent increase in the germination parameters considering the increased incubation time. However, plant growth stages presented different results, with stimulation in root weight and decreased shoot weight with increased incubation time. Still, the length of both plant roots and shoots decreased with the increase in incubation.

It should also be stated that the current study aimed to determine the effects on seed germination and early-stage growth of plant parameters. However, as an organic entomofertilizer, the study of insect frass must be more deeply evaluated. The focus of future studies on the valorization and efficacy of OP-BSF_{frass} is necessary. Previous work demonstrated that organic fertilizer application methods (e.g., solid and liquid application) and N mineralization time could affect crop productivity (Lee, 2010; Li et al., 2017). The incorporation period for entomofertilizer may vary depending on these factors, but it is generally recommended to incorporate it a few weeks before planting. This allows time for frass to break down and for nutrients to become available in a form that plants can use, such as nitrates, nitrites, and ammonia (Watson et al., 2021; Innangi et al., 2017). These results can be a starting point for understanding how OP-BSF_{frass} (or insect frass, in general) can affect different

plants (or crops of interest) with the incubation time and finding the right application time for each application context. Interestingly, in the early plant growth parameters, any statistical effect considering OP-BSF_{frass} application was only seen after 32 days of incubation, where stimulation was obtained for relevant OP-BSF_{frass} doses. However, it should be noted that few adverse effects were observed only at high concentrations. Considering this observation, our results confirmed that, in general, the effects of OP-BSF_{frass}, considering the incubation time, depended on the plant species and parameters evaluated.

As stated, OP-BSF_{frass} did not induce adverse effects on soil invertebrates, a consistent trend across both incubation durations of OP-BSF_{frass} in soils, confirming the positive effects previously observed in plant growth parameters in frass-amended soils (Borkent and Hodge, 2021), despite the adverse impact on other soil invertebrates when exposed to other olive oil industry subproducts, such as the case of olive oil mill wastes (Kovačević et al., 2022). Results from the current studies demonstrated that frass does not affect the survival and reproductive capacity of a model invertebrate *E. crypticus,* and plant germination at concentration ranges close to the realistic conditions for applying this organic fertilizer. These results comply with a recent study focusing on applying BSF frass bioconverted from vegetable waste and cereal on soil media. The study evaluated the early development of the target organisms *Allium cepa* (onion), *Brassica rapa* (turnip), and *Solanum lycopersicum* (tomato), and also the survival and reproduction of invertebrates *E. crypticus* and *F. candida,* which proved the safety under moderate application rate of BSF frass into the soil (Malheiro et al., 2024). However, several factors can influence the composition of BSF frass, such as the dynamics of insect feed nutrients, microbial content, and bioactive compounds of the insect frass (Abd Manan et al., 2024). Based on these findings, case-specific evaluation of the safety and efficiency of different frass is recommended. The current safety evaluation of this frass application in soils, especially knowing the phytotoxic affinity of OP, indicated that this approach allows future soil applications of this specific type of frass. Efficacy of this frass application should be carefully evaluated in the future to optimize the promotion and valorization of OP, producing a high-quality and valuable product with an impact on the mitigation of conventional fertilizers as waste mitigation.

In summary, the current study provides valuable information to inform regulatory authorities, farmers, and other stakeholders regarding the safety of applying olive pomace-based frass as an entomofertilizer in agricultural practices. More information related to the environmental risks of frass application in soil needs to be collected in the future. For example, the ecotoxicological effects on soil invertebrates and plants can offer a first screening approach to the safety of using this OP frass in soils before large-scale application. In addition, it can serve as a starting point for developing the production sector of (organic) fertilizers nationally/locally, reducing the carbon footprint of fertilizer transportation considering the intensive olive oil production in Portugal and other Mediterranean European countries. However, further studies involving a broader range of soil types, different soil model organisms (and thus potential ranges in species sensitivity) and endpoints, soil functional approaches, and environmentally relevant scenarios, including long-term field trials, are required to implement BSF-bioconverted OP frass successfully.

4. Conclusion

This study illustrates the potential use of BSF frass derived from the bioconversion of OP as an environmentally friendly soil amendment. Our results indicate no significant adverse effects on the tested soil health indicators - soil-dwelling invertebrates and plants. Despite minor plant growth reductions at the highest applied frass concentration (9.6% w/w) not relevant in the context of frass application in the field, this study provides unique evidence of an environmentally safe alternative for OP management in Portugal and the broader Mediterranean region. The present work provides a foundation for further integrating OP-frass into the European olive industry value chain, focusing on soil health and sustainability. It aligns with the EU Soil Strategy 2030, which advocates for soil protection and sustainable resource use. Further research involving a broader range of soil organisms and conditions and long-term field trials is necessary to fully address the safety and effectiveness of OP-BSF_{frass} in agricultural practices.

CRediT authorship contribution statement

Amid Mostafaie: Writing – original draft, Methodology, Investigation, Formal analysis. **Ana Rita R. Silva:** Writing – review & editing, Validation, Investigation. **José N. Pinto:** Writing – review & editing, Investigation. **Marija Prodana:** Writing – review & editing, Validation. **Ivã G. Lopes:** Writing – review & editing. **Daniel Murta:** Resources. **Bryan W. Brooks:** Writing – review & editing. **Susana Loureiro:** Writing – review & editing, Validation, Supervision. **Diogo N. Cardoso:** Writing – review & editing, Validation, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2025.124151.

Abbreviations

FAO	Food and Agriculture Organization
OP	Olive Pomace
BSF	Black Soldier Fly
OP-BSF_{frass}	Olive Pomace-based Frass
WHC	Water Holding Capacity
SGR	Seed Germination Rate
GI	Germination Index
RSG	Relative Seed Germination
FW	Fresh Weight
LOQ	Limit of Quantification

Data availability

Data is available at Zenodo. https://doi.org/10.5281/zenodo.11259827

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