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ORIGINAL PAPER

Insect Frass as a Novel Organic Soil Fertilizer for the Cultivation of Spinach (*Spinacia oleracea*): Effects on Soil Properties, Plant Physiological Parameters, and Nutrient Status

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Abstract

Soils naturally low in organic matter need to be amended with recycled organic materials that would boost soil health. In this work, we tested insect frass, the excrements of the yellow mealworm, *Tenebrio molitor*, as a novel organic soil amendment in a spinach cultivation. In a 60-day pot experiment with spinach (*Spinacia oleracea*), we added frass at rates of 0, 0.25, 0.5, and 1%, as well as an inorganic nitrogen-phosphorus-potassium (NPK) fertilizer as a positive control. We found that organic matter increased significantly from 2.7% in the unamended control to 3.2% in the treatment of 1% frass, showing that frass has a valuable role in boosting soil carbon (C). Also with increasing frass rates, N uptake by plant was enhanced, due to the fact that nitrogen use efficiency (NUE) remained unchanged. These findings exhibit the fact that added N was utilized satisfactorily. However, frass effectiveness was not successful in the case of added P and K: the likely reason is the already very high P and K available contents in the unamended soil. Spinach growth was significantly favored by the amendment of frass in a rate-dependent manner: the higher biomass accumulation was found under 1% frass—six-fold higher than NC, while even at 0.25% the yield was significantly higher. Plants at this treatment had the highest chlorophyll a + b content throughout the course of the experiment and also exhibited the highest photosynthetic efficiency and performance of the plant photosynthetic apparatus under frass treatments. The index of total photosynthetic efficiency (PI_{total}) also fared better in the frass-added treatments upwards from 0.25%. We conclude that frass is a highly valuable soil conditioner, given the amble organic C and N that adds to soil, resulting in boosted growth of spinach. As this report is a preliminary study, we suggest that future works should expand frass testing to (a) more plants, where a series of important plant physiology features must be evaluated, and (b) the role of frass in trace element availability.

Keywords Chlorophyll content · Insect farming · Organic fertilizer · Photosynthetic performance · Soil fertility · Yellow mealworm

1 Introduction

Insect farming, i.e., the production of insects for food and feed applications, has gained considerable scientific and business interest during the last decade (Van Huis et al. 2021). Apart from insect meal and insect oil, the insect rearing process results in the production of large quantities of insect frass (Chavez and Uchanski 2021; Poveda 2021). Frass is the “manure” of the farmed insects, and several studies have demonstrated the positive effect of this organic material on soil fertility (Gebremikael et al. 2022; Houben et al. 2021); recently, the potential of frass as plant growth and health promoter has been identified (Barragán-Fonseca et al. 2022; Xiang et al. 2022). The exploitation and

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valorization of this organic residual side-stream can offer an additional income to insect producers (Beesigamukama et al. 2022a); the market of insect frass fertilizers is expected to exceed \$300 million by 2029, growing at a compound annual growth rate (CAGR) of 24.3% from 2022 to 2029 (Meticulous Research 2022). To set the regulatory framework for frass production and its placing in the market as biofertilizer and harmonize its production among EU members, EU recently adopted Regulation 2021/1925 (European Commission 2021), which dictates a sanitizing treatment prior to use, in line with the standards for other processed animal manures. Frass contains nitrogen-phosphorus-potassium (NPK) in high concentrations and also organic matter (Gärttling and Schulz 2022); for example, Houben et al. (2021) reported that the frass of the yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), used in their pot experiment contained a remarkable total N content of 5%, much higher than that expected in any other organic amendment, such as manure (Koutroubas et al. 2020) or biochar (Farid et al. 2022). Its composition, however, varies highly, depending on the insect species (Beesigamukama et al. 2022b), as well as the feeding substrates (Fischer and Romano 2022).

Frass would be suitable for the production of leafy vegetables (cultivation in smaller areas compared to cereals): in the literature, there are reports concerning barley (*Hordeum vulgaris*; Houben et al. 2020), maize (*Zea mays*; Beesigamukama et al. 2020), lettuce (*Lactuca sativa*; Esteves et al. 2022), ryegrass (*Lolium multiflorum*; Menino et al. 2021), and bean (*Phaseolus vulgaris*; Poveda et al. 2019). Frass would be valuable especially in low-fertility-soils, deprived of organic matter. The same is the case with frass-derived micronutrients, as reported in a study that assessed five trace nutrients in three different frass samples, i.e., deriving from black soldier fly, buffalo worm, and mealworm. Values ranged for the 3 frass samples as follows: Boron (B) = 8.09–10.99, copper (Cu) = 8.97–14.57, iron (Fe) = 14.88–17.65, manganese (Mn) = 6.01–19.42, and zinc (Zn) = 7.75–14.99 (units in mg kg^{-1}) (Watson et al. 2021). Moreover, amended organic matter to soil through the addition of frass would generously boost soil carbon (C; Liu et al. 2022), especially in Mediterranean areas which are in desperate want of enhanced organic matter; this would increase soil health. Frass could thus be part of a wider plan of carbon farming (Moruzzo et al. 2021).

In contrast to frass of the black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae) that has been more intensively investigated (Beesigamukama et al. 2020, 2022a, 2022b; Xiang et al. 2022), to the best of our knowledge, the frass produced from *T. molitor* has rarely been studied as a soil amendment for promoting the growth of vegetables in low-fertility and low-organic-matter-content soils, such as those of the Mediterranean region. In this study, we chose a typical agricultural soil of that area and we cultivated spinach (*Spinacia oleracea*), a highly nutritious leafy vegetable never studied before in such a setting. Apart from soil fertility parameters and plant growth, we chose to examine also plant chlorophyll content and photosynthetic performance, two factors that have rarely been reported in the literature in frass-amended soils. For example, in an early work, Kolb et al. (1999) added frass at two rates in potted fir seedlings and found no effect on its photosynthetic performance. The organized examination of the effect of an underexplored (yet very promising) soil improver (i.e., insect frass) to the above-mentioned plant physiological parameters, along with plant growth and soil fertility indices in a test plant never studied before (i.e., spinach), indicates the novelty of this work. Thus, in this work, we explored the effects of added frass to soil nutrient status, to nutrition in spinach, as well to its growth and physiological features. The hypothesis tested was that frass will have an overall benefit in both soil and plant examined characteristics. The aims were to assess the nutrition status of a *T. molitor*-produced frass as a potential soil fertilizer for the cultivation of spinach. We consider this study as an initial step towards a study of a wider scale concerning the beneficial effects of frass in deprived soils; hence, we concentrated our efforts in the most important macronutrients, N, P, and K. In this effect, we studied growth-related and physiological features of the test plant, along with their nutrient content as affected by the relative nutrient status in soil.

2 Materials and Methods

2.1 Insect Rearing and Frass Collection

Tenebrio molitor was bred in the pilot-scale rearing unit of the Laboratory of Entomology and Agricultural Zoology at the University of Thessaly, Greece. Larvae were reared in plastic insect breeding trays (60 × 40 × 14.5 cm) (Beekenkamp Verpakkingen BV, Maasdijk, The Netherlands) under constant conditions, i.e., 27 ± 0.5 °C, 60 ± 5% relative humidity and continuous darkness. Wheat bran was used as feeding substrate, whereas agar (20 g L^{-1}) was provided to larvae three times per week as a moisture source. At harvest time, late-instar larvae were separated from the substrate by sieving through a 1-mm sieve, whereas the substrate was further sieved at 500 μm. This yielded the residual feed in the sieve (of no further use in this study) and the frass passing through the sieve (the actual material used). Frass was collected in plastic bags and stored at room temperature until further analysis and use.

2.2 Experimental Design

A loamy soil (with clay content of 20%, sand 50%, calcium carbonate ($CaCO_3$) = 20%) was selected from the Velestino University Farm in September 2021. As for frass, it contained total N = 4.98%, total P = 2.63%, total K = 1.65% and organic C = 45%, and had pH = 7.8. One kilogram of soil, which made a volume of 800 mL, was weighted and mixed with 800 mL of perlite for better aeration and growth conditions. This procedure was repeated 50 times, and the mixtures were placed into 50 pots of a capacity of 2 L. Ten of these pots were mixed with *T. molitor* frass at 0.25% w/v (2.5 g of frass per pot); there were two other rates of frass, 0.5% and 1%, replicated in 10 pots each. Apart from these three frass treatments, there was one unamended control (thereafter referred to as "negative control") and one "positive control," where inorganic fertilizer was added. The maximum frass dosage of 1% is equivalent to 40 t ha^{-1} in a soil with bulk density of 1.33 g cm^{-3} and at an incorporation depth of 30 cm. This dosage is higher than what would be normally applied in real field conditions, but it is necessary in the settings of a pot experiment so that trends may be identified and examined. These 50 pots comprising 5 treatments (negative control, positive control, 0.25% frass, 0.5% frass, and 1% frass, each replicated 10 times) were watered at 65% of their water holding capacity and left to equilibrate for 1 week. Pots were watered frequently according to their needs, so that soil moisture may be retained stable. After that, seedlings of spinach (*S. oleracea*) were transplanted in the pots (one seedling per pot) and placed outdoors, under ambient environmental conditions. That day was considered the commencement of the experiment, i.e., day 1, at late October 2021. In the positive control, N, P and K were amended with fertilizers as follows: 100 mL pot^{-1} of a solution containing 1 g of the fertilizer 30–10-10 L^{-1}, added on day 4. Also on day 10, we added 50 mL pot^{-1} of a solution containing 4 g of the fertilizer 26–0-0 L^{-1}; the same addition as that of day 10, we performed on days 30 and 50. These additions amended a total of N = 180, P = 4.37, and K = 8.30 mg kg^{-1} soil throughout the course of the 60-day experiment, equivalent to 720 kg N ha^{-1}, 40 kg P_2O_5 ha^{-1}, and 40 kg K_2O ha^{-1}, respectively, assuming a depth of incorporation of 30 cm and bulk density 1.33 g cm^{-3}. Bulk density was selected because it is the actual value determined in the field, while the depth or incorporation represents the usual depth of ploughing.

The plant growth lasted for 60 days, until late December 2021. During the course of the experiment, the pots were watered regularly so that their moisture may be retained stable, as stated earlier, and pots were rotated at regular intervals so that microsite differences in sunlight and temperature may be compensated. During the experiment, we performed the measurements of in vivo chlorophyll *a* fluorescence and chlorophyll (chl) *a* and *b* content in the growing plants (see Sect. 2.3). At the end of the experiment, all aboveground plant biomass was cut 2 cm above soil surface. Biomass was immediately weighed so that fresh weight may be recorded. Then, the biomass was thoroughly washed with tap water to rinse any adhered dust, then washed with deionized H_2O, and placed in pre-weighed clean paper bags.

Soil from the pots was then sampled as follows: at three points on its surface, a core of soil going all the way down to the depth of the pot was taken; these three cores per pot were mixed together into one composite sample representing the respective pot. Soil samples were put in paper bags and placed in an oven at 100 °C until totally dry (typically overnight). Soil samples were then sieved through a sieve with openings of 2 mm and placed in clean plastic vials until analysis (see Sect. 2.3). After the soil samples were dealt with, plant roots were carefully washed with running tap water so that all adhered soil particles may be rinsed. Roots were subsequently washed with d. H_2O and then placed into pre-weighed paper bags. All 100 paper bags with the washed plant biomass (50 with aboveground biomass plus 50 with roots) were then put in a force-draught oven at 70 °C until no weight loss was recorded (typically for 72 h). After that, dry biomass was recorded for all samples. Dried plant parts were then milled to fine powder and stored in clean plastic vials until further analysis (see Sect. 2.3).

2.3 Soil and Plant Analyses

2.3.1 Non-destructive Analyses (in the Course of Plant Growth)

Chlorophyll *a* in vivo fluorescence was monitored at predawn on fully dark-adapted leaves. The pre-dawn measurement was chosen in order to detect any long-term adaptation responses of the photosynthetic apparatus in a period of the year that photoinhibitory conditions (cold and clear days) are possible. All measurements took place on a 10-day basis throughout the experimental period with Handy PEA + fluorimeter (Hansatech Instruments Ltd, UK) on 20 replicates per treatment (2 mature leaves/plant). The illumination of leaves with 3000 μmol photons m^{-2} s^{-1} for 2 s was used to assess the OJIP transients which subsequently were analyzed with PeaPlus Software v.1–13 (Hansatech Instruments Ltd, UK). Here, we present only the PI_{total}, the index of total photosynthetic efficiency, which is derived by the following equation (Strasser et al. 2000):

$PI_{total} = (RC/ABS) \cdot (\varphi P_o/1 - \varphi P_o) \cdot (\psi E_o/1\text{-}\psi E_o) \cdot (\delta R_o/1\text{-}\delta R_o)$.

RC/ABS: reaction center (RC) per absorption flux (for Photosystem II antenna chls).

φP_o: Maximum quantum yield of primary photochemistry.

ψE_o: Probability that a trapped exciton moves an electron into the electron transport chain to intermediate acceptors.

δR_o: Probability that a trapped exciton moves an electron into the electron transport chain from intermediate receptors to final acceptors of Photosystem I.

Total chlorophyll content of spinach leaves was determined with a portable chlorophyll meter (SPAD-502, Minolta-Konica Ltd.), after extracting the corresponding actual chlorophyll concentrations from a calibration curve specifically prepared for spinach. Leaves of various chlorophyll values were extracted with mortar and pestle with 80% acetone and chl a + b concentration per unit area were estimated according to the equations of Lichtenthaler and Wellburn (1983).

2.3.2 Analyses After the End of the Experiment

Soil samples were analyzed for pH (1:2.5 H_2O), electrical conductivity (EC; 1:5 H_2O), organic C (wet oxidation with 0.17 N $K_2Cr_2O_7$ back-titrated with 0.5 M $FeSO_4$), extractable N (1:10 at 2 M KCl shaken for 2 h) in the form of nitrate (NO_3)-N measured in a UV spectrophotometer at 210 and 270 nm, Olsen-P (1:20 at 0.5 M $NaHCO_3$ shaken for 30 min, and analyzed with ammonium vanadomolybdate/ascorbic acid blue in a visual spectrophotometer at 880 nm), and K (as exchangeable, 1:10 at 1 M CH_3COONH_4 pH 7, shaken for 1 h, and analyzed in a flame photometer). All analyses were performed according to Rowell (1994).

Plant samples were analyzed for P and K with dry ashing. A weight of 0.5 g of plant tissues (aboveground and roots alike) was weighed into porcelain crucibles, ashed at 500 °C for 5 h, and the ash was extracted with 20 mL of 20% HCl into 50-mL volumetric flasks, where the volume was made up to the mark with d. H_2O. Phosphorus was then analyzed with the ammonium vanadomolybdate/ascorbic acid blue method in a visual spectrophotometer at 880 nm. Potassium was measured in the same extract in a flame photometer. Nitrogen was measured according to Kjeldahl: 1 g of plant material was weighed into a 300-mL digestion tube with 20 mL of concentrated H_2SO_4 and digested at 420 °C until clear. Then, the digest was distilled with 40% NaOH into NH_3 being transferred into a solution containing 4% H_3BO_3, and the distilled solution was then titrated with 0.1 N H_2SO_4.

Based on these primary data, we calculated the following indices:

Nutrient uptake (mg of nutrient in plant per pot) = Nutrient in plant (mg of NPK per kg plant) × kg plant dry weight per pot.

We then extrapolated the uptake to a field level for reasons of better comparison with other studies, by multiplying nutrient uptake in pots with 4×10^6, soil weight at 1 hectare, assuming an effective depth of soil root-zone of 30 cm and bulk density of 1.33 g cm^{-3}. Nitrogen was expressed in kg N ha^{-1}, while P in kg P_2O_5 ha^{-1} (by further multiplying the uptake at pot level with 142/62), and potassium in kg K_2O ha^{-1} (by further multiplying with 94/78).

NUE (nitrogen use efficiency) = (N uptake in treatments − N uptake in negative control) / Added N (either by fertilizer in positive control or as total N by frass).

2.4 Data Quality Control and Statistical Analysis

Data quality control was addressed with the systematic use of blanks and in-house reference materials of soil and plant. In all data, we conducted a one-way analysis of variance (ANOVA) and post hoc was performed according to Duncan at the level of significance of $p < 0.05$. The statistical package used was IBM SPSS Statistics 26.

3 Results

The addition of frass caused a slight but significant increase in soil pH in the 0.25% treatment (pH = 8.23) compared to the negative control (pH = 7.56; significant at $p < 0.001$); however, further frass addition resulted in the pH value being reduced back to the control (pH at 1% frass = 7.44; Fig. 1A). Soil electrical conductivity (EC; Fig. 1B) was relatively low at negative control (248 µS cm^{-1}), but it increased significantly at 1% frass to 396 µS cm^{-1}. As for soil organic matter (SOM), although there was an unexpected decrease at 0.25% frass relative to the controls, there was a notable increasing trend with added frass at higher dosages. At 1% frass (where SOM was 3.42%), SOM had a significant increase relative to

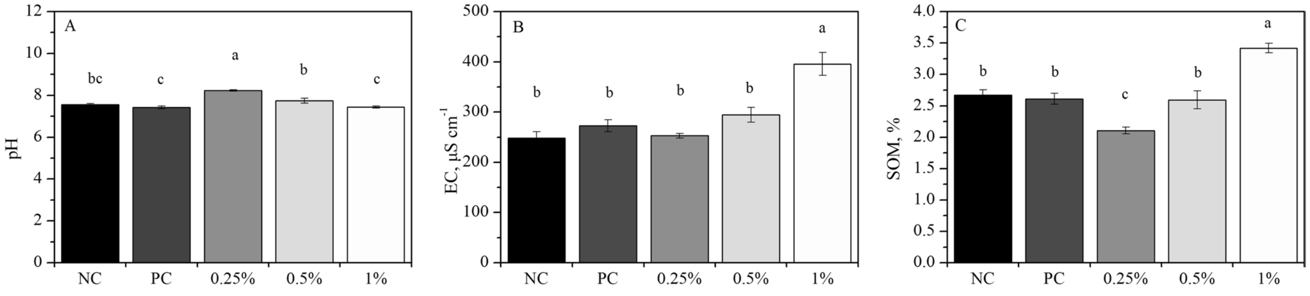


Fig. 1 Soil properties in the various frass treatments concerning **A** soil pH, **B** soil electrical conductivity (EC), and **C** soil organic matter (SOM)

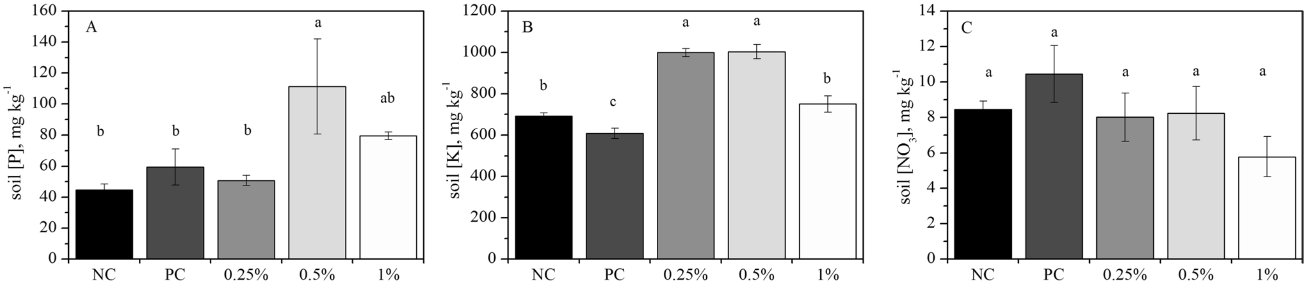


Fig. 2 Soil nutrients in the various frass treatments concerning **A** Olsen-P (presented as [P]), **B** exchangeable K (presented as [K]), and **C** residual N as NO_3-N (as [NO_3])

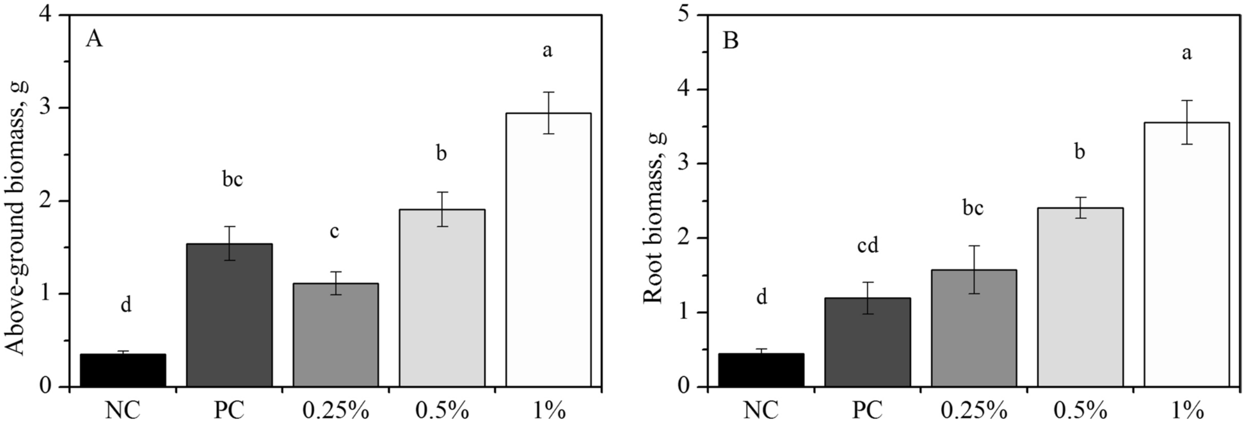


Fig. 3 Plant biomass as produced in the various frass treatments concerning dry matter **A** aboveground tissues and **B** roots

the negative control (2.67%; Fig. 1C). As for the measurements concerning the nutrient status in soil, we found that at negative control, both Olsen-P (44.72 mg kg^{-1}; Fig. 2A) and exchangeable K (692 mg kg^{-1}; Fig. 2B) were relatively high; it is interesting to note that both P and K levels were rather decreased at 1% frass. As for NO_3-N (Fig. 2C), our results show no significant increase in residual soil N with added frass.

The application of 1% frass significantly improved spinach growth in both the above- (Fig. 3A) and below-ground parts (Fig. 3B) compared to the negative control (NC) and positive control (PC), by 88% and 48%, respectively. At NC, plants exhibited the lowest values of biomass, while the aerial biomass accumulated at PC was similar to that in the 0.25% and 0.5% treatments. As for chlorophyll content, spinach leaves in the NC showed a pronounced downtrend already from day 10 of the experiment (Fig. 4). An analogous but considerably less intense trend was also evident in 0.25%-treated plants after day 20. All other treatments sustained high levels of total chlorophylls throughout the experimental period. PI_{total} derived from fluorescence measurements is an index of the total photosynthetic efficiency, incorporating PSI- and PSII-related components and is presented in Fig. 5. An early indication of nutrient deficiency stress on the photosynthetic performance at NC was already evident from day 10, and PI_{total} remained at low levels until

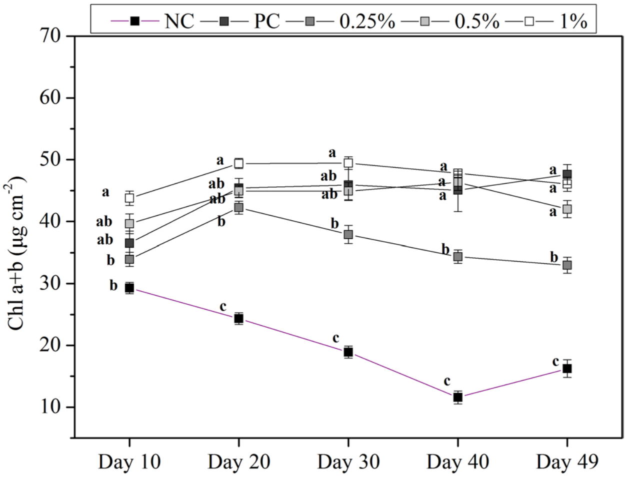
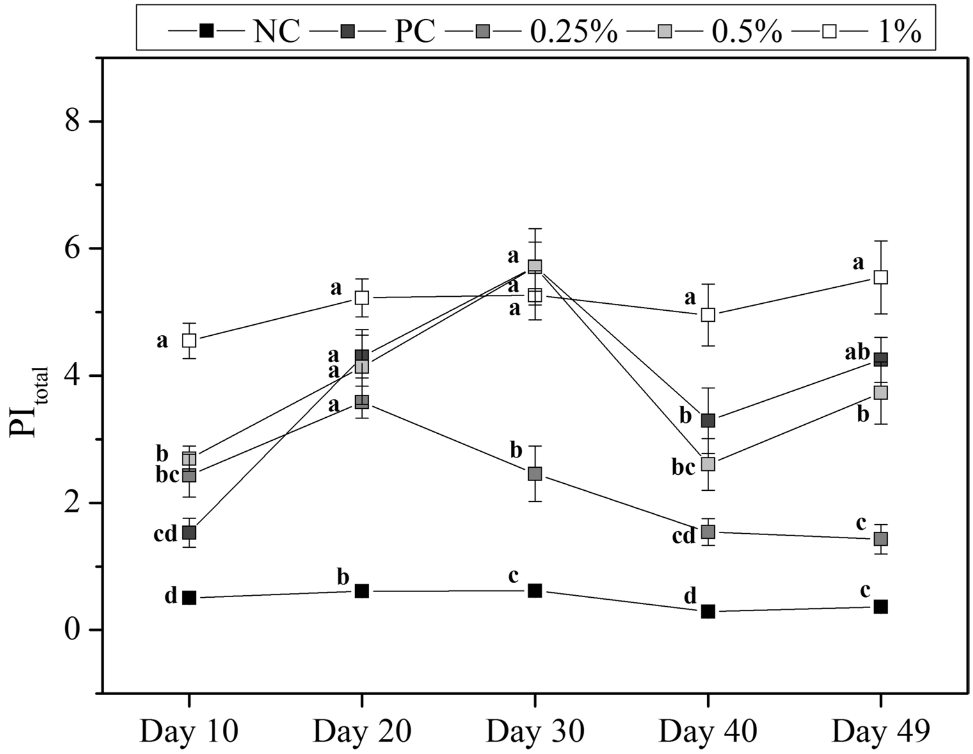


Fig. 4 Concentration of total chlorophylls (presented as chl a+b) in spinach leaves of the various frass treatments throughout the experimental period

the end of the experiment. At the other end, 1%-treated plants showed the maximum PI_{total} throughout the growth period, with all other treatments holding an intermediate position between these two extremes. Interestingly, the profile of chlorophyll content exhibited similar fluctuations with PI_{total} over the course of the experiment, highlighting the contribution of chlorophyll decline in the impaired photosynthetic activity and efficiency of NC and partially of the 0.25%-treated plants.

According to the elemental analysis of spinach tissues, the significant improvement of growth and photosynthetic efficiency under 1% cannot be attributed to enhanced P (Fig. 6A) and K leaf content (Fig. 6B). Indeed, P and K in the aboveground biomass did not seem to have increased with added frass and this was largely the case also with roots. Contrary to what was found regarding the P and K elemental content in plants, their uptake (an indicator taking also into consideration aboveground plant yield) was found

Fig. 5 The fluctuation of photosynthetic efficiency (PI_{total}) in the various frass treatments during the experimental period



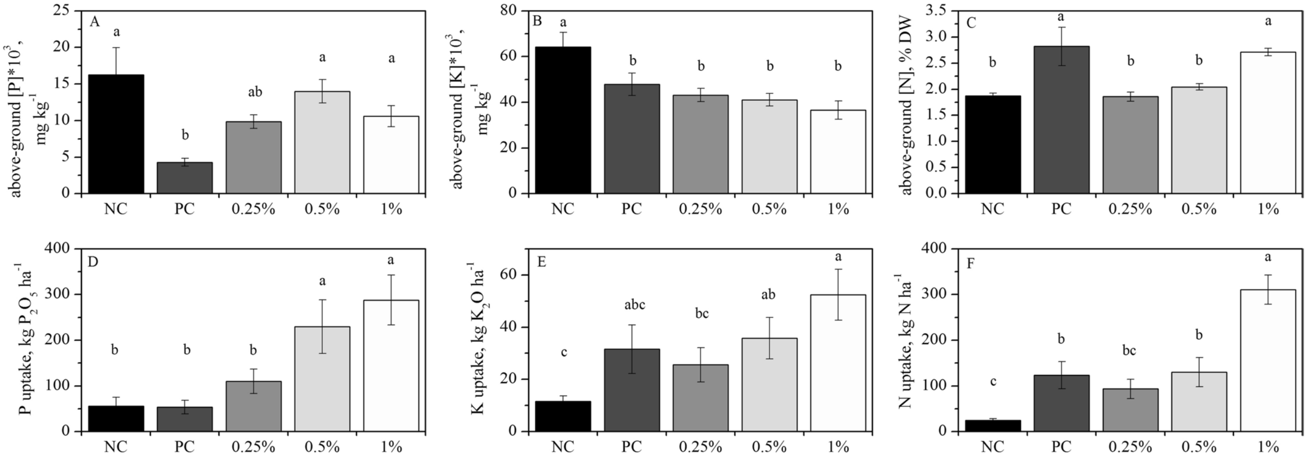


Fig. 6 Plant nutrients in plant aboveground biomass in the various frass treatments: **A** phosphorus concentration ([P], mg kg^{-1}); **B** potassium concentration ([K], mg kg^{-1}); **C** nitrogen concentration in g per 100 g plant ([N], %); **D** phosphorus uptake (kg P_2O_5 ha^{-1}); **E** potassium uptake (kg K_2O ha^{-1}); and **F** nitrogen uptake (kg N ha.$^{-1}$)

to increase significantly and gradually with added frass: P uptake increased from 56.0 (negative control) to 288 (at 1% frass) (kg P_2O_5 ha^{-1}; Fig. 6D) and K from 116.6 to 524 (kg K_2O ha^{-1}; Fig. 6E). As for N uptake (Fig. 6F), similar to P and K, there was a significant increase, but in this case, the increase was much more notable: from 24.8 (negative control) to 310.6 (at 1% frass; units in kg N ha^{-1}), with N uptake at 1% frass exhibiting a twofold increase compared to that at 0.5% (130.1 kg N ha^{-1}).

4 Discussion

Bearing in mind that frass was alkaline, it is rather expected that soil pH increased with frass addition. This means that frass is an excellent amendment for acidic soils, as they tend to be neutralized. At higher application rates, it seems that the frass-borne added N, in the form of NH_4-N, was readily nitrified to NO_3-N. Nitrification is a process typically occurring in well-moisturized, alkaline soils with sufficient NH_4-N levels—all three conditions were present in our soils. Nitrification causes a pH decrease, as 1 mol of oxidized N from NH_4^+ to NO_3^- releases 2 mol of H^+ in the soil solution (Guan et al. 2022). EC indicated a soil without salinity pressures, but EC increased at 1% frass. This shows that added frass indeed burdens soil with salinity due to the frass-borne nutrients (as is also the case with the addition of other organic materials; Zhang et al. 2022). However, such addition is rather harmless in soils with low initial salinity levels, such as is the case here. As for SOM, at field level, taking into account an incorporation depth of 30 cm and a bulk density of 1.33 g cm^{-3}, we found an increase of organic matter of 0.75% (i.e., from SOM 2.67 to 3.42%); this would result in an addition of 17.4 t C ha^{-1}, a major contribution to soil C. Such a figure, which is laboratory-measured, concurs with the amount that would be calculated based on the organic C content in frass (45%) and the amendment dose (1% in a hectare, weighing 4×10^6 kg): the calculated amount would then be 18 t of added C ha^{-1}. Such an effect exhibits the benefit of added frass to soil, similar to that of other organic materials added as conditioners or fertilizers to soil (Wang et al. 2022).

The levels of extractable P and K were well above their critical limits (i.e., implying satisfactory available P and K levels). This is frequently the case with tested soils that have been receiving adequate amounts of fertilizers. The P and K levels further increased with added frass, as was rather expected, due to the high P and K content in frass. The levels of these two nutrients decreased at 1% frass probably due to the high relative assimilation of these nutrients in the test plant. Indeed, P and K uptake was much increased at 1% relative the other treatments (see below in the relevant discussion).

As for soil NO_3-N, it must be noted that it records the residual N remaining in soil at the end of the experiment, after all soil available N has been assimilated by plants. The fact that NO_3-N did not increase significantly with frass is a rather rare finding in similar studies, as usually residual N in soils added with organic materials is found to be increased (Maucieri et al. 2019). In our case, there are two important inter-related factors that would explain this finding: (a) enhanced N assimilation by plant, and (b) non-decreased N use efficiency with added frass, as will be discussed below.

The improvement of crop growth, as assessed by plant yield of the aboveground and root biomass, by frass amendment has been previously reported for barley (Houben et al. 2020), maize (Beesigamukama et al. 2020), pakchoi (Agustiyani et al. 2021), ryegrass (Houben et al. 2021), and chard (Poveda et al. 2019). These works have emphasized on certain parameters that mediated the final effect on crop productivity, being either the insect's diet (Poveda et al. 2019) or the rate of frass application (Houben et al. 2021; Beesigamukama et al. 2020). Our results corroborate the latter findings, since an application rate-dependent increase in both aerial and root biomass was evident in spinach. Beesigamukama et al. (2020) working with 2.5 (equivalent to 0.06% of a soil weighing 4000 t per ha)—7.5 t ha^{-1} (0.19%) of black soldier fly frass found that all rates of frass resulted in increased growth parameters of maize as well as grain yield as compared with commercial organic fertilizer and NC. They suggested, however, different rates according to whether frass will be used to cover the supply of all nutrients in maize cropping system or to provide adequate N to sustain maize productivity. On the other hand, Houben et al. (2021) worked with 5 and 10 t ha^{-1} (0.12% and 0.25%, respectively) of mealworm frass and reported that only the second rate was effective in improving ryegrass growth. This result was attributed to the improved nutrients supply to the soil due to fast N mineralization and stimulated soil microbial activity. All the above-mentioned experiments found that frass resulted in comparable or slightly increased crop growth compared with chemical fertilizers, proving the potential of frass to partially or completely substitute mineral NPK fertilizers. On the contrary, spinach in the present work was significantly favored by 1% frass input which resulted to 48% increase of both aerial and root biomass compared to PC. Species-specific responses are always valid in such experiments (Poveda 2021), yet insect species and diet also play important roles, not excluding the influence of the environmental conditions during crop growth; for example, spinach was grown in open air, not in controlled environment.

Chlorophyll content has been rarely determined in frass-related works, the majority of which focus on agronomic traits such as growth and yield. In a work with field-grown maize, the frass application of 7.5 t ha^{-1} produced plants with the highest chlorophyll concentration in comparison with other organic amendments (Beesigamukama et al. 2020). Poveda et al. (2019) reported that all mealworm diets tested in their experiment outweighed chemical fertilizers in the response of chard chlorophyll content. Although pakchoi growth was greatly favored by black soldier fly frass of 5–15% over NPK fertilizer, no effects on chlorophyll content were evident (Agustiyani et al. 2021). The photosynthetic performance of plants cultivated under frass input has not been evaluated yet in the relevant literature; thus, to the best of our knowledge, this is the first report of the state and efficiency of the photosynthetic apparatus under frass treatments. The chlorophyll a in vivo fluorescence is a state-of-the-art tool which rapidly and non-destructively tracks the dynamics of photosynthetic performance, through the evaluation of the responses of the photosynthetic apparatus to growth conditions and possible stresses. Nutrient deficiency is usually reflected in marked reductions of chlorophyll concentrations, which are in turn accompanied by significant decrease in chl*a* fluorescence (Kalaji et al. 2014). Nevertheless, the latter may also be attributed to sub-optimal levels of certain micronutrients, not determined in the present study, such as Fe, which is not only involved in chlorophyll biosynthesis, but is also structural factor of the photosynthetic apparatus (Rai et al. 2021).

As for the elemental content of P and K in spinach, it was found that they did not increase with added frass. One contributing factor may be the fact that the nutritional level of plants even in the negative control was rather satisfactory: P was 1.6% and K 6.4%, both of which are levels indicating rather unexpectedly high nutrient contents (Hannet et al. 2021). This is in agreement with the finding of available P and K in soil, as was discussed earlier. We may further hypothesize that there are also other factors not evaluated in the present study, which resulted in the improved spinach performance under the highest application rate: Frass-derived micronutrients or even C that was mineralized may have accounted for this outcome. Houben et al. (2021) performed an incubation experiment for 32 days and demonstrated that fast decomposition of easily degradable organic compounds enriched soil with C and N, while frass significantly stimulated soil microbial activity. Corroborating the latter, Poveda et al. (2019) showed that bacteria and fungi with plant growth promoting (PGR) characteristics were present in frass and concluded that these microorganisms played an important role in improving chard growth. Although this was the case with P and K contents in plant, N was found to be significantly higher at 1% frass (2.71%) and at positive control (2.82%) compared to the negative control (1.87%), with the former two treatments having no significant difference between them (Fig. 6C). This increase was likely caused due to the fact that available N in the negative control was low—N is a very mobile element and is expected to decrease down to almost zero after each growing season; unlike P and K which have a high residual effect in soil (Chai et al. 2022).

As for the uptake of P and K, it is noteworthy that 0.5% frass and 1% did not differ in either nutrients, indicating that 0.5% would suffice to give the desirable boosted uptake of P and K. Nitrogen uptake at 1% had a two-fold increase compared to that at 0.5% frass. This is a remarkable achievement of our organic amendment, indicating the high importance regarding its use as a source of N to plants. This was the case due to the fact that N use efficiency (NUE) did not seem to be decreased with added frass (13.7% at negative control; 14.4% at 1% frass; data not shown), although the effectiveness of frass as a source of plant available N is rather low (as judged upon NUE) compared to the positive control (where NUE was 20.2%; expectedly high for an inorganic fertilizer treatment; de Paz et al. 2022).

5 Conclusions

We tested frass, a novel organic soil amendment, as a source of nutrients in a spinach cultivation. Frass is very high in total nitrogen, phosphorus, potassium, and organic C, making it especially beneficial for low-organic-matter soils, such as those found in the Mediterranean region. We showed that added organic matter to soil was vast, amounting up to an equivalent of 17.4 t C ha^{-1}—this indicates the valuable role of frass in increased soil C. Also added N was utilized by plant in a satisfactory manner—with increasing frass rates, the residual N in soil in the form of NO_3-N was not increased, while N uptake in plant was enhanced, due to the fact that nitrogen use efficiency (NUE) remained unchanged (although NUE is usually expected to decrease with added soil N). Significant improvement of spinach growth and photosynthetic efficiency under 1% frass treatment was evident, while total chlorophyll content was sustained at high levels throughout the experimental period. Nutritional effectiveness of frass was not as successful concerning P and K, given the fact that their levels in the unamended soil were already rather enhanced. We conclude that frass is a promising soil amendment: it is a highly valuable source of organic C and N to soils deprived from organic matter, and this would increase soil health and soil C, while improving the growth of leafy vegetables (here, spinach). We further suggest that frass should also be tested in other plants, taking into account more detailed plant physiology parameters, where frass-amended trace nutrients will also be evaluated. Also field-based studies should be conducted with more conservative frass dosages which would be closer to real conditions. Taking into consideration that even from the treatment of 0.25% of frass the beneficial effects on growth and physiological features were evident, it is likely that at field scale, the necessary rates will be lower than those used here. We perceive this work as an initial step towards a full evaluation of frass, given the promising findings reported in this work.

Author Contribution V. Antoniadis: designed the experiment, did computations of the data, and wrote the manuscript with contribution from E.L. and C.I.R. A. Molla: conducted the experiment and did the laboratory analyses. A. Grammenou: conducted the experiment and did the laboratory analyses. V. Apostolidis: conducted the experiment and did the laboratory analyses. C.G. Athanassiou: designed and overviewed the experiment. C.I. Rumbos: contributed in the writing of the manuscript. E. Levizou: designed the experiment, did data analysis, did computations of the data, and contributed in the writing of the manuscript. All authors read and approved the final manuscript.

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Data Availability Data are available upon request.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Our study does not contain any data from an individual person.

Conflict of Interest The authors declare no competing interests.

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