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# Mealworm Frass as a Potential Organic Fertilizer in Synergy with PGP-Based Biostimulant for Lettuce Plants

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**Abstract:** This study explores the potential use of frass, the larval excrement residue obtained from mealworm rearing, as organic fertilizer for crops. Its high organic matter content means that its joint application with a biostimulant based on efficient microorganisms, favoring its mineralization, is of interest. An experiment with lettuce plants (*Lactuca sativa* L.) was conducted with two factors and six replicates under greenhouse conditions. The first factor was frass amendment at 0%, 1%, 2.5%, and 5% of the peat substrate, and the second factor was a *Bacillus*-based BS at two levels, with and without efficient microorganism application. The results reveal that frass shows great potential as an organic fertilizer, providing macronutrients and increasing lettuce aerial biomass, although its effect is mediated by the application rate. Rates of 2.5% or higher proved negative for lettuce plant growth, especially root development, probably due to an increased incidence of potentially pathogenic fungi. The negative effect of medium–high frass rates was counteracted by the addition of a PGP-based biostimulant, enhancing lettuce plant nutrient uptake, aerial biomass, and quality in terms of succulence, but also favoring microbial diversity in the rhizosphere, increasing the incidence of beneficial microorganisms, and decreasing potentially pathogenic fungi. This positive synergy observed between frass and the PGP-based biostimulant is of interest for the design of new organic fertilization strategies.

**Keywords:** *Tenebrio molitor*; organic amendment; efficient microorganisms; sustainable agriculture; biofertilization; *Lactuca sativa* L.



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## 1. Introduction

The ability of the soil to recycle nutrients is essential for fertility maintenance, and microorganisms are primarily responsible for it due to their involvement in nutrient cycling [1]. However, agriculture based on chemical fertilizers underestimates the need to maintain a good level of organic matter in the soil, which affects the conservation of its physico-chemical properties and, therefore, its production capacity. The current management of mineral fertilizers thus jeopardizes the sustainability of agriculture, as it contributes to the progressive deterioration of soils, which can lead to soil depletion and even salinization or desertification [2].

World agriculture is currently seeking to achieve sustainability by trying to reduce the use of non-renewable resources, preserving natural diversity, and stimulating the proliferation of a vigorous population of microorganisms to regain soil balance [3]. A model based on minimal use of external inputs is proposed, which leads to a reduction in the use of synthetic fertilizers while promoting the use of soil protection and conservation

techniques. In this context, one of the challenges facing modern agriculture is to propose alternatives to the use of mineral fertilization, in line with the concept of circular economy. The European legislative proposals on waste, adopted in 2015, set a clear common EU target for increasing recycling from farming and agricultural waste. In this regard, the high nutritional value of insects and their resource efficiency in converting organic matter into protein makes their rearing for human consumption a topic of increasing interest. Compared to other livestock, insect farming has a much smaller ecological footprint [4] due to its low feed-to-meat ratio. The use of insects as protein sources would be a major resource in favor of food security. In this regard, the new EU food regulation, Regulation 2015/2283 (in application since 1 January 2018), clarifies the legal status of insects and their derived products. More recently, in January 2021, the European Food Safety Authority confirmed the safety of dried yellow mealworm (*Tenebrio molitor* larvae) as a novel food under Regulation (EU) 2015/2283, making it the first legally approved and endorsed insect-based food in the EU. Consequently, insect production is expected to grow considerably in the coming years, due to the increasing need to find alternative protein sources. This will lead concomitantly to a rise in waste production derived from this insect farming, in the form of insect feces, or frass. This byproduct of the rearing process is a mixture of larval excrement, undigested organic waste, and shed exoskeletons, which has the potential to be used as a soil amendment or organic fertilizer [5]. It is known that frass deposition in natural conditions increases soil fertility as result of its high nutrient and labile C content [6,7]. Frass has excellent potential as a partial or complete substitution for mineral NPK fertilizer [8–10] due to its fast mineralization rate and its high content of readily available nutrients. It is similarly effective in supplying N, P, and K, thus sustaining biomass production as an NPK fertilizer [11], also containing micronutrients such as Cu and Zn, unlike many NPK-based mineral fertilizers. Furthermore, it potentially contains microorganisms that promote plant growth when the frass has not been sterilized [11]. Nevertheless, there is currently very little information on using the frass produced by insect farms as organic fertilizer to improve soil fertility and crop yield. To date the potential of frass as a fertilizer has been proved in a few studies [9,11–13], but there is a need to determine frass application rates and optimal timing according to crop demand. This knowledge gap highlights the urgent need to focus research efforts on the potential of frass as fertilizer before a significant rise in the industry takes place [11]. This research is also framed by the necessity to find alternatives to conventional mineral fertilization that fit better with the principles of circular economy. In addition, in the case of lettuce plants, organic fertilization is a good strategy for avoiding the nitrate accumulation in lettuce leaves that results from applying mineral fertilizers [14], so frass is a good alternative to NPK fertilization in lettuce.

The new European fertilizer regulation (EU 2019/1009) promotes agriculture with fewer synthetic fertilizers, favoring the reuse of organic waste, and encourages alternatives capable of improving the efficiency of crops and the availability of nutrients that already exist in the soil, such as biostimulants. In reducing the use of synthetic products, the development of products based on microorganisms, in particular bacteria with plant-promoting properties, is of great importance. In this sense, biostimulants based on plant growth promoters (PGPs), which comprise countless species of bacteria (PGPBs) and fungi (PGPFs), are proposed as an alternative to increase crop yield and quality while avoiding the environmental problems associated with excessive chemical fertilization. In recent years, research has focused on efficient microorganisms that promote plant growth, colonizing the extracellular or intracellular rhizosphere environment of plants and improving crop development through direct and indirect mechanisms [15]. Those mechanisms include nutrient solubilization, nitrogen fixation, phytohormone secretion [16–18], and even increasing plants' resistance to abiotic or biotic stress [19–23]. A good level of organic matter in the soil favors the activity of efficient microorganisms, and increases in their population accelerate the organic matter degradation rate [24], making nutrients available to plants faster than with non-stimulated mineralization.

Considering the potential of frass as an organic fertilizer on the one hand and the ability of PGP-based biostimulants to improve plant nutrition on the other, the combined application of frass and biostimulants formulated with PGPs can be expected to greatly increase plant yields. This study seeks to investigate the fertilizer potential of mealworm (*Tenebrio molitor*) frass in lettuce, applied alone and in combination with a PGP-based biostimulant, with a goal of facilitating the development of crop management protocols to enable farmers to adopt good practices to ensure agricultural sustainability by increasing crop yield and quality, while minimizing costs and environmental pollution. To our knowledge, this is the first study that reports data on the combined application of frass and efficient microorganisms.

## 2. Materials and Methods

### *2.1. Frass and Biostimulant*

*Tenebrio molitor* excrement (frass) was provided by Insekt Label Biotech S.L. Frass was obtained after growing mealworm larvae fed with whole wheat flour supplemented with vegetables in open trays for 9 weeks. After harvesting and sieving, the frass was air-dried for 1 week and sieved (1 mm) before application or analyses. The physicochemical characteristics of the frass are stated in Table 1. Elemental nutrient concentrations were determined via an Optima 8000 inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer, Baesweiler, Germany). Nitrate and ammonium contents were measured using an AA3 HR Nutrient Autoanalyzer (SEAL Analytical GmbH, Norderstedt, Germany).

**Table 1.** Physicochemical characteristics of frass (n.d., not detected; MPN, most probable number).

| | | | |
|---|---|---|---|
| Dry matter (%) | 89.95 | Total calcium (mg/L) | 105 |
| Organic matter (%) | 86.4 | Sulphate (mg/L) | 338 |
| pH (1/5 *v*/*v*) | 5.8 | Phosphate (mg/L) | 5780 |
| Organic C (%) | 50.2 | Magnesium (mg/L) | 460 |
| Total N (%) | 3.64 | Carbonate (mg/L) | <5 |
| C/N | 13.8 | Bicarbonate (mg/L) | <5 |
| Ammonium (mg/L) | 192 | Chloride (mg/L) | 314 |
| Nitrate (mg/L) | 27.9 | Potassium (mg/L) | 3440 |
| Conductivity (mS/cm) (1/5 *v*/*v*) | 18.2 | Sodium (mg/L) | 804 |
| Density (g/cm$^3$) | 1.59 | Boron (mg/L) | 2.08 |
| Humic acids (%) | 7.52 | Manganese (µg/L) | 2760 |
| Fulvic acids (%) | 19.6 | Iron (µg/L) | 4750 |
| Total humic extract (%) | 27.1 | Zinc (µg/L) | 1200 |
| *Escherichia coli* (MPN/g) | n.d. | Copper (µg/L) | 2730 |
| *Salmonella* (25 g) | <1 MPN/g | | |

The PGP-based biostimulant applied, SullicaB® (Corteva AgriSciences™, Spain), is a water-soluble product based on efficient microorganisms developed in a liquid medium, stable at room temperature and formulated for direct application. It contains $12 \times 10^7$ colony-forming units (CFU) per ml of product of four different *Bacillus* strains: *B. licheniformis* (40%), *B. safensis* (20%), *B. pumilus* (30%), and *B. velezensis* (10%). Together, they provide the ability to solubilize soil P and K, fix atmospheric $N_2$, and produce indole acetic acid. C and N organic contents are less than 1% and 0.3%, respectively, and the ammonium content is less than 0.018 mg/L, i.e., no nutrient was added by applying the biostimulant. Indeed, neither plant hormones (gibberellins, cytokinins, and auxins) nor vitamins, except B1 (1.21 mg kg$^{-1}$) hormones, were detected in the PGP-based biostimulant. This product is registered in the Register of Fertilizing Products of the Ministry of Agriculture, Fisheries and Food of the Government of Spain in the category of non-mycorrhizal microorganisms (n. F0004881/2031).

### 2.2. Pot Experimental Design

The greenhouse experiment was designed with lettuce (*Lactuca sativa*, Batavia type). Lettuce seeds were sown in expanded polystyrene trays on peat substrate. They grew with irrigation on demand, only with water, for one month, and were then transplanted to 2 L pots filled with the different substrate mixtures, where they were grown under the different conditions assayed for seven weeks. The trial consisted of two factors with six repetitions. The first factor, frass, included four levels of frass with increasing concentrations in peat substrate (% *v*/*v*): 0% or peat substrate alone, 1% frass, 2.5% frass, and 5% frass. The second factor, biostimulant (BS), comprised two levels: irrigation with water and irrigation with BS. In the BS treatment the seedling roots were immersed for 15 min in a solution of 10% BS before transplanting. Two weeks later, 250 mL of BS diluted to 1% was applied per pot, following the manufacturer's recommendation. As a result, eight treatments were assessed, with six pots each. Three of them were used for biometrical analysis and the other three for physiological and biochemical parameters. The plants were watered on demand until harvest, for the seven weeks of duration of the assay.

### 2.3. Plant Sampling and Analyses

At the beginning and end of the experiment, the pH and electrical conductivity of the substrate mixture were analyzed. The substrate was mixed with deionized water at a ratio of 1:5 (*v*/*v*), stirred for 30 min at 100 rpm, and left to decant for another 30 min. At the end of the experiment three plants per treatment were used for morphological characterization: plant height, root diameter at neck level, and canopy diameter. Roots, stems, and leaves were then weighed separately and dried at 80 °C for 48 h. The dried material was ground and sieved through 0.12 mm mesh stainless steel, homogenized, and re-dried for at least 2 h at 80 °C. Then, 0.5 g samples were weighed out for analysis. Samples were wet-digested in a mixture of 1% $HNO_3$ + 2% $HClO_4$ (85:15, *v*:*v*) under a temperature gradient ranging from ambient to 190 °C for 12 h. The resulting solution was used to determine mineral content via ICP-OES (THERMO ICAP 6500 DUO). The nitrogen contents of the subsamples (100 mg) of the dried homogenate were measured with an elemental analyzer (LECO CN 828). For physiological and biochemical determinations, the three remaining plants per treatment were used. Leaf succulence was calculated using three leaf disks of 1 cm diameter per plant and expressed as the difference between fresh and dry weight divided by the area (mg $H_2O$ $cm^{-2}$ leaf). Relative water content (RWC) was calculated using three leaf disks of 1 cm diameter per plant. After fresh weight determination, the discs were floated in the sampling can in distilled water for 3 h at room temperature (about 15 °C) with no lighting. Following surface drying with absorbent paper toweling and turgid weight determination, the discs were oven-dried at 85 °C overnight and reweighed. Electrolyte leakage was measured to assess the stability of leaf cell membranes. This technique is based on the increase in cellular membrane permeability and concomitantly greater electrolyte diffusion from cells when leaf tissue is injured by a stress situation [25]. Five leaf disks of 0.8 cm diameter per plant and five root pieces of 1 cm in length and identical diameter per plant were thoroughly rinsed with distilled water, placed in 10 mL of deionized water, and maintained at 20 °C for 24 h, when the electrical conductivity of the solution was measured (T1). The samples were then autoclaved for 15 min at 120 °C and their electrical conductivity was measured again (T2). Electrical conductivity (EC, %) was expressed in relative units according to the following equation: (T2/T1) × 100. Chlorophyll a, chlorophyll b, and total carotenoids were determined using two disks of 6 mm diameter per plant extracted with 2 mL dimethyl sulfoxide (DMSO) for 2 h at 80 °C. Absorbances at 750, 665, 649, and 480 nm were determined, and the different pigment concentrations were calculated as in Wellburn [26]. From the remaining fresh leaf and root material of the three plants, aliquot samples were frozen for further biochemical determinations, as follows. Aliquots of frozen leaf tissue (0.1 g fresh weight) were ground in a cold mortar using 1 mL of aqueous buffer (50 mM Tris–HCl (pH 7.6), 1 mM $MgCl_2$, 1 mM EDTA, 4 µM leupeptine, and 14.3 mM β-mercaptoethanol). The homogenates were centrifuged at 16,100× *g* for 20

min at 4 °C. The supernatant was collected and used to determine soluble proteins and nitrate content. Leaf soluble protein content was determined by the protein dye-binding method of Bradford [27], and leaf nitrate content was determined as in Cataldo et al. [28]. Aliquots of fresh root tissue (about 1 g of fresh root) were used in duplicate per plant to determine acid and alkaline phosphatase activities by adding 4 mL of buffer (pH 6.5 for acid and pH 11 for alkaline phosphatases) and 1 mL of 0.1 M disodium phenyl phosphate as substrate. The mixture was incubated at 37 °C for 1 h and then 1 mL of 0.5 M $CaCl_2$ and 0.5 M NaOH was added to stop the reaction. The mixture was filtered through Whatman n°2 filter paper and absorbance was then measured at 420 nm. The absorbance of filtrates was compared with *p*-nitrophenol standards. For each assay, a control was included to account for non-enzymatic substrate hydrolysis.

### *2.4. Determination of Rhizosphere Cultivable Microorganism Determination*

From the three plants per treatment used for physiological and biochemical determinations, aliquot samples of 1 g of fresh root weight were washed with sterile water and homogenized in 100 mL of sterile saline solution (0.9% NaCl, pH 7.2), and 1:10 serial dilutions were prepared up to dilution $10^{-5}$. From each dilution, 100 µL was spread on Petri dishes with different growing media. Filamentous fungi and yeasts were isolated on Rose Bengal (RB) medium with chloramphenicol, while Luria–Bertani medium (LB) was used to isolate general bacteria. For each treatment, dilution, and culture medium, three dishes were prepared in replicate and incubated at 28 °C in darkness for four days for LB plates and seven days for RB plates. The main morphotypes (MFs) were differentiated and the number of colony-forming units (CFU $g^{-1}$ of root) was determined for each MF.

### *2.5. Statistical Analysis*

Statistical analyses were carried out using SPSS v 26.0 software (Chicago, IL, USA). Data normality and variance homogeneity were checked. Plant parameters for the different treatments assayed were compared using ANOVA variance analysis, as well as Duncan's test for comparison of mean values at a significance level of $p < 0.05$. Student's *t* test was also used to compare irrigation with and without biostimulants within each frass level in the substrate. PRIMER 7 software [29] was used to analyze the cultivable microbiota communities, with square root overall transformed data. The effect of frass and BS factors on microbial communities was assessed with a Permutational Multivariate Analysis of Variance (PERMANOVA), based on a Bray–Curtis dissimilarity matrix. To show the similarity of the cultivable microbial communities of the different frass treatments a Multidimensional Scaling (MDS) using bootstrap averages analysis was performed. Principal Coordinate Analysis (PCoA) was performed to show the similarity of microbial communities due to BS application. The Shannon Diversity Index was also calculated for each treatment.

## 3. Results

### *3.1. Chemical Characteristics of the Substrate*

Frass amendment had a significant effect on the physicochemical parameters of the substrate (Figure 1), as the addition of frass strongly increased the substrate electrical conductivity (EC) proportional to the rate applied. It also increased the initial pH value of the substrate from 4.9 to 5.2, independent of the frass rate (Figure 1). By the end of the experiment, pH values had increased by 1–2 points, while the EC values decreased, especially at low frass rates. When the BS was applied, no differences in substrate pH or EC were observed with respect to the untreated substrate.

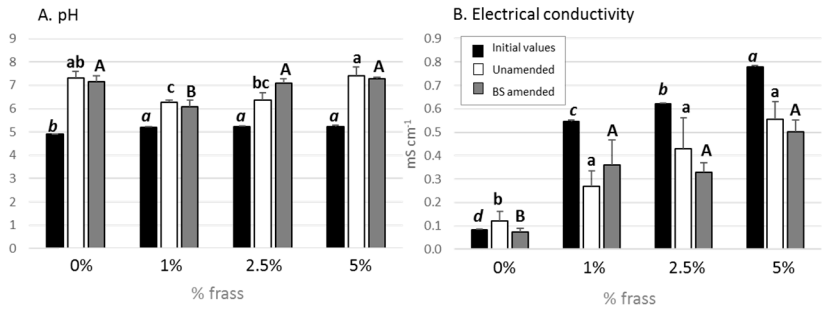


**Figure 1.** Substrate pH (**A**) and electrical conductivity (**B**) values at different frass rates (0%, 1%, 2.5%, and 5%). Black bars = initial substrate conditions; white bars = untreated substrate at the end of the experiment; grey bars = BS-treated substrate at the end of the experiment. Different letters indicate significant differences using the Duncan test ($p < 0.05$; $n = 3$) within each condition (italic lowercase for initial substrate conditions, lowercase letters for unamended control substrate, and uppercase letters for BS-amended substrate).

### *3.2. Lettuce Plant Biometrical Parameters*

Both frass and BS addition influenced the biometric parameters of lettuce plants, as indicated by partial $\eta^2$ values (Table 2). Frass addition increased shoot fresh weight at low rates and decreased root fresh weight and root collar diameter at medium–high rates but showed no effect in terms of dry matter (Table 2, Figure 2). Lettuce plant height, canopy diameter, and leaf succulence also remained stable independent of the frass rate applied.

BS amendment promoted shoot development, leading to plants with greater shoot biomass and greater succulence in pots without frass application, but not affecting plant height or diameter. However, the joint application of frass and BS led to lettuce plants with double the aerial biomass whatever the frass rate applied, and significantly increased canopy diameter (Figure 2). The combined application also improved plant height at medium–high frass rates and increased plant succulence at low–medium rates. Root fresh weight decreased with frass application, but this decrease was somewhat mitigated with the combined application of BS at the 5% frass rate (Figure 2).

### *3.3. Lettuce Plant Biochemical Parameters*

The two factors tested, frass and BS, significantly affected the photosynthetic pigment content of lettuce plants, and partial $\eta^2$ values indicate that the effect is similar for both factors (Table 2). Frass amendment at a rate of 1% increased the total chlorophyll content by 85% and the total amount of carotenoids by 60%. However, medium–higher frass rates led to values similar to frass-unamended plants (Figure 3). BS addition also led to a positive effect, increasing total leaf chlorophyll content by 82% and the amount of carotenoids by 61% in frass-unamended lettuce plants. The combined application of frass and BS tended to increase leaf pigment content, with that increase being significant for total chlorophyll content at a medium–high frass rate and for total carotenoids at the 2.5% frass rate.

Regarding N assimilation by lettuce plants, partial $\eta^2$ values observed for nitrogen compound contents, such as nitrate, soluble protein, and even total N content in leaf and roots (Table 2), indicate that only frass amendment shows a significant effect. Frass amendment increased soluble protein and leaf nitrate concentration by 95% and 30%, respectively, independent of the frass rate (Figure 3). The application of the BS, whether alone or in combination with frass, had no effect on N compound concentration.

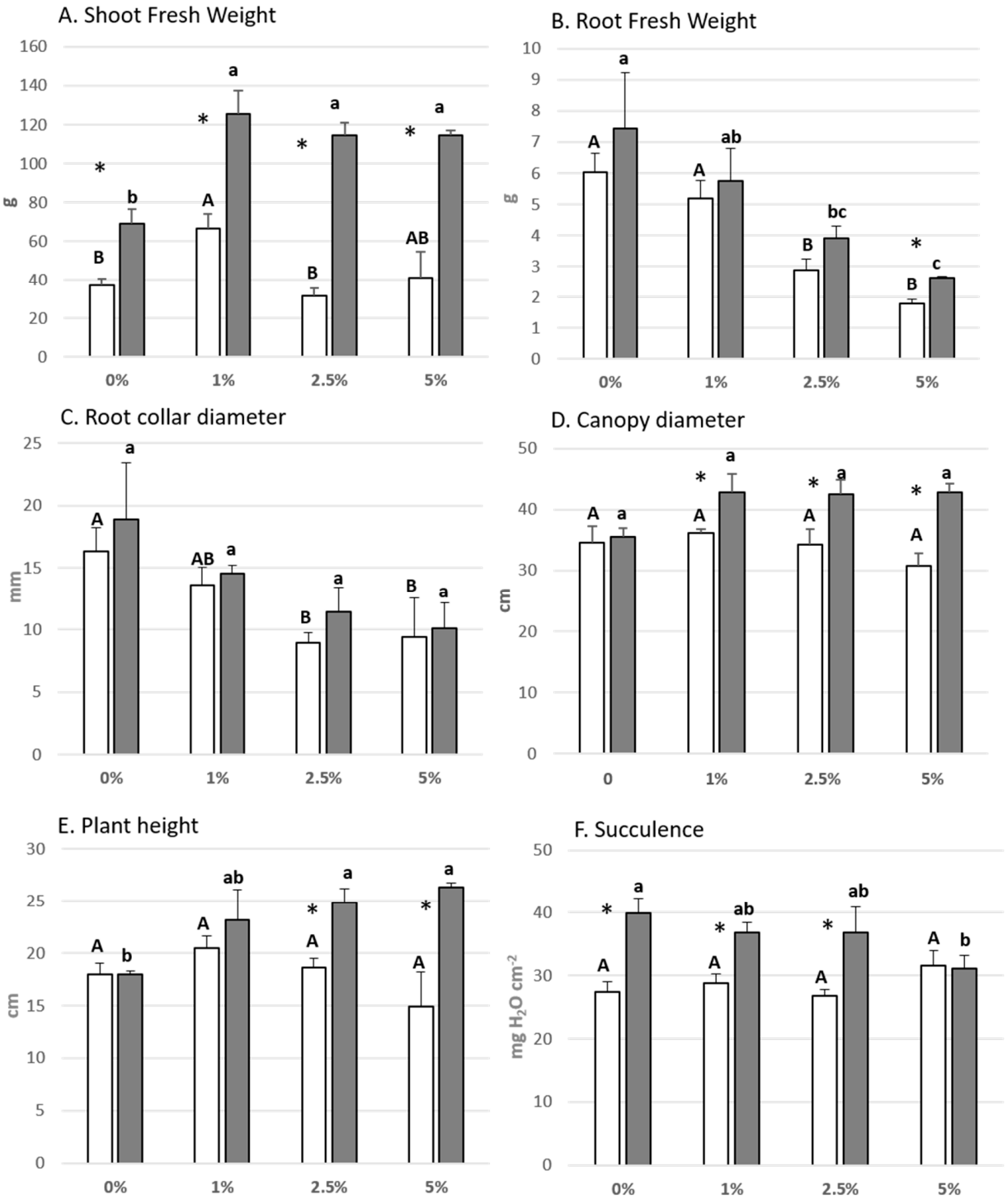


**Figure 2.** Biometric parameters of lettuce plants cultivated with different frass rates (0%, 1%, 2.5%, and 5%), with BS (grey bars) and without BS (white bars): (**A**) = shoot fresh weight; (**B**) = root fresh weight; (**C**) = root collar diameter; (**D**) = canopy diameter; (**E**) = plant height, and (**F**) = succulence. Different letters indicate significant differences ($p < 0.05$; $n = 3$) between each frass dose (uppercase letters for control substrate and lowercase letters for BS-amended substrate). An asterisk means significant differences using Student´s *t* test between control and BS within each frass rate.

**Table 2.** Significance (sig) and size effect determined as partial eta-squared ($\eta^2$p) of each factor (frass and BS) and their interaction for the different variables measured ($n$ = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = non-significant; FW = fresh weight; DW = dry weight; Chl = chlorophyll; carot = carotenoids; EL = electrolyte leakage; WRC = water relative content; EC = electrical conductivity.

| | | Frass | BS | BS x Frass | | | Frass | BS | BS x Frass |
|---|---|---|---|---|---|---|---|---|---|
| **Shoot FW (g)** | *sig* | ** | *** | * | **Chl a (μg cm$^{-2}$)** | *sig* | ** | *** | ns |
| | $\eta^2p$ | 0.650 | 0.884 | 0.425 | | $\eta^2p$ | 0.588 | 0.635 | 0.164 |
| **Shoot DW (g)** | *sig* | ns | ** | ns | **Chl b (μg cm$^{-2}$)** | *sig* | ** | *** | ns |
| | $\eta^2p$ | 0.127 | 0.535 | 0.131 | | $\eta^2p$ | 0.587 | 0.554 | 0.361 |
| **Root FW (g)** | *sig* | *** | ns | ns | **Chl tot (μg cm$^{-2}$)** | *sig* | ** | *** | ns |
| | $\eta^2p$ | 0.772 | 0.202 | 0.024 | | $\eta^2p$ | 0.591 | 0.631 | 0.174 |
| **Root DW (g)** | *sig* | ns | ns | ns | **Chl a/b (μg cm$^{-2}$)** | *sig* | * | ** | * |
| | $\eta^2p$ | 0.205 | 0.054 | 0.081 | | $\eta^2p$ | 0.435 | 0.42 | 0.444 |
| **Height (cm)** | *sig* | ns | ** | * | **Carot (μg cm$^{-2}$)** | *sig* | ** | *** | ns |
| | $\eta^2p$ | 0.282 | 0.513 | 0.429 | | $\eta^2p$ | 0.579 | 0.495 | 0.035 |
| **Leaf DW/FW** | *sig* | ns | ns | ns | **Leaf prot (μg mg$^{-1}$ FW)** | *sig* | *** | ns | ns |
| | $\eta^2p$ | 0.285 | 0.022 | 0.035 | | $\eta^2p$ | 0.687 | 0.026 | 0.11 |
| **Root FW/DW** | *sig* | *** | * | ns | **Root prot (μg mg$^{-1}$ FW)** | *sig* | ** | ns | ns |
| | $\eta^2p$ | 0.851 | 0.206 | 0.196 | | $\eta^2p$ | 0.589 | 0.057 | 0.236 |
| **Root diameter (cm)** | *sig* | * | ns | ns | **Leaf $NO_3^-$ (nmol mg$^{-1}$ FW)** | *sig* | ** | ns | * |
| | $\eta^2p$ | 0.471 | 0.056 | 0.015 | | $\eta^2p$ | 0.6 | 0.079 | 0.455 |
| **Canopy diameter (cm)** | *sig* | ns | *** | ns | **Root $NO_3^-$ (nmol mg$^{-1}$FW)** | *sig* | ** | ns | * |
| | $\eta^2p$ | 0.231 | 0.566 | 0.298 | | $\eta^2p$ | 0.602 | 0.024 | 0.403 |
| **Leaf N (%)** | *sig* | *** | ns | ns | **Leaf EC (%)** | *sig* | *** | *** | *** |
| | $\eta^2p$ | 0.82 | 0.029 | 0.225 | | $\eta^2p$ | 0.812 | 0.327 | 0.715 |
| **Root N (%)** | *sig* | * | ns | ns | **Root EC (%)** | *sig* | *** | ns | ns |
| | $\eta^2p$ | 0.424 | 0.053 | 0.15 | | $\eta^2p$ | 0.914 | 0.007 | 0.12 |
| **Ca (μg g$^{-1}$ DW)** | *sig* | *** | ns | ns | **Acid phosphatase (μmol PNP g$^{-1}$ FW h$^{-1}$)** | *sig* | *** | *** | * |
| | $\eta^2p$ | 0.819 | 0.162 | 0.241 | | $\eta^2p$ | 0.635 | 0.582 | 0.347 |
| **K (μg g$^{-1}$ DW)** | *sig* | *** | ns | ns | **Alcaline phosphatase (μmol PNP g$^{-1}$ FW h$^{-1}$)** | *sig* | ** | *** | ns |
| | $\eta^2p$ | 0.925 | 0.043 | 0.332 | | $\eta^2p$ | 0.356 | 0.512 | 0.156 |
| **Mg (μg g$^{-1}$ DW)** | *sig* | *** | ns | ns | **WRC (%)** | *sig* | * | ns | ns |
| | $\eta^2p$ | 0.926 | 0.001 | 0.315 | | $\eta^2p$ | 0.197 | 0.029 | 0.062 |
| **Na (μg g$^{-1}$ DW)** | *sig* | *** | ns | ns | **Succulence** | *sig* | ns | *** | * |
| | $\eta^2p$ | 0.883 | 0.007 | 0.292 | | $\eta^2p$ | 0.029 | 0.355 | 0.183 |
| **P (μg g$^{-1}$ DW)** | *sig* | *** | ns | ns | **Substrate pH** | *sig* | *** | ns | ns |
| | $\eta^2p$ | 0.922 | 0.025 | 0.024 | | $\eta^2p$ | 0.632 | 0.007 | 0.211 |
| **S (μg g$^{-1}$ DW)** | *sig* | *** | ns | ns | **Substrate EC** | *sig* | *** | ns | ns |
| | $\eta^2p$ | 0.907 | 0.115 | 0.107 | | $\eta^2p$ | 0.685 | 0.016 | 0.105 |

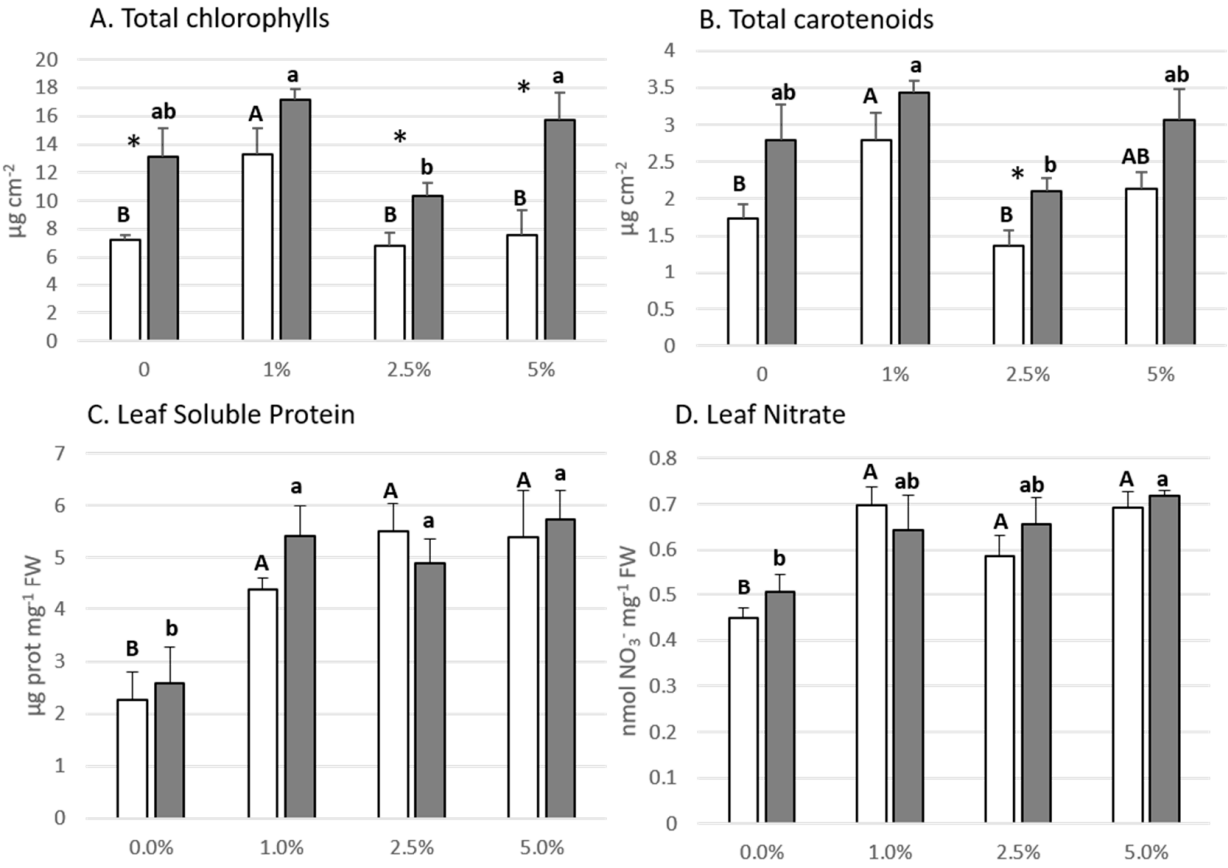


**Figure 3.** Biochemical parameters of lettuce plants cultivated with different frass rates (0%, 1%, 2.5%, and 5%), with BS (grey bars) and without BS (white bars): leaf total chlorophyll content (**A**), total carotenoid content (**B**), leaf soluble protein content (**C**), and leaf nitrate content (**D**). Different letters indicate significant differences ($p < 0.05$; $n = 3$) between each frass dose (uppercase letters for control substrate and lowercase letters for BS-amended substrate). An asterisk means significant differences using Student´s *t* test between control and BS within each frass rate.

As observed for N, partial $\eta^2$ values reveal that a high proportion of variance in terms of leaf P, K, Mg, and S contents can be explained by the addition of frass (Table 2), while BS addition did not influence leaf tissue concentration of these macronutrients. However, when taking into account the total aerial biomass, the total extraction of macronutrients from soil by lettuce plants follows the same trend as shoot biomass. The total macronutrient extraction (Figure 4) is thus influenced not only by the frass rate but also by the application of the BS. These extractions significantly increased at the low frass rate (1%), were equal to those of the frass-unamended plants at a medium rate (2.5%), and decreased at the high rate (5%), except for N extraction (Figure 4). BS addition tended to increase macronutrient extraction in the frass-unamended plants, with this increase being significant only in the case of K and N. The combined application of BS and frass strongly increased macronutrient extraction contents compared to the application of frass alone, whatever the frass rate.

An analysis of the integrity of the root and leaf cell membranes revealed that the application of frass explained a high proportion of the variance of leaf and root electrolyte leakage (EL) (Table 2), while BS application barely explained 30% of the variance in leaf EL and had no effect on root EL (Table 2). Root EL increased with the addition of frass, whatever the rate applied (Figure 5). Due to the low root biomass obtained at 5% frass treatment, there was not enough material to determine EL in this treatment. By contrast, EL increased in leaf tissue depending on the frass rate, being 2.5 times higher at the low frass rate of 1% and six times higher at the high frass rate of 5% (Figure 5). BS addition did

not influence EL in the leaf or root. However, the combined application of frass and BS strongly reduced leaf EL at the high frass rate of 5%.

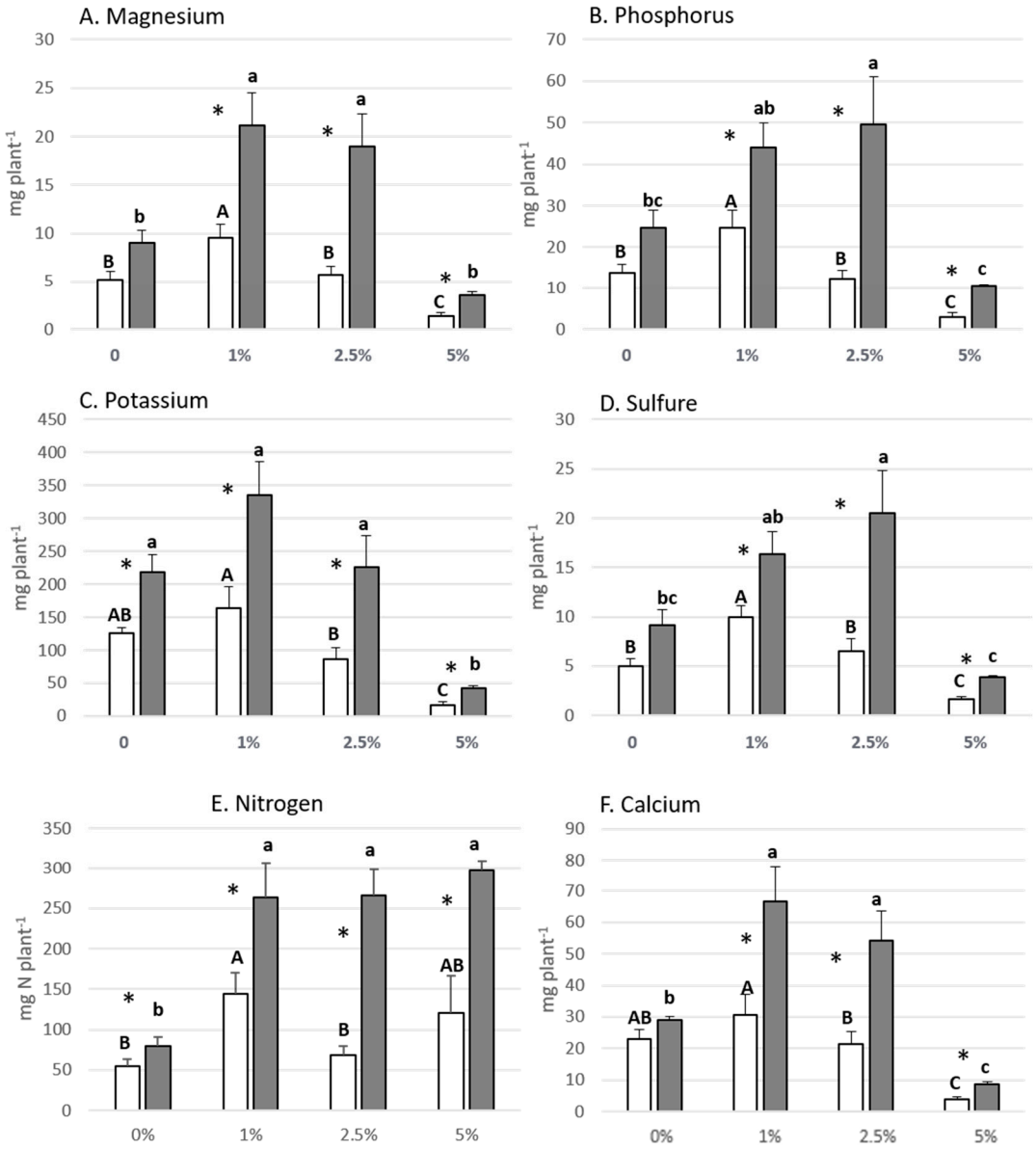


**Figure 4.** Total macronutrient extraction by lettuce plants cultivated with different frass rates (0%, 1%, 2.5%. and 5%), with BS (grey bars) and without BS (white bars): magnesium extraction (**A**), phosphorus extraction (**B**); potassium extraction (**C**); sulfur extraction (**D**); nitrogen extraction (**E**) and calcium extraction (**F**). Different letters indicate significant differences ($p < 0.05$; $n = 3$) between each frass dose (uppercase letters for control substrate and lowercase letters for BS-amended substrate). An asterisk means significant differences using Student's *t* test between control and BS within each frass rate.

Changes in root acid and alkaline phosphatase activities were induced by both frass and BS addition, with each factor explaining a similar proportion of the variance (Table 2). The small quantity of root material obtained at the 5% levels of frass treatment prevented the measurement of these activities at this high frass rate (Figure 5). Phosphatase activities increased with the frass rate, except in the case of acid phosphatase at the 2.5% rate. BS application in frass-unamended plants significantly increased acid phosphatase activity, while the combined application of BS and frass significantly increased acid phosphatase

activity at both 1% and 2.5% frass rates, and alkaline phosphatase at the 1% frass rate (Figure 5).

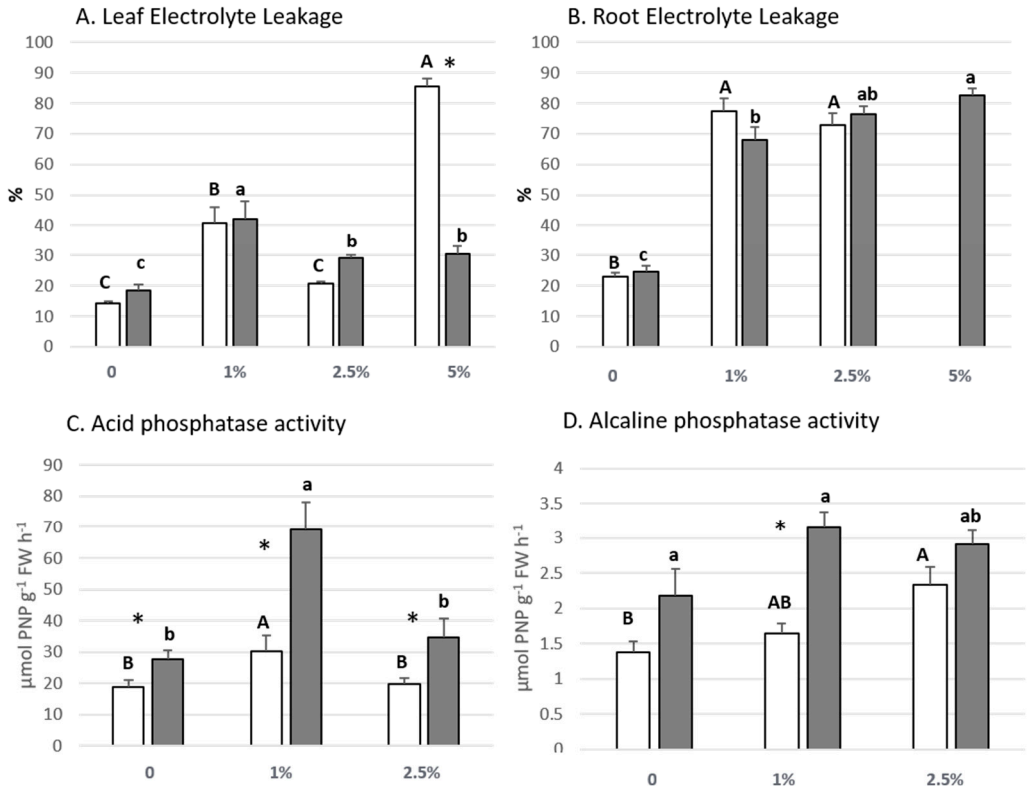


**Figure 5.** Electrolyte leakage in plant tissues and root phosphatase activity of lettuce plants cultivated with different frass rates (0%, 1%, 2.5%, and 5%), with BS (grey bars) and without BS (white bars): leaf electrical conductivity (**A**); root electrical conductivity (**B**); root acid phosphatase activity (**C**); and root alkaline phosphatase activity (**D**). Different letters indicate significant differences ($p < 0.05$; $n = 3$) between each frass dose (uppercase letters for control substrate and lowercase letters for BS-amended substrate). An asterisk means significant differences using Student's *t* test between control and BS within each frass rate.

### *3.4. Rhizosphere Cultivable Microbiota Analysis*

The cultivable rhizosphere microbiota were quantified and classified into different morphotypes (MFs). In the total treatments, 14 MFs of filamentous fungi were identified up to the genera level via microscopic observations: *Absidia*, *Acremonium*, *Cladosporium*, *Fusarium* (2 MFs), *Aspergillus* (4 MFs), *Penicillium* (3 MFs), *Trichoderma*, and *Rhizopus*. In addition, 9 MFs of yeasts (MFY1-MFY9) and 15 MFs of bacteria (MF1-MF15) were distinguished (Figure 6). The PERMANOVA analysis revealed highly significant effects of both frass and BS amendment on the cultivable bacteria, filamentous fungi, and yeasts of the lettuce plant´s rhizosphere (Table 3).

To view the effect of frass rate and BS amendment on the different microbial communities, a Multidimensional Scaling Analysis (MDS) using bootstrap averages and a Principal Coordinate Analysis (PCoA) were performed, both based on the Bray–Curtis similarity index and using fourth-root transformed species data (Figure 7). The addition of frass significantly influenced the composition of the microbial communities, with the stress values of the analyses being 0.09 for bacteria, 0.15 for filamentous fungi, and 0.06 for yeasts, implying a good representation of the data in reduced dimensions. For rhizosphere bacteria, clear differences were observed depending on the frass rate (Figure 7). For filamentous fungi and yeasts, the dissimilarities were clearest between the frass-unamended treatment and the frass-receiving treatments.

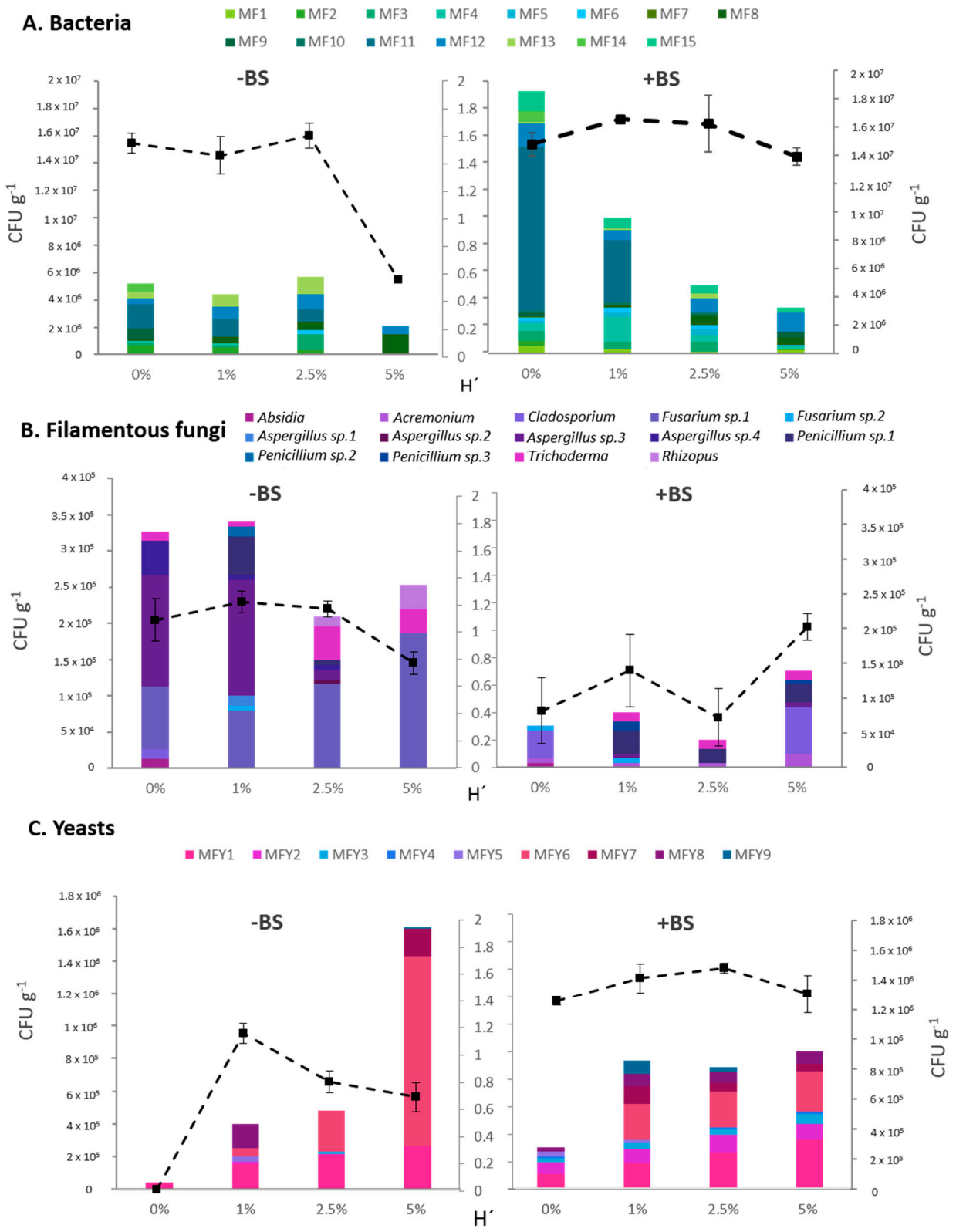


**Figure 6.** Cultivable bacteria (**A**), filamentous fungi (**B**), and yeasts (**C**) isolated from the rhizosphere of lettuce plants cultivated with different frass rates (0%, 1%, 2.5%, and 5%), with BS (right) and without BS (left). Bars show microorganism quantities in colony-formation units (CFU) per gram of lettuce root as the sum of the quantity of all MFs (represented by different colors) found in each treatment. MF, bacterial MFs; MFY, yeast MFs. Dotted line graphs show changes in the Shannon Diversity Index (H′) for each treatment. The values represent mean ± SE ($n$ = 9).

**Table 3.** Results of the PERMANOVA analysis to test the effect of frass and BS on the rhizosphere cultivable microbial communities (bacteria, filamentous fungi, and yeasts).

| Bacteria | | | | | Filamentous Fungi | | | | | Yeasts | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Source** | **Df** | **Sum Sq** | **Pseudo-F** | ***p* (Perm)** | **Source** | **Df** | **Sum Sq** | **Pseudo-F** | ***p* (Perm)** | **Source** | **Df** | **Sum Sq** | **Pseudo-F** | ***p* (Perm)** |
| Frass | 3 | 14,569 | 7.721 | **0.001** | Frass | 3 | 16,108 | 3.1693 | **0.002** | Frass | 3 | 14,431 | 13.122 | **0.001** |
| BS | 1 | 10,647 | 16.93 | **0.001** | BS | 1 | 21,498 | 12.689 | **0.001** | BS | 1 | 8077.7 | 22.035 | **0.001** |
| Frass x BS | 3 | 2958.7 | 1.568 | **0.151** | Frass x BS | 3 | 6642.9 | 1.307 | **0.261** | Frass x BS | 3 | 5439.2 | 4.9458 | **0.001** |
| Residuals | 16 | 10,063 | | | Residuals | 16 | 27,107 | | | Residuals | 16 | 5865.4 | | |
| Total | 23 | 38,237 | | | Total | 23 | 71,356 | | | Total | 23 | 33,813 | | |

The microbial communities of the rhizosphere of the BS-treatment were well separated in the PCoA ordination, which was confirmed by the main PERMANOVA test (Figure 7 and Table 3). The PCoA identified two main coordinates that showed a clear stratification of the control and BS-treated rhizospheric communities in the 2D domain (Figure 7). In total, 67.5% of the variation in the bacterial community structure, 59.6% in the filamentous fungal community structure, and 74.1% in the yeast community could be explained by the two axes. BS addition structured the microbiota communities: bacterial and filamentous fungal communities from the BS-amended rhizosphere clustered at a noticeable distance from communities grown in the control rhizosphere. The yeast community was more widely dispersed, leading to distinct but overlapping clusters.

The differences observed in the MDS and in the PCoA can be seen more explicitly in Figure 6, which shows the amount and composition of each microbial community, reflecting diversity by means of the Shannon Diversity Index (Figure 6). The addition of frass had no effect on bacterial abundance and composition at a low–medium frass rate, giving rise to a Shannon Index similar to that of the control substrate at around 1.5 (Figure 6A). By contrast, the 5% frass rate decreased both bacterial abundance and diversity, resulting in a Shannon Index of 0.6, with only MF8 and MF12 present in the rhizosphere of this treatment. BS amendment increased bacterial abundance four times to $1.9 \times 10^7$ CFU $g^{-1}$, with diversity values remaining similar to those of the control. This increase in bacterial abundance was mainly due to the increase in MF11, although five new MFs also appeared in the rhizosphere of this treatment (MFs 5, 6, 7, 10, and 15) that were not present in the controls. The combined application of BS and frass maintained bacterial diversity, with Shannon Index values of around 1.6, even at the higher frass rates, while bacterial abundance dramatically decreased as the frass rate increased (Figure 6A). Regarding filamentous fungi, the addition of frass slightly increased fungal diversity at low and medium rates, but that diversity decreased at 5% of frass. The presence of genera such as *Trichoderma* increased with frass addition, but the rhizosphere for the 5% treatment was mainly dominated by *Fusarium* and *Rhizopus* genera (Figure 6B). BS amendment significantly reduced both the amount and diversity of filamentous fungi, promoting the growth of genera such as *Cladosporium* and *Acremonium* and reducing the presence of *Fusarium* and *Aspergillus*. The combined application of frass and BS maintained *Trichoderma* but avoided the incidence of *Fusarium* and *Rhizopus* genera. The rhizosphere of frass-unamended control plants was very poor in terms of yeast abundance and diversity, with only one MF being isolated in this treatment (MFY1) (Figure 6C). Frass addition led to an increase in both yeast abundance and diversity, mainly due to the presence of MFY6. BS addition increased both yeast abundance and diversity, and the dual application of frass and BS further increased this effect. With the combined application, yeast diversity remained high even at the highest frass rate, preventing the disappearance of yeast MFs.

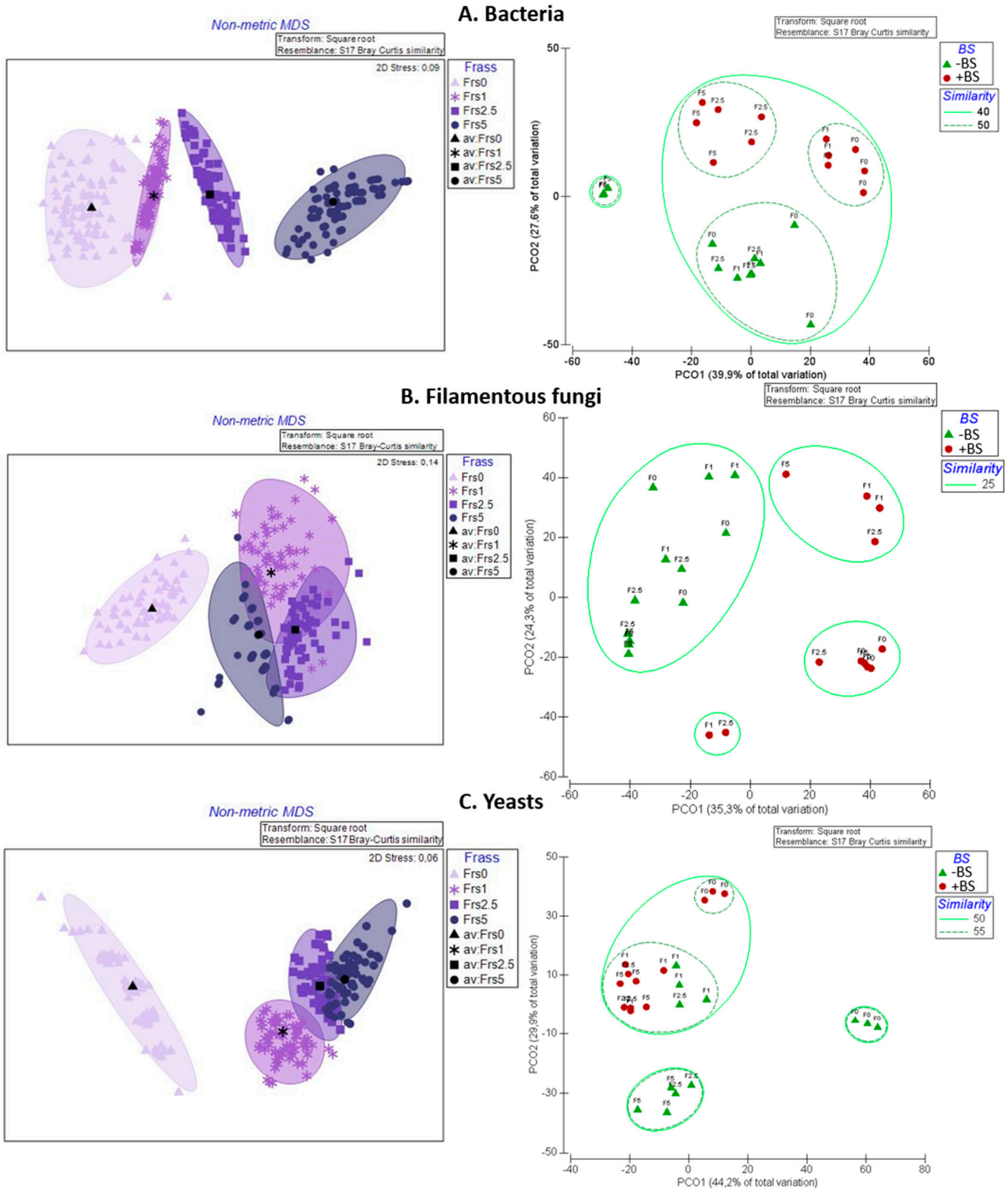


**Figure 7.** Analysis of the cultivable microbiota of lettuce plants rhizosphere. Left: Multidimensional Scaling (MDS) using bootstrap averages of the different microbiota communities ((**A**) bacteria, (**B**) filamentous fungi, and (**C**) yeasts), compared in terms of frass rates (0% light purple, 1% violet, 2.5% dark violet, and 5% grey) and based on the Bray–Curtis index. Metric MDS ordination uses (100 per group) bootstrap averages of the centroid of each sample to show where 95% of the centroid averages lie within multivariate space. Right: Principal Coordinate Analysis (PCoA) based on the Bray–Curtis index comparing cultures regarding BS amendment (without BS substrate (green) vs. BS-amended substrate (brown)).

## 4. Discussion

### *4.1. Use of Frass as an Alternative Organic Amendment*

The effectiveness of organic fertilizers for vegetable production mainly depends on their nutrient content, especially N content, and on the rate of nutrient release [30–32]. Mealworm frass shows high potential for use as organic fertilizer, since its concentrations of N, P, and K are as high as those found in other organic fertilizers, such as raw manure [11]. Increases in ryegrass [9], barley [11], and chard [12] biomass have been observed after frass amendment. Consistent with the studies cited, a significantly higher lettuce aerial biomass was obtained in this experiment after 1% frass application, due to an improvement in plant N uptake and assimilation. This was corroborated by improved foliar concentrations of nitrate, soluble protein, and total chlorophylls compared with frass-unamended control plants.

The application of organic fertilizers does not always result in a short-term increase in nutrient availability for crops, due to microbial immobilization [33], among other factors. However, this is not the case with frass, as it has a high content of labile organic matter and a fast mineralization rate [8,34]. In this sense, Houben et al. [11] demonstrated that 37% of the total organic nitrogen is mineralized by 7 days after frass is added to the substrate, with that figure increasing to 55% after 91 days. These observations are consistent with the increased biomass and quality found here in frass-amended lettuce plants after the seven weeks of the experiment. However, the nutritional benefits of frass are not limited to N but also include other elements, as reflected in the higher P, K, Mg, and S leaf extractions of the frass-amended plants compared with the unamended controls.

Frass shows promising results as organic fertilizer and may be a good candidate to replace totally or at least partially the mineral NPK fertilizer applied to crops. However, as Chavez and Uchanski [8] point out in their review, there are major differences between the few existing studies on this subject, and it is essential to find the right timing and optimal frass application rate. Our data show that high frass rates have a negative effect on lettuce plants. A general decrease in biometric parameters such as shoot and root fresh weight and root collar diameter was observed at 2.5% and 5% frass rates. At the same time, biochemical parameters such as chlorophyll and carotenoid content decreased as the frass rate increased. More markedly, tissue electrolyte leakage, used as a pivotal parameter of stress injury, increased in leaf tissue in line with the frass rate and was high independent of the frass rate in root tissue. This suggests that lettuce plants that receive frass rates of 2.5% or higher suffer some degree of stress that results in a clear inhibition of plant development. Watson et al. [13] report a negative consequence of 2.5% frass application due to nitrite accumulation, which is detrimental to microbial activity and inhibits root growth [35]. This would explain the dramatic decrease in root biomass and the reduction in abundance and diversity of both bacteria and filamentous fungi at high rates of frass. In contrast, some studies regarding black soldier fly frass report unfavorable effects on the growth of maize [36] and Japanese mustard spinach [37] due to nutrient deficiency and high salinity, while the application of 3% of mealworm frass almost completely inhibits ryegrass germination [5]. In our study, the Na content of the frass was high (Table 1) when it was added to the substrate in quantities lower than 5%, but the EC remained below 1 mS/cm. According to FAO classification, the characteristic EC range of soils without salinity problems is between 0 and 2 mS/cm. The addition of frass, therefore, did not lead to a dangerous increase in EC. Another indicator that supports the idea that salinity is not a problem is microbial activity in the rhizoplane. Although microbial properties can be negatively affected by high salt loads [38,39], in our experiment the microbial biomass was not negatively affected at a low–medium frass rate. The same was observed by Watson et al. [5]. The increased activity of acid and alkaline phosphatases in the rhizoplane of lettuce plants suggests increased microbial activity due to frass amendment. In this sense, some authors [10,12,21] argue that the differences in the productivity of frass-grown plants are not exclusively due to the nutritional composition of the feces and suggest that their associated microbiota may be the main driver of plant growth promotion. Osimani

et al. [40] analyzed the bacterial microbiota of mealworm frass and revealed high loads of *Enterobacteriaceae*, lactic acid bacteria, and several species of mesophilic aerobes. In our lettuce plants, rhizosphere bacterial abundance did not change at low and medium frass rates, but bacterial communities in the frass at 2.5% and 5% clustered far from the frass-unamended control and the frass at 1%. At the same time, filamentous fungi and especially yeasts showed well-differentiated communities compared to frass-unamended controls. Overall, three bacterial MFs and two filamentous fungal MFs disappeared when frass was applied, while one bacterial MF, four filamentous fungal MFs, and four yeast MFs appeared. The genera appearing with frass addition included some interesting genera described as PGPs, such as *Bacillus* and *Trichoderma*. As observed by Watson et al. [13], the application of frass stimulates microbial activity and the growth of rhizosphere saprotrophic fungi associated with the later stages of organic matter decomposition. However, increasing frass rates also increased the incidence of potentially pathogenic genera, such as *Fusarium* and *Rhizopus*, which would explain the observed decrease in root biomass in treatments at 5% of frass. Note that Regulation (EC) 1774/2002 of the European Parliament states that the use of animal excreta in agriculture requires some type of transformation, such as composting or sanitization, to ensure the absence of *Enterobacteria*, *Salmonella*, or sporulating toxigenic bacteria. This transformation process is expected to have an impact on the microbiota associated with the frass, probably reducing the incidence of pathogenic fungal strains. However, the sanitization process would also eliminate any other beneficial microorganisms with plant growth promotion capabilities, which is why the combined application of frass and PGP-based BS may be a good alternative to NPK fertilizers.

### *4.2. Use of a PGP-Based Biostimulant to Improve Plant Yield and Quality*

The use of efficient microorganisms as plant growth promoters in agriculture is increasingly widespread. The main scientific challenge is the complexity of the physiological effects of PGP-based biostimulants, since the primary effects are to introduce physiological responses in the plant, many of them bearing on primary metabolism, growth, and development [17]. The growth-promoting capacity of the *Bacillus*-based BS used in this study was reflected in the biometric and plant quality parameters, which were better in the 0% frass BS-amended plants than in the BS-unamended control plants. This indicates that lettuce plant growth was improved without adding any mineral or organic fertilization and that microorganisms present in a PGP-based BS can mobilize nutrients that would not be available for control lettuce plants in the substrate. Indeed, microorganisms present in the BS led to an improvement in root phosphatase activity and influenced the rhizosphere microbiota composition. Thus, BS application increased the abundance of bacteria (*Bacillus* and *Pseudomonas* spp. among them) by seven times and that of yeasts by four times, and decreased that of filamentous fungi, probably due to the greater development observed in the other microorganism groups. *Bacillus* strains have been described as able to solubilize P [41] and K [42] and produce auxins [43] and siderophores [44], thus enhancing root development and nutrient uptake, whereas *Pseudomonas* has also been described as a PGP and biocontrol agent [44,45]. Both genera include strains able to fix $N_2$, making N available to the plant [46,47]. Therefore, even if nitrogen is not directly added as fertilizer, plants extract more nitrogen than the BS-unamended control plants. In addition, although the incidence of filamentous fungi decreased with the BS, genera such as *Cladosporium* appeared. This genus has been described as producer of auxins, gibberellins, and siderophores [48,49], which may also promote plant growth. Thus, the changes observed in microbial functional groups, with increasing bacterial and yeast abundances and decreased filamentous fungal diversity and abundance, could explain differences in lettuce plant yield and quality when the BS was added.

### *4.3. Use of the Combination of Frass and PGP-Based Biostimulant as an Alternative to Improve Plant Growth and Nutritional Quality*

Peat is a low-fertility substrate, so the powerful ability of the PGP-based BS to solubilize nutrients became more evident when it was added together with a source of organic matter, such as frass. The increased beneficial effect of the BS when frass was incorporated into the substrate can be explained by the increase in C availability due to frass mineralization. C is the factor that most determines microbial growth, favoring the diversity and activity of rhizosphere microorganisms [50]. Thus, an increase in these populations will accelerate the rate of organic matter degradation, as mentioned by [51], making nutrients available to plants quickly and, to a large extent, favoring plant growth and quality. This was reflected in the total extraction of macronutrients from the substrate, with a strong increase in the extraction of N, P, K, Mg, and S, due to greater nutrient mineralization and solubilization in the rhizosphere.

Interestingly, this study shows that the combined application of frass and PGP-based biostimulant favors lettuce plant growth, resulting in plants with higher shoot biomass than when they are applied separately. This means that there is a strongly positive synergy between the two products, since the application of BS completely prevented the loss of aerial biomass caused by medium–high frass rates. Moreover, although the inhibitory effect of root growth observed due to frass also occurred in BS-amended plants, this effect was lower, increasing water and nutrient absorption capacity and, consequently, providing better aerial development. The described ability of several *Pseudomonas* and *Bacillus* species to act as phytohormone producers [52–54] could explain the differences in shoot development and the smaller decrease in root biomass in 5% frass. Thus, the positive synergy observed means that plants that received the BS counteracted the negative effect of medium and high frass rates on shoot biomass. Moreover, when frass was applied in combination with the BS, beneficial microorganisms such as *Trichoderma* or *Acremonium* increased. The growth-promoting effect of *Trichoderma* sp. on lettuce plants has been demonstrated in several papers [55–57], while the PGP ability of *Acremonium* spp. has been observed in rice [58] and *Allium tuberosum* [59]. *Acremonium* [60] and *Trichoderma* [61,62] also show antagonistic behavior against several phytopathogenic fungi, inhibiting their growth by different mechanisms, such as hyper parasitism, competition for nutrients and space, and antibiosis. The application of a BS favors functional diversity in the rhizosphere and a greater presence of these biocontrol fungi, which may explain the mitigation of the deleterious effect that high frass rates have on plant roots, thus demonstrating again the positive synergy of the combined application of frass and a PGP-based BS.

Not only yield but also quality was positively affected by this synergistic combined application, as succulence values and total chlorophyll content were higher than in the treatments without BS. In this sense, nitrogen is a structural element of chlorophyll, and there is a close relationship between available nitrogen and chlorophyll concentration in leaves [63], so it can be deduced that the nitrogen provided with frass led to a greater N assimilation by plants, which was much more effective with the help of the BS. The application of N is considered to increase leaf nitrate content [64], which poses a risk for human consumption, but the use of the BS, alone or combined with frass, increased lettuce growth without causing nitrate accumulation in leaves. As observed with chlorophylls, the carotenoid content also tended to increase with the application of frass and BS, an effect previously described for BS-treated tomatoes [65] and lettuces [66]. Thus, a greater content of photoprotective pigments may increase the antioxidant capacity of lettuce plants and result in a more protected photosynthetic apparatus. These results, taken together, indicate that the combined use of frass and BS can improve the nutraceutical quality of lettuce. This addresses the current consumer demand for foods that not only meet nutritional needs but also provide health benefits and effects related to the nutritional quality of vegetables.

## 5. Conclusions

Yellow mealworm frass shows high potential as an organic fertilizer for lettuce plants, both in providing nutrients due to its mineralization and as a beneficial amendment for rhizosphere microbial abundance and diversity, enabling lettuce plants to grow in a low-fertility substrate such as peat. However, although frass is found to increase macronutrient uptake by plants, its fertilizer potential is interestingly mediated by its application rate. Frass rates of 2.5% or higher resulted here in a loss of lettuce yield and quality, presumably due to the high incidence of fungal pathogenic strains. The negative effect of higher doses of frass can be counteracted with the addition of a PGP-based biostimulant, which improves plant yield and quality and shows a positive synergy with the application of frass, making the nutrients from frass available for plant growth but also influencing rhizosphere-associated microbiota. The combined application of frass and the PGP-based BS led to a decrease in fungal pathogenic genera and a rise in beneficial microorganisms. This positive synergy observed between frass and the PGP-based BS in terms of yield and quality makes them good options for developing new fertilization strategies that fit with more sustainable agriculture in the framework of a circular economy.

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