applied sciences

Article

Hermetia illucens Frass Fertilization: A Novel Approach for Enhancing Lettuce Resilience and Photosynthetic Efficiency under Drought Stress Conditions

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Citation: Sawinska, Z.; Radzikowska-Kujawska, D.; Kowalczewski, P.Ł.; Grzanka, M.; Sobiech, Ł.; Skrzypczak, G.; Drożdżyńska, A.; Ślachciński, M.; Świtek, S. *Hermetia illucens* Frass Fertilization: A Novel Approach for Enhancing Lettuce Resilience and Photosynthetic Efficiency under Drought Stress Conditions. *Appl. Sci.* **2024**, *14*, 2386. https://doi.org/10.3390/app14062386

Academic Editors: Achille Antenucci and Stefano Dughera

Received: 6 February 2024
Revised: 28 February 2024
Accepted: 11 March 2024
Published: 12 March 2024

Abstract: Agriculture is faced with the need to reduce mineral fertilizers in order to reduce costs but also to meet political goals. Resilience-enhancing climate change, especially in the face of increasingly frequent and prolonged droughts, has become another issue. The dynamically increasing production of insects for feed and food purposes has become one of the answers to this challenge. This study assesses the fertilizing efficacy effect of frass derived from Black Soldier Fly (*Hermetia illucens*) production on lettuce (*Lactuca* L.) growth, including aspects such as yield, photosynthesis activity, photosystem II performance (chlorophyll fluorescence), mineral profile, and antioxidant properties. Additionally, the properties of the soil were assessed by measuring the gas exchange between the soil and the atmosphere. The lettuce plants grew under two water regimes—optimal irrigation and induced drought. The efficiency of frass fertilization was compared with the control and traditional cattle manure. The results indicate that *H. illucens* frass (HI frass) used as a fertilizer increased the content of essential nutrients in plants—such as potassium and iron. As the dosage of frass increased, the content of vitamin B2 (riboflavin) doubled. The plants that were subjected to drought and properly fertilized showed greater resistance; therefore, a reduction in the synthesis of polyphenolic compounds was observed. Fertilizer had a positive effect on the efficiency of photosynthesis. This study underscores the promising impact of unconventional organic fertilizers, such as *H. illucens* frass, on enhancing plant performance, especially in challenging environmental conditions. Fertilizers obtained from insect production can be green chemicals in a sustainable food production model.

Keywords: sustainable agriculture; bio fertilizers; regenerative agriculture; photosynthesis

1. Introduction

Today's agriculture faces many challenges, which include the declining profitability of production [1], climate change [2], extreme weather events [3], and droughts [4]. Despite this, agriculture production must provide enough calories for the growing human population [5], considering increased societal and environmental concerns [6,7]. Feeding 8.5 billion people would not have been possible without the agrotechnological progress that has taken place in last decades.

The progress that has been made in plant nutrition began in the 19th century and it involved many discoveries and the introduction of innovative practices at the time. The introduction of legumes into crop rotations increases the nitrogen supply [8]. The Broadbalk experiment, the oldest long-term field experiment to this day, provided evidence of the importance of manure and phosphorus fertilization [9]. Liebig proposed the nutrient availability theory [10]. However, the era of fertilizer use on a massive scale, increasing crop yields, began in the 20th century. This also led to the introduction of synthetic fertilizers and an increase in their accessibility [11].

Unfortunately, the intensification of production has very quickly led to negative consequences for the natural environment [12–15]. High doses of fertilizers have led to their release from agricultural soils into the environment, resulting in the eutrophication of water [16,17], alteration of biodiversity and remodeling of the agricultural landscape, climate change [18] or biodiversity change [19,20].

Currently, the situation with the use of fertilizers is changing again. Their use has moved from developed to developing countries; nowadays, 60% of the global use of fertilizers occurs in Asia [21,22], and rising fertilizer prices [23] limit the ability of farmers to purchase them. In many parts of the world, the problem is not the excess, but the shortage of fertilizers—especially valuable natural fertilizers—as a result of the increasing number of farms without animal production [24], and at the same time, the concentration of production in specific places [25], which is unfavorable for the natural environment [26,27]. Tightened legal regulations are also important. This is particularly visible in the EU, which implements the Farm to Fork policy, one of the aims of which is the sustainable use of fertilizers [28–30].

Reconciling environmental challenges and maintaining efficient production requires the introduction of new production systems and technologies. An example of this new approach is insect production, which is developing dynamically in both scientific research and practice [31–33]. Insect production has a lot of advantages: a low emission score [34], the possibility of using waste for their feed [35], and the low demand for water and land for production [36]. Insect protein production is also considered to have a smaller ecological footprint and is associated with the possibility of using waste. The insect protein market is developing very well and the number of products sold is increasing [37]. This is followed by legal regulations. The European Commission has introduced statutory regulations which facilitate the production and trade of insects and products derived from their production [38]. Also, research on insect production is currently developing dynamically. Insects that can be used in production include the bearberry (*Tenebrio molitor*), the mealworm (*Alphitobius diaperinus*), the house cricket (*Acheta domesticus*), or house fly (*Musca domestica*) [39]. The most important species, among others used commercially, is the Black Soldier Fly (*Hermetia Illucens*) [40].

Although we can make great use of waste in the production of insects, the production itself also generates waste. This includes the remains of exoskeletons but also, above all, insect excrements [41]. How to manage this waste is an intensive area of research today. It can be a source of nutrients for plants, providing nitrogen and other nutrients [42]. Frass obtained from the production of Black Soldier Fly and used for fertilizer purposes even has a special name: Black Soldier Fly Frass Fertilizer (BSFFF) [43]. This natural fertilizer may cause an increase in the plant's biomass and the concentration of minerals contained in it [44]. Fertilizer obtained from insects can also be a valuable source of organic material, which is particularly important for farms that do not have animals and have not used organic fertilizers for years [45]. However, the number of articles devoted to the role of frass as fertilizers is relatively small [46].

Photosynthesis is the most important process occurring in a plant and its measurement can be a key indicator not only of the plant's health but also its adaptation to stressful conditions. Drought stress in particular has a strong impact on the photosynthesis process, because the first defense mechanism of plants against water shortage is the closure of stomata, which further leads to a chain of processes that reduce the efficiency of photosynthesis and, consequently, a decrease in yield. The nutrition supplied to plants determines their health and also affects the effectiveness of photosynthesis [47,48].

In this work, we investigated how waste from *H. illucens* production affects plant yield and chemical parameters, and whether its use increases the resistance of plants to drought stress. We conducted our research on lettuce plants, which are one of the most important vegetable species in the world. Lettuce belongs to the Astaraceae family and is a temperate climate plant that today also grows in other climatic conditions, both in fields and in greenhouses. It is also the most consumed vegetable in the world. The largest producer is China, which grows stem lettuce, which is not very popular in other parts of the world, such as Europe. The United States contributes 22% of production, where it ranks third in terms of consumption [49]. It is the main salad plant, usually eaten raw, which allows for better utilization of the nutrients contained in it by the body. It is used in sandwiches and salads. However, the use of lettuce can be much broader. Oil can be pressed from the seeds, and even cigarettes can be made from the leaves. Wild forms were used to produce Lacturarium, used as a sleep aid in ancient Egypt [50].

Lettuce is a plant that is widely used in agricultural research. One of the reasons for this is the short cultivation cycle and relatively easy agrotechnics. Many studies focus on improving the yield and nutritional value of this plant [51], as it is a source of nutrients but also compounds beneficial to human health. Among lettuces, leafy lettuce contains a larger amount of minerals, vitamins and bioactive compounds than crunchy lettuce. The content of folic acid is similar to that of other vegetables. Young lettuce is particularly popular [49]. The best quality of lettuce is achieved through appropriate fertilization and proper irrigation [52]. The value of lettuce in human nutrition is determined by its nutritional properties—a high content of vitamin C, polyphenols, antioxidants and fiber. Research indicates the role of lettuce in preventing many diseases. Due to these factors, lettuce is an economically important plant [53].

We expect that the use of frass in plant fertilization will lead to similar effects to typical natural fertilizers, in particular, manure. Fertilized plants should have a higher nutrient content and tolerate drought better. The results may provide evidence that insect frass is also as effective as classic natural fertilizer, or maybe even better. Fertilizers of this type may also contribute to a circular economy. Whether their use increases the resistance of plants to drought stress is investigated.

2. Materials and Methods

2.1. Experiment Design, Plant Material and Growing Conditions

The pot experiment was carried out at the Department of Agronomy of the Poznań University of Life Sciences, Poznan, Poland (52.482854, 16.900465). Lettuce (*Lactuca sativa*) was grown in pots in greenhouse conditions. The experimental factors were a) fertilization and b) water conditions. Four levels of fertilization were used: control (no fertilization), manure 10 (10 g of granulated cattle manure applied to each pot) and two variants with frass applications: HI frass 10 (10 g granulated frass from *Hermetia illucens* per pot) and Hi frass 12.5 (12.5 g granulated frass from *Hermetia illucens* per pot). The dose was determined on the basis of a preliminary study, which indicated the optimal amount of fertilization. The water regime had two levels labeled as watered and drought. Half of the plants were irrigated as needed until harvest (watered). In the other half of the plants, a drought effect was induced by ceasing watering six days before harvesting (drought). The experiment was carried out in 4 replications.

The soil used for the experiment was ordered from Biobizz Worldwide SL (Industrial Systems s.r.o., Prague, Czech Republic). According to the manufacturer's information, it contained the following concentration of nutrients: min. 2.8% nitrogen, min. 2.8% phosphorus, min. 2.0% potassium and minimum 0.8% magnesium. The organic matter content was at least 60%, and the soil had a pH of 6.2. Before filling the pots, this soil was mixed.

After filling the pots with soil mixed with fertilizers, they were placed on a table and flooded with water for 48 h in the amount of 100 mL/pot, after which lettuce seeds were sown. Further irrigation was carried out using a hand sprinkler to maintain constant soil moisture. Pots with plants were placed in a greenhouse where light and thermal conditions were controlled during the experiment. The length of the day was 16 h and was provided by natural light and additionally by light from a 400 W sodium lamp (Elektro-Valo Oy Netafim, Avi: 13473, HPS, Ussikaupunki, Finland). The temperature during the experiment was 22 °C.

2.2. Drought Implication

Forty days after sowing, watering was stopped in half of the pots with plants in order to induce drought stress. To determine the state of drought, soil moisture was measured with a moisture meter (ThetaProbe, Ejkelkamp, Giesbeek, The Netherlands). After 6 days of no watering, the pots reached a humidity level of 6–8%, while the control pots had a soil moisture level of 20–22%. Lettuce plants were measured at the commercial maturity stage—leaf rosette (46 days after sowing). All measurements (biometric and physiological) were measured in triplicate, alternating between plants under drought stress and plants growing under optimal water conditions.

2.3. Photosynthesis and Chlorophyll Fluorescence

To assess the activity of photosynthesis and chlorophyll fluorescence, the plants were placed in a phytotron, where dark conditions were induced for a period of 6 h before measurements to silence photosynthesis. The conditions in the phytotron were as follows: temperature of 25 °C and air humidity of 70%. The next step was to measure photosynthetic activity using the LCpro-SD Gas Exchange Measurement System, manufactured by ADC BioScientific Ltd., Hoddesdon, UK). The parameters measured were A—CO_2 assimilation level (μmol m^{-2} s^{-1}), E—transpiration (mmol m^{-2} s^{-1}), Gs—stomatal conductance (mol m^{-2} s^{-1}) and Ci—intercellular CO_2 concentration (vpm). The plant in each pot was measured, selecting the youngest fully developed leaf.

An OS5p fluorometer manufactured by Optisciences Inc. (Hudson, NH, USA) was used to measure chlorophyll fluorescence. The device allows you to measure chlorophyll fluorescence after dark and light adaptation. The following parameters were determined: F0—minimum fluorescence, Fm—maximum fluorescence, Fv/Fm—maximum photochemical efficiency of PSII, yield—quantum yield of photosynthetic energy and ETR—electron transport rate (units not nominated). As in the case of photosynthetic activity, the measurement was carried out on the youngest fully developed leaves of the plants after 6 h of dark adaptation. Kinetic test was selected from the instrument menu. The following settings were used: The modulation source was set to 22 (out of a possible operating range of 1 to 32), which was the highest possible intensity that did not induce variable fluorescence. The saturation flash was set to 30 (out of a possible setting of 1 to 32). The measurement cycle was set to two saturation pulses at an interval of 180 s. The measurement was made in 3 biological and 2 technical repetitions.

2.4. Plant Harvesting and Yield

After measuring photosynthetic activity and chlorophyll fluorescence, the plants were harvested. Then, the fresh weight was determined using a laboratory scale. The samples were then frozen for subsequent determination of chemical parameters, including mineral composition, antioxidant activity and polyphenol profile.

2.5. Mineral Profile of Lettuce Leaves

Freeze-dried plant samples were subjected to mineralization according to the innovative method described previously [54]. It involved the use of a high-pressure/high-temperature system using microwave energy. The plant material was placed in vessels covered with modified Teflon; 60% nitric acid and 30% hydrogen peroxide were added to it. The whole thing was then placed in a steel jacket whose interior was exposed to microwave energy. Mineral components were determined using a spectrometer (ICP OES technique). The following elements were determined: calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn) and lead (Pb). The phosphorus (P) content was determined using the spectrophotometric method (using molybdenum). The determination was carried out on an SBT spectrophotometer (spectral slit 1.4 nm and wavelength 700 nm).

2.6. Antioxidant Activity and Polyphenolic Compounds in Lettuce Leaves

Plant materials were extracted using an 80% methanol solution. The freeze-dried lettuce leaves at an amount of 1 g of DM was mixed with 15 mL of 80% methanol solution and shaken for 30 min, then centrifuged for another 20 min at a speed of $4000\times g$. The supernatant was filtered through a 0.22 µm filter and then stored at −80 °C until analysis.

The colorimetric method according to Singleton et al., 1999 [55], using Folin–Ciocalteu reagent, was used to determine the total amount of polyphenols. The measurement was made on a Multiskan Go spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland). The antioxidant capacity of lettuce was assessed using the Trolox Equivalent Antioxidant Capacity (TEAC) test proposed by Re et al. [56] with the radical cation ABTS. The ferric reducing ability of lettuce leaves, using the FRAP assay proposed by Benzi and Strain [57], was also used to measure the antioxidant power. It is expressed as a TEAC value.

The analysis of polyphenolic compounds was performed using high-performance liquid chromatography. For this purpose, an Agilent 1260 Infinity II liquid chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) was used. The operating parameters were as follows. A wavelength of 280 nm was used to determine vanillin and *p*-hydroxybenzoic acid, 320 nm for caffeic and ferulic acid, and 255 nm for chlorogenic acid.

2.7. Soil Respiration

Pots remaining after lettuce harvest with the underground part of the plant intact were subjected to soil respiration intensity testing. For this purpose, the LCpro-SD Gas Exchange Measurement System (manufactured by ADC BioScientific Ltd., UK) with a special cylinder enabling the measurement was used. The cylinder was placed on the pot, which served to cut off the soil surface from external air. Gas exchange between the soil and the atmosphere was measured. The operating parameters of the device were as follows: the concentration of CO_2 supplied to the measuring chamber, the air flow and the H_2O concentration were set to those in the environment. NCER-Net CO_2 Exchange Rate (µmol m^{-2} s^{-1}) and Ce-Soil Respiration (vpm) were measured.

2.8. Statistical Analysis

Statistical analysis was performed based on two-way analysis of variance. In order to determine homogeneous groups, the Post-hoc Tukey was used with a significance level of $\alpha = 0.05$. Statistical calculations were performed in ARM and Statistica (Dell Software Inc., Aliso Viejo, CA, USA).

3. Results

3.1. Plant Yield

Fertilization is the most important factor determining the growth and yield of plants. The use of natural fertilization did not result in a significant increase in yield (Table 1). The lettuce yield in the watered treatments was higher than under drought conditions. The highest yield (30.4 g/pot) was collected from the plants fertilized with HI frass at a dose of 12.5 g/pot. In the drought treatment, the highest lettuce yield was harvested without fertilization, and the lowest was harvested with cattle manure.

Table 1. Lettuce leaf biomass under different fertilizers and water conditions.

Water Regime	Fertilizer	Dose (g/L)	Leaf Biomass (g)
Watered	Control	-	29.6 ab
	Cattle manure	10	25.7 abc
	HI frass	10	36 a
	HI frass	12.5	30.4 ab
Drought	Control	-	12.6 bc
	Cattle manure	10	9.5 c
	HI frass	10	9.9 bc
	HI frass	12.5	10.5 bc

Tukey HSD (p = 0.05) 17.69. Letters a–c indicate statistically different mean values (α = 0.05).

3.2. Photosynthetic Activity

The exposure of plants to drought reduced the values of CO_2 assimilation compared to the watered. However, using a higher dose of HI frass to fertilize plants growing in watered conditions as well as in a condition of drought led to a higher value of the A (CO_2 assimilation) parameter. Drought also caused a reduction in other photosynthesis parameters: E (transpiration) and Gs (stomal conductance) (Table 2).

Table 2. Lettuce photosynthetic activity under different fertilizer and water conditions.

Water Regime	Fertilizer	Dose (g/L)	A (umol/m × s)	E (mmol/m × s)	Gs (mol/m × s)	Ci (vpm)
Watered	Control	-	3.30 bc	0.97 bc	0.04 bc	219 ab
	Cattle manure	10	4.91 ab	1.43 ab	0.06 abc	220 ab
	HI frass	10	6.14 a	1.71 ab	0.08 ab	216 ab
	HI frass	12.5	6.62 a	2.22 a	0.11 a	246 a
Drought	Control	-	0.51 d	0.17 c	0.00 c	233 ab
	Cattle manure	10	0.84 d	0.23 c	0.00 c	204 ab
	HI frass	10	1.51 cd	0.33 c	0.01 c	157 b
	HI frass	12.5	1.06 d	0.27 c	0.01 c	166 ab

Letters a–d indicate statistically different mean values (α = 0.05). Photosynthetic activity was determined by measuring the following parameters: A—CO_2 assimilation, E—transpiration, Gs—stomatal conductance and Ci—intercellular CO_2 concentration. A: LSD Fertilizer: 1.893; LSD Water regime: 2.705; LSD Fertilizer × Water regime: NS, E: LSD Fertilizer: 1.067; LSD Water regime: 0.128; LSD Fertilizer × Water regime: NS, Gs: LSD Fertilizer: 0.063; LSD Water regime:0.075; LSD Fertilizer × Water regime: NS, Ci: LSD Fertilizer: 83.530; LSD Water regime: 4.785; LSD Fertilizer × Water regime: NS.

3.3. Chlorophyll Fluorescence

Drought stress influenced the chlorophyll fluorescence parameters at all levels of fertilization. A significant increase in the values of the fluorescence parameters measured after dark adaptation (Fm, Fv/Fm) and a decrease in those after light adaptation (Yield and ETR) were observed as a result of drought (Table 3). As a result of the closing of the stomata during drought stress, the demand for photochemical energy decreased, which resulted in a decrease in the quantum efficiency of the photochemical reaction in PSII, as evidenced by the yield parameter, as well as the electron transport rate (ETR). However, drought stress was not severe and did not cause permanent damage to the photosynthetic apparatus. On the contrary, the increased Fm and Fv/Fm values indicated the mobilization of plants to adapt to the conditions of limited water availability. An increase in the value of this parameter means a disruption occurred in the energetic antennas related to the capture of light or its transport between photosynthetic pigments (mainly chlorophyll a). Similarly, the highest (most favorable) values of the parameters Fm and Fv/Fm were observed for the plants fertilized with HI frass. Significantly higher quantum yield of photosynthetic energy (yield) values were demonstrated in the plants fertilized with HI frass, but only under conditions of optimal irrigation. In turn, the electron transport rate turned out to be faster in plants fertilized with HI frass, both in drought and optimal irrigation conditions.

Table 3. Lettuce chlorophyll fluorescence under different fertilizer and water conditions (unnominated units).

Water Regime	Fertilizer	Dose (g/L)	F0 (-)	Fm (-)	Fv/Fm (-)	Yield (-)	ETR (-)
Watered	Control	-	206 abc	1006 d	0.79 e	0.20 b	29.3 c
	Cattle manure	10	208 ab	1010 d	0.80 d	0.21 b	29.9 c
	HI frass	10	192 c	1016 d	0.81 bc	0.23 a	32.3 b
	HI frass	12.5	198 bc	1021 d	0.81 cd	0.23 a	33.2 a
Drought	Control	-	212 ab	1081 c	0.80 cd	0.10 c	13.5 f
	Cattle manure	10	214 a	1129 b	0.81 bc	0.10 c	14.1 ef
	HI frass	10	200 abc	1135 ab	0.81 b	0.10 c	14.9 de
	HI frass	12.5	197 bc	1146 a	0.82 a	0.10 c	15.2 d

Letters a–f indicate statistically different mean values (α = 0.05). F0: LSD Fertilizer: 14.85; LSD Water regime: 8.138; LSD Fertilizer × Water regime: NS, Fm: LSD Fertilizer: 16.12; LSD Water regime: 460.900; LSD Fertilizer × Water regime: NS, Fv/Fm: LSD Fertilizer: 0.009; LSD Water regime: 0.000; LSD Fertilizer × Water regime: NS, Yield: LSD Fertlizer: 0.014; LSD Water regime: 0.000; LSD Fertilizer × Water regime: 0.012, ETR: LSD Fertlizer: 0.991; LSD Water regime: 0.908; LSD Fertilizer × Water regime: NS.

3.4. Mineral Profile

The use of fertilization with both frass and cattle manure resulted in a significant increase in the potassium level in the collected plant material. The opposite effect, i.e., a decrease in the content as a result of fertilization, was observed for calcium. In the case of magnesium, sodium, copper and zinc, no differences were noted in their concentration between different fertilizers. The iron content in the drought conditions was higher where HI frass was use (Table 4).

Table 4. Lettuce mineral profile under different fertilizer and water conditions.

Mineral	Fertilizer	Dose (g/L)	Watered (mg/g)	Drought (mg/g)
Ca	Control	-	95.1 ± 6.4 a,A	82.3 ± 7.8 a,B
	Cattle manure	10	78.7 ± 6.3 c,B	84.4 ± 6.0 a,A
	HI frass	10	83.7 ± 7.3 bc,A	75.2 ± 5.5 b,A
	HI frass	12.5	87.4 ± 8.0 b,A	78.8 ± 5.5 b,A
Mg	Control	-	36.9 ± 3.1 a,A	34.2 ± 2.8 a,A
	Cattle manure	10	33.2 ± 2.8 a,A	32.1 ± 2.8 a,A
	HI frass	10	31.7 ± 2.7 a,A	30.8 ± 2.4 a,A
	HI frass	12.5	34.4 ± 2.8 a,A	32.4 ± 3.0 a,A
K	Control	-	37.6 ± 2.5 b,B	49.5 ± 3.8 b,A
	Cattle manure	10	57.8 ± 4.0 a,A	60.2 ± 5.0 a,A
	HI frass	10	56.3 ± 4.7 a,A	59.9 ± 4.2 a,A
	HI frass	12.5	68.7 ± 5.0 a,A	65.5 ± 4.4 a,A
Na	Control	-	27.9 ± 1.5 a,A	25.7 ± 1.6 a,A
	Cattle manure	10	25.9 ± 1.8 a,A	24.6 ± 1.5 a,A
	HI frass	10	23.7 ± 1.5 a,A	25.0 ± 1.6 a,A
	HI frass	12.5	23.1 ± 1.6 a,A	24.5 ± 1.5 a,A
Cu	Control	-	0.28 ± 0.04 a,A	0.22 ± 0.03 a,A
	Cattle manure	10	0.20 ± 0.04 a,A	0.25 ± 0.03 a,A
	HI frass	10	0.22 ± 0.04 a,A	0.28 ± 0.04 a,A
	HI frass	12.5	0.18 ± 0.03 a,B	0.31 ± 0.04 a,A
Fe	Control	-	0.37 ± 0.02 a,B	0.43 ± 0.02 b,A
	Cattle manure	10	0.38 ± 0.02 a,A	0.39 ± 0.02 b,A
	HI frass	10	0.33 ± 0.02 a,B	0.47 ± 0.03 b,A
	HI frass	12.5	0.39 ± 0.02 a,B	0.73 ± 0.02 a,A

Table 4. *Cont.*

Mineral	Fertilizer	Dose (g/L)	Watered (mg/g)	Drought (mg/g)
Mn	Control	-	0.31 ± 0.02 a,A	0.30 ± 0.02 a,A
	Cattle manure	10	0.11 ± 0.01 b,B	0.29 ± 0.02 a,A
	HI frass	10	0.13 ± 0.02 b,B	0.30 ± 0.02 a,A
	HI frass	12.5	0.30 ± 0.02 a,A	0.30 ± 0.02 a,A
Zn	Control	-	0.07 ± 0.01 a,A	0.06 ± 0.01 a,A
	Cattle manure	10	0.06 ± 0.01 a,A	0.05 ± 0.01 a,A
	HI frass	10	0.06 ± 0.01 a,A	0.07 ± 0.01 a,A
	HI frass	12.5	0.07 ± 0.01 a,A	0.07 ± 0.01 a,A
Pb	Control	-	0.60 ± 0.02 a,A	0.43 ± 0.03 a,B
	Cattle manure	10	0.45 ± 0.02 b,A	0.38 ± 0.03 a,A
	HI frass	10	0.31 ± 0.02 c,B	0.52 ± 0.02 a,A
	HI frass	12.5	0.34 ± 0.02 c,B	0.64 ± 0.02 a,A
P	Control	-	21.6 ± 1.2 b,A	23.2 ± 1.5 ab,A
	Cattle manure	10	27.4 ± 1.5 a,A	19.9 ± 1.2 c,B
	HI frass	10	22.4 ± 1.0 b,A	21.6 ± 1.4 bc,A
	HI frass	12.5	25.9 ± 1.5 a,A	25.0 ± 1.3 a,A

The results noted with different lowercase letters differ statistically at the level of α = 0.05 between the fertilizers used. The results noted with different uppercase letters differ statistically at the level of α = 0.05 between watering methods and appropriate fertilizers. Statistical difference for each mineral and water condition calculated separately.

3.5. Antioxidant Activity and Polyphenolic Compounds

Table 5 shows the results of antioxidant activity (measured by ABTS and FRAP methods) and total polyphenol content (expressed as ferulic acid equivalent (FAE)). The use of fertilization resulted in a decrease in the concentration of polyphenols in the plant tissues. This effect was observed both in the drought and optimal irrigation conditions. Increasing the frass dose from 10 to 12.5 g/pot significantly reduced the content of polyphenols from 14.2 to 13.1 mg/g dm. Due to the concentration of polyphenols in the plant, a decrease in the antioxidant activity was observed. The plants were better nourished, which caused less stress and, as a result, fewer antioxidant compounds.

Table 5. Antioxidant activity.

Water Regime	Fertilizer	Dose (g/L)	FAE (mg/g dm)	TEAC $_{ABTS}$ (mmol Trolox/g dm)	TEAC $_{FRAP}$ (mmol Trolox/g dm)
Watered	Control	-	16.6 ± 0.7 a,A	97 ± 18 a,A	33.68 ± 1.82 a,A
	Cattle manure	10	14.1 ± 0.9 b,A	94 ± 13 a,A	19.57 ± 2.26 b,A
	HI frass	10	14.2 ± 0.7 b,A	75 ± 15 ab,A	11.92 ± 4.01 c,A
	HI frass	12.5	13.1 ± 0.6 c,A	58 ± 9 b,A	10.53 ± 1.06 c,A
Drought	Control	-	14.2 ± 1.0 a,B	66 ± 16 ab,B	14.63 ± 1.79 a,B
	Cattle manure	10	11.1 ± 1.3 b,B	52 ± 11 bc,B	10.44 ± 1.50 ab,B
	HI frass	10	10.3 ± 1.5 b,B	53 ± 17 bc,B	9.81 ± 0.67 b,B
	HI frass	12.5	10.2 ± 1.4 b,B	45 ± 14 c,A	4.61 ± 2.26 c,B

The results noted with different lowercase letters differ statistically at the level of α = 0.05 between the fertilizers used. The results noted with different uppercase letters differ statistically at the level of α = 0.05 between watering methods and appropriate fertilizers. Statistical difference for each parameter and water condition calculated separately.

Since polyphenols have growth-regulating properties, they support the processes of plant adaptation to stressful conditions. One of the most important polyphenols is salicylic acid, the concentration of which increases in unfavorable conditions. As a result of the action of this compound, the concentration of auxins in the plant decreases, leading to growth inhibition.

The content of water-soluble vitamins was determined in water–acetonitrile extracts (1:1 v/v) using high-performance liquid chromatography (HPLC). For qualitative and quantitative identification, the internal standards method was used. The results were converted into mg per g of plant dry matter. Among the analyzed vitamins, spectra were obtained allowing for the identification of only riboflavin (vitamin B2). Frass turned out to be a fertilizer that influenced the riboflavin content. Its highest concentration (0.013 mg/g) was recorded on plants fertilized with frass at a dose of 12.5 g/pot. Drought caused a decrease in the concentration of this compound in plant tissues (Table 6).

Table 6. Lettuce polyphenolic compounds.

Water Regime	Fertilizer	Dose (mg/L)	Riboflavin (mg/g)	*p*-HyDroxybenzoic Acid (µg/g)	Chlorogenic Acid (µg/g)	Ferulic Acid (µg/g dm)	*o*-Coumaric Acid (µg/g dm)
Watered	Control	-	0.006 c,A	0.472 c,A	2303 a,A	4548 a,A	1.011 b,B
	Cattle manure	10	0.006 c,A	0.704 a,A	1401 b,A	2825 b,A	1.136 a,B
	HI frass	10	0.009 b,A	0.733 a,A	1190 c,A	2545 b,A	1.135 a,B
	HI frass	12.5	0.013 a,A	0.576 b,A	26.3 d,B	692 c,B	1.079 b,B
Drought	Control	-	0.004 a,A	0.460 a,A	531 b,B	1667 a,B	1.650 a,A
	Cattle manure	10	0.004 a,B	0.386 c,B	568 a,B	1318 c,B	1.463 b,A
	HI frass	10	0.004 a,B	0.412 b,B	426 c,B	1292 c,B	1.639 a,A
	HI frass	12.5	0.005 a,B	0.463 a,B	570 a,A	1506 b,A	1.514 ab,A

The results noted with different lowercase letters differ statistically at the level of α = 0.05 between the fertilizers used. The results noted with different uppercase letters differ statistically at the level of α = 0.05 between watering methods and appropriate fertilizers. Statistical difference for each parameter and water condition calculated separately.

3.6. Soil Respiration

Fertilizers such as frass can bring many benefits to the environment, contributing to the improvement of the soil and increasing access to nutrients and water for the plant. Drought stress causes the Net CO_2 Exchange Rate (NCER) to decline. The value of this indicator is influenced by the fertilizer used. The highest soil respiration rate (Ce) under the conditions of optimal hydration occurred in the treatments fertilized with HI frass, which had a 57% and 54% higher NCER for the dose of 10 and 12.5 g/L, respectively, than the cattle manure. Similarly, under drought conditions, the plants fertilized with Hi frass at a dose of 10 and 12.5 g/L showed a higher Ce than those fertilized with cattle manure, by 44 and 58% (Table 7).

Table 7. Soil respiration under different fertilizer and water conditions.

Water Regime	Fertilizer	Dose (g/L)	Ce (vpm)	NCER (µmol m^{-2} s^{-1})
Watered	Control	-	11.9 c	1.2 c
	Cattle manure	10	82.9 ab	8.7 ab
	HI frass	10	130.1 a	13.6 a
	HI frass	12.5	127.5 a	13.4 a
Drought	Control	-	1.7 c	0.18 c
	Cattle manure	10	20.2 c	2.1 c
	HI frass	10	29.0 c	3.1 c
	HI frass	12.5	31.9 bc	3.4 bc

Letters a–c indicate statistically different mean values (α = 0.05). Ce: LSD Fertilizer: 5.422; LSD Water regime: 30.249; LSD Fertilizer × Water regime: NS, NCER: LSD Fertlizer: 1.443; LSD Water regime: 31.597; LSD Fertilizer × Water regime: NS.

4. Discussion

Lettuce is one of the most popular vegetable plants used in the human diet. Leaves contain polyphenolic compounds, vitamins and calcium and iron. When eaten raw, lettuce can be a source of antioxidants in the human diet, preventing chronic diseases. It can be grown both in the field and in greenhouses. It generally belongs to a temperate, cool climate and is a good vegetable in such conditions [58]. It is expected that the production of insects for protein purposes will likely increase significantly in the coming years. Along with this, the production of frass excrement from this production, which will be used for agriculture, will also increase. The use of frass in lettuce fertilization is one of its possible uses [59]. It has also been used as an addition to soilless substrate in the production of tomatoes, lettuce and basil [60].

In the conducted experiment, frass application did not influence the fresh mass of lettuce. There could be many reasons for this—the short vegetation period of the plant, which did not allow for the observation of the fertilization effect when too small or too large doses were used. We also find similar results from other authors. High doses of frass fertilization may inhibit plant growth and reduce plant yields. This is due to the toxicity of NH_4. This effect may also be allelopathic [61]. The research showed that frass had various effects on the yield and mineral composition of lettuce leaves. Also, no increase in the yield was observed even at higher doses [62]. *Brassica rapa* plants gained greater biomass if the frass was N-rich, while the biomass was reduced if the frass was N-poor [63]. Treating frass may also be good to increase nitrogen availability, through anaerobic digestion or composting [64]. Frass can also have a stimulating effect on the number of soil microorganisms: the number of bacteria, archaea and fungi, carbon mineralization and nitrification, which may ultimately translate into the availability of post-food nutrients for the plant [65].

In addition to the yield, an essential part of the work was also to investigate the physiological condition of the plants. The study of chlorophyll fluorescence has become possible thanks to the popularization of mobile chlorophyll fluorometers. However, interpretation and measurement are not easy. The measurement principle is based on the following phenomenon: Light energy absorbed by chlorophyll molecules can either be used to drive photosynthesis, or the excess can be dissipated as heat or emitted back as light—chlorophyll fluorescence. An increase in the efficiency of one causes a decrease in the intensity of the other [66]. By measuring the fluorescence intensity, it is possible to determine the efficiency of photochemistry. Measuring fluorescence has long been used to monitor the photosynthetic efficiency. They can be used for the non-invasive assessment of the operational quantum efficiency of electron transport, and the efficiency is related to CO_2 assimilation. Measurements can provide the information used [67]. Drought stress caused a significant decrease in CO_2 assimilation, which was an expected reaction from plants as a result of stomatal closure as a defense against water loss. For the same reason, a decrease in the level of transpiration and stomatal conductance was noted. However, the decrease in these parameters (E and Gs) was not significant at all levels of fertilization. It was observed that plants fertilized with HI frass retained CO_2 assimilation and transpiration at a higher level than those fertilized with manure and those not fertilized at all in both water regimes. This proves these plants had a better physiological condition, exhibited more efficient photosynthesis and some adaptation to drought.

The results of the chlorophyll fluorescence confirm a better condition of the photosynthetic apparatus in these plants; although the measurement results do not indicate statistically significant differences, a clear tendency is visible. Lower minimum fluorescence values were recorded in the plants fertilized with HI frass, which indicates the greater efficiency of the operation of energy antennas and photosynthetic pigments. Higher maximum fluorescence values, in turn, indicate the higher efficiency of electron acceptors in photosystem II (PSII). The Fv/Fm parameter, according to some sources [58,59], is not sensitive to drought stress, but it can be seen in optimally watered plants and fertilized plants that the values of the maximum photochemical efficiency of PSII are slightly higher, although their values do not indicate stress at any of the experimental levels. Significant differences in the values of the fluorescence parameters under light—yield and ETR—were demonstrated only in the optimally watered plants. They indicate faster electron transport through photosystems as well as the higher quantum yield of the photosynthetic energy in plants fertilized with Hi frass. However, the differences are small.

Therefore, we conclude that HI frass fertilizer slightly improved the health and physiological condition of lettuce plants, which allowed for more effective defense against drought stress. Any improvement in plant health seems to be valuable in the era of climate change and water scarcity.

Plants are exposed to biotic and abiotic stress throughout their life. As a result of secondary metabolism, plants produce chemical compounds that can protect the plant against abiotic stress factors such as light, UV radiation, temperature and heavy metals. Some of these substances have antioxidant properties that are beneficial to humans. By supplying them to the body, we reduce oxidative stress and reduce disease incidence [68]. Also, in plants, the action of polyphenols defends against stress factors. This is the most studied group of metabolites, containing 8.000 types. Their structure is very diverse, and based on their structure, they can be divided into phenolic acids, flavonoids, stilbenoids and lignans. They may occur in plants as free forms or conjugated with other particles. By measuring the content of polyphenols, they can be taken as a marker of biological activity [69]. The content of polyphenolic compounds in plants is often correlated with fertilization. In a study conducted on herbs, it was found that limiting the supply of ingredients resulted in an increase in phenolic compounds. For example, basil fertilized with the lowest doses of nitrogen was characterized by a higher concentration of rosmarinic and caffeic acids. Fertilization in this respect also affects antioxidant activity [70]. Many chemically diverse compounds perform antioxidant functions in the plant.

Many factors influence the content of polyphenolic compounds in a plant. Leaf vegetables are characterized by a high variability of concentrations. Good sunlight and temperature favor the growth of polyphenolic compounds. In the research conducted by Wieczorek [71], the concentration of polyphenols in lettuce leaves varied greatly, ranging from 7 to over 250 mg/100 g of food. Iceberg lettuce turned out to be a low source of phenolic compounds. Compared to lettuce, a cultivated vegetable, wild plants fared much better, the concentration of which was on average 13 times higher than in lettuce. Also, in this study, it was verified that in the cultivation of basil, antioxidant activity and polyphenol concentration depend on the fertilization applied [72].

The literature states that lettuce leaves contain vitamins from group B, such as B1, B2, B3, B6 and B9. In the analyzed samples, it was possible to determine only vitamin B2, riboflavin, the concentration of which increased due to the use of Frass fertilizer from insect production. It has been found that fertilization affects the concentration of vitamins and microelements in plants [73].

Riboflavin itself is necessary for the development and proper growth of the plant. Research shows that riboflavin increases the resistance of plants to drought. Its administration to 4-week-old tobacco plants resulted in increased drought resistance [74].

The content of ingredients in plant organs may change depending on many factors, including environmental conditions. The availability of ingredients in the substrate usually translates into their greater concentration in the plant's organs. For this reason, balanced fertilization and providing the plant with all nutrients is not only important from the point of view of good nutrition of the plant but also the balance of nutrients in the food obtained.

Fertilizer obtained from various species of insects is characterized by an appropriate concentration, content of nutrients and their availability. Compared to other insects, the frass obtained from the black fly is characterized by a higher concentration of nitrogen and potassium [45]. Frass can be as effective in fertilization as classic NPK mineral fertilizers thanks to quick mineralization and easily available nutrients and can complement or even completely replace mineral fertilization [59]. In the case of fertilizing the *Gongronema latifolium* plant with inorganic NPK fertilizer, this led to an increase in the concentration of potassium, sodium, calcium, magnesium, phosphorus and nitrogen in the leaves, and the concentration of nutrients increases with increasing fertilization. In the case of fertilizing the *Gongronema latifolium* plant with inorganic NPK fertilizer, this led to an increase in the concentration of potassium, sodium, calcium, magnesium, phosphorus and nitrogen in the leaves; the concentration of nutrients increases with increasing fertilization [75].

Magnesium accumulation depends on many factors. These include genetic factors, but also environmental factors such as soil and climate. The plant absorbs nutrients from the soil solution through various mechanisms. Potassium, calcium and magnesium ions have antagonistic effects. The uptake of potassium by a plant depends on its concentration in the soil solution, and the concentration also affects the uptake mechanism. The role of potassium in the plant is manifold and it serves, among others, as a support for photosynthesis. It also increases the plant's resistance to unfavorable environmental conditions—water shortage—by regulating the opening of stomata. Calcium in the plant is responsible for the construction of cell walls [76].

5. Conclusions

Residues from industrial insect production, called frass, can be an alternative to conventional natural fertilizers, such as cattle manure, which are difficult to obtain in various regions of the world. The use of insect frass increased the concentration of nutrients such as potassium and iron in lettuce plants. At the same time, an increase in the content of vitamin B2 and a decrease in the synthesis of polyphenolic compounds were observed. The application of this fertilizer allowed for an increase in the efficiency of photosynthesis. Plants fertilized with HI frass showed greater resistance to drought stress, which was confirmed by the results of chlorophyll fluorescence measurements. The use of the test fertilizer also contributed to an increase in the value of soil respiration parameters, which indicates a beneficial effect on the lettuce root system. HI frass is therefore a promising alternative to currently used fertilizers.

Author Contributions: Conceptualization: S.Ś. and Z.S. Methodology: S.Ś., Z.S., D.R.-K. and M.G. Formal Analysis: Z.S. and P.Ł.K. Investigation: S.Ś., Z.S., D.R.-K., M.G., P.Ł.K., Ł.S., G.S., A.D. and M.Ś. Writing—original draft preparation: S.Ś. Writing—review and editing: Z.S., D.R.-K. and P.Ł.K. supervision: P.Ł.K. and Z.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Narodowe Centrum Badań i Rozwoju grant number POIR.01.01.01-00-1503/19, entitled Development of a technology for the production of organic fertilizer (in the form of pellets/granules) based on the Hermetia illucens frass and testing its impact on selected plants.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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