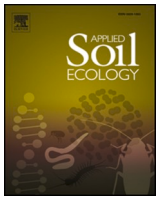
Applied Soil Ecology 167 (2021) 104037

Contents lists available at ScienceDirect

Applied Soil Ecology

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



Biometric, chemical, and microbiological evaluation of common wheat (*Triticum aestivum* L.) seedlings fertilized with mealworm (*Tenebrio molitor* L.) larvae meal

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ARTICLE INFO

Keywords:
Insect meal
Amino acids
Rhizosphere microorganisms
Biometry
Nitrogen

ABSTRACT

Alternative organic fertilizers are being developed to minimize the adverse environmental impact of chemical plant protection agents. The interest in industrial-scale insect farming has increased in recent years. Mealworm larvae are a rich source of protein and fatty acids. This study focuses on mealworm larvae, which are characterized by a rapid increase in biomass and a high nutritional value. In the present experiment, mealworm larvae were processed into fertilizer with a high content of organic nitrogen. The fertilizer's effect on wheat growth, soil and rhizosphere microorganisms, including phytopathogenic fungi of the genus *Fusarium*, and N-cycle, was analyzed. Mineral nitrogen fertilizer and mealworm larvae meal used as fertilizer caused a similar increase (~40%) in the total nitrogen content of the soil. Due to its mineral content, mealworm larvae meal contributed to an increase in the concentrations of P, K, and Mg in soil. The amino acid quality was high (0.89). Increasing the load of *Bacillus* spp. after using the meal was negatively correlated with the *Fusarium* spp. load in the wheat rhizosphere. In the case of meal fertilization, ammonification was noticed, and organic nitrogen was successively mineralized. The fertilizer produced from mealworm larvae offers a viable alternative to mineral fertilizers. It improves the health and nutrient status of wheat seedlings and stimulates the growth of *Bacillus* bacteria that enhance the availability of soil nutrients to plants and prevent seedling damping off. Further research is needed to confirm the applicability of the mealworm fertilizer in other field crops.

1. Introduction

In modern crop production, the main focus is on increasing yields to cater to continued population growth and increasing demand for livestock and the scarcity of arable land. Agricultural intensification increases the use of mineral fertilizers and chemical crop protection agents, which, when applied excessively, can have adverse environmental consequences. Crop monocultures and repeated application of the same active ingredients contribute to resistance development in pathogen populations. Biological products and biotechnological treatments can be applied as safer alternatives to improve crop yield and quality and protect plants against pests and pathogens. Agricultural chemicals can be replaced with biotechnological treatments and appropriate fertilizers containing organic substances that directly or indirectly eliminate undesirable bacteria, fungi, weeds, or pests. Organic fertilizers can increase yields and reduce the incidence of plant diseases without exerting a harmful impact on the natural environment (Compant et al., 2005; Bonilla et al., 2012; Robačer et al., 2016).

A serious problem is posed by the presence of plant pathogenic fungi in agroecosystems. The most dangerous genus is the *Fusarium* which infects plants at various stages of growth and induces diseases with different symptoms, including stem rot, Fusariosis, and Fusarium head blight (FHB). *Fusarium* spp. produce dangerous mycotoxins (i.e., deoxynivalenol, nivalenol, T2, fumonisin, zearalenone, vomitoxin). These endophytic fungi can exist as saprotrophs and cause asymptomatic infections; however, they can also have lethal consequences for the

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https://doi.org/10.1016/j.apsoil.2021.104037
Received 17 November 2020; Received in revised form 16 February 2021; Accepted 8 April 2021
Available online 5 May 2021

colonized plants. Moreover, they cause soil-borne diseases of seedlings (Magan et al., 2002; Leslie and Summerell, 2006; Alonso et al., 2013; Przemieniecki et al., 2014, 2019a, 2019b).

Soil microorganisms play important roles by stabilizing biological processes in the soil solution, increasing soil nutrients' availability, contributing to humus formation, promoting plant growth, and protecting plants against pests and phytopathogens. Beneficial bacteria can protect plants against phytopathogens directly or indirectly through various strategies. They colonize ecological niches and suppress the abundance of phytopathogens by stimulating plant immunity and producing such substances as chitinase, gluconase, cellulase, protease, lipase, siderophores, and antibiotics. Considerable research has been dedicated to the production of antibiotics by microorganisms. In agricultural ecosystems, fungistatic compounds are produced mainly by: *Pseudomonadaceae*, *Actinomycetes*, and *Bacillus* spp. The development of the above bacterial groups is determined by a wide range of environmental factors, including plant cover, soil C:N ratio, soil moisture content, soil nutrient levels, soil structure, and the accompanying microbiota (Ahemad and Kibret, 2014; Kooch et al., 2018). For instance, *Pseudomonas fluorescens* produces the antibiotic 2,4-DAPG, which effectively inhibits the development of pathogens, in particular soil fungi that cause root diseases. This bacterial species is ubiquitous in long-term monocultures (Svercel et al., 2009; Ahemad and Kibret, 2014). Phytopathogens are also effectively eliminated by bacteria of the genus *Bacillus*. These bacteria produce various antibiotics (lipopeptides) and chitinase, which prevent *Fusarium* spp. pathogens from colonizing plants. Research indicates that spore-forming *Bacillus* bacteria are most effective under field conditions (Cavaglieri et al., 2005; Gajbhiye et al., 2010; Zhao et al., 2014).

Plant cultivation requires the constant availability of nutrients. Except for water, nitrogen is a factor limiting plant growth; however, the excess of available mineral forms causes rapid volatilization (N-NH_4) or leaching (N-NO_3) of nitrogen available to plants. A safer solution is to use organic and natural fertilizers because, with the participation of microorganisms, nutrients from these fertilizers are transformed into mineral forms and gradually released into the soil.

In the case of nitrogen: nitrification, denitrification, ammonification, and N_2 fixation are the four basic microbial processes involved in the supply, leaching, and conversion of nitrogen in soil systems. The current state of nitrogen in the soil can be measured, however the determination of its stability, utilization, and fate requires analyzing a load of functional genes (indirectly microorganisms) responsible for nitrogen transformations in the soil environment. *Tenebrio molitor* larvae are omnivorous, resistant to adverse environmental conditions, and characterized by a rapid biomass growth. These larvae have numerous industrial applications, including in food and feed production, and in the management of waste that is difficult to process. Mealworms are capable of digesting polyethylene, polystyrene, and cellulose waste. Researchers have recently hypothesized that mealworms' ability to adapt to different foods is determined by their gut bacteria (Przemieniecki et al., 2020). Mealworm larvae have also been used as a protein source in livestock diets. Animal feeds containing mealworm larvae meal have a higher protein content and a more desirable fatty acid composition than commercial feeds. Mealworm larvae are characterized by a high protein content, low dry matter content, and rapid biomass increase on waste substrates; therefore, they can be used as fertilizer after pre-treatment (Siemianowska et al., 2013; Yang et al., 2015; Brandon et al., 2018; Dabbou et al., 2019).

Very popular new class fertilizer are insects frass, especially from *T. molitor* (Poveda et al., 2019; Dulaurent et al., 2020; Houben et al., 2020). Previous studies on plants (Poveda et al., 2019) have shown a high fertilizing value of mealworm frass. In addition, this fertilizer increase plant resistance to stress. The microbiome analysis revealed the presence of plant growth promoting bacteria. Mealworm frass is as efcient as mineral NPK fertilizer to improve plant growth, and can improve microbiological properties of soil (Poveda et al., 2019; Houben et al., 2020). The advantage of laravae meal over its frass is the much higher content of NPK (several times higher in the case of N, Siemianowska et al., 2013, Houben et al., 2020) and the content of chitin, potentially supporting the plant immune system. This study attempted to use larvae meal form *T. molitor* as an alternative to frass fertilizer.

The research hypothesis: The application of meal from the larvae into the soil has a positive effect on assimilable nitrogen content, chemical parameters, and rhizosphere microorganisms.

This study aimed to determine the effect of high-protein insect meal on the growth of seedlings, microbiological and chemical parameters, and the fate of nitrogen in the wheat rhizosphere.

2. Material and methods

2.1. Chemical composition of mealworm larvae meal

Mealworm larvae were obtained from a continuous breeding system. They were fed residues from oatmeal production. After molting, the larvae were separated mechanically and freeze-dried under vacuum at a temperature of −55 °C for 24 h in the Christ Delta 2–4 freeze dryer.

The protocol for analyzing the contents of protein, fat, carbohydrates, and other biogenic elements in the mealworm larvae meal was described in detail in our previous study (Siemianowska et al., 2013). The content of amino acids, excluding tryptophan, was determined in the dried meal using the Ingos AAA-400 automatic amino acid analyzer (Ingos, Czech Republic). The analytical procedure was conducted according to the manufacturer's instructions. The samples were hydrolyzed in 6 M HCl for 24 h at a temperature of 110 °C. The hydrolysate was cooled, filtered, rinsed, and evaporated in a water bath. The dry residue was dissolved in a buffer with a pH of 2.2. The resulting sample was used in the ninhydrin test with the use of buffers having pHs of 2.6, 3.0, 4.25, and 7.9. The ninhydrin solution was treated with a buffer with a pH of 5.5. Amino acids were separated on an Ostion ANB Ingos ion-exchange column with a length of 370 mm (Ingos, Czech Republic). The column temperature was 58–74 °C, and the reactor temperature was 120 °C. The content of sulfur-containing amino acids (methionine and cysteine) was determined by oxidation and hydrolysis using formic acid and hydrogen peroxide (9:1), at a temperature of 110 °C for 23 h. The sample was cooled and prepared according to the protocol for acid hydrolysis. The applied buffers had pHs of 2.6 and 3.0. The column temperature was 60 °C, and the reactor temperature was 120 °C. Amino acids were analyzed in duplicate. Tryptophan content was determined according to the Polish Standard PN-77/R-64820.

The content of essential amino acids was determined based on FAO guidelines for model protein (chicken egg white, FAO, 1985). The results were expressed as the percentage ratio of mealworm meal amino acids to chicken egg white amino acids. The chemical score of essential amino acids was calculated using the following formula:

$$CS = \frac{D}{M}$$

D – concentration of essential amino acid in dietary protein (mg g^{-1} of mealworm protein)
M – concentration of the same amino acid in model protein (mg g-1 of egg protein)

Amino acid scores were expressed as the total score for all essential amino acids.

2.2. Experimental design

The soil was obtained from the Agricultural Experimental Station in Bałcyny (*Bałcyny Ltd.*, Poland) from a site with a high incidence of infections caused by *Fusarium* fungi. Soil samples were transported to the laboratory of the University of Warmia and Mazury in Olsztyn. The pot

experiment was established on humus horizon of Haplic Luvisol developed from silty sandy loam of average quality class IVa. Soil type according to the WRB classification (WRB, 2015). The soil was passed through a 2 mm mesh sieve, brought to 60% maximum water capacity, and placed in pots (2 kg pot^{-1}). In the mineral nitrogen (ammonium nitrate fertilizer - ammonia: nitrate 50:50 w/w) or mealworm larvae meal treatments, fertilizers were applied by mixing with soil to a depth of 25 cm in sufficient amounts to meet the nitrogen requirements of spring wheat (both fertilizer in dose 180 kg N ha^{-1}). Unfertilized pots served as the control treatment. Spring wheat cv. Bombona was sown manually by placing 6 seeds at a depth of 2 cm in each pot. In this cause pots filled with soil (without sown plants – background control) to which insect meal or nitrogen (both fertilizer in dose 180 kg N ha^{-1}) and no additive were applied. The experiment was conducted in a phytotron for 30 days, in 5 replications (5 pots per treatment). The experimental conditions were described in our previous study (Gorczyca et al., 2018) with modification. Seedlings were allowed to grow up in a phytotron under controlled conditions at 22°C day and 18°C night temperature, a 12 h photoperiod with a light intensity of 220 μmol photons $m^{-2}{\cdot}s^{-1}$ PPFD, and 80% RH. After germination, 5 plants were left in the pot (technical replication). After wheat vegetation, the plant and the root system were taken out of the pots, next the bulk soil was discarded by shaken hands, and next adhering rhizosphere was collected in sterile condition with knives. Finally roots remains were discart and rhizosphere samples were frozen at −20 °C for further analysis. In this expertiments setup was used background control.

The soil and rhizosphere at the beginning of the experiment were also collected for the calculation of the bioavailability rate of macro and microelements (Supplement A). The ratio of concentration of bioavailable nutrients (BN) was calculated according formula:

$$BN\ (\%) = 100 - \left(\frac{T30*100}{T0}\right)$$

T0 – concentration of macro or microelement at the beginning of experiment
T30 – concentration of macro or microelement after 30 day of incubation

2.3. Chemical analysis of soil

Before analysis the soil samples were passed through a 2 mm sieve and ground in a mortar. The pH of the soil was determined in 1 mol dm^3 KCl (the soil: solution ratio 1:2.5 w/v) with a potentiometer. Magnesium was extracted in 0.0125 M $CaCl_2$ solution (soil to solvent ratio – 1:10) according to the method proposed by Schachtschabel (1954). Phosphorus and potassium levels were determined with the Egner-Riehm method (Egnér et al., 1960) with buffered calcium lactate and lactic acid (pH 3.55). The total nitrogen content was determined with the Kjeldahl method (Kjeldahl, 1883), and the total organic carbon content - with the Tyurin method (Tyurin, 1931). A mixture of potassium dichromate and concentrated sulfuric acid ($K_2Cr_2O_7 + H_2SO_4$) served as the oxidizing agent. Organic carbon was oxidized by heating for 5 min in the presence of a silver sulfate catalyst. 0.1 M Mohr's salt solution ($FeSO_4 + NH_4 + 2SO_4 \cdot 6H_2O$) was used as the reducing agent.

2.4. Biometric parameters of wheat seedlings and the severity of seedling infections

The height of wheat seedlings (to the leaf sheath of top leaf), the average length of the first two leaves (measuring beginning at the end of the leaf sheath), and root length were measured manually. The dry weight of above-ground biomass and roots was determined by freeze-drying under vacuum at −55 °C for 24 h in the Christ Delta 2-4 freeze dryer and the prepared plant material was weighed on scale AS 110.R2 (Radwag, Poland). Chlorophyll content was measured with the SPAD 502 Plus Chlorophyll Meter (Konica Minolta, Japan). Ten-gram samples of the rhizosphere soil were collected for microbiological analyses. Disease incidence was determined based on the method proposed by Khan et al. (2007).

2.5. Quantitative polymerase chain reaction (qPCR)

2.5.1. Isolation of genetic material and qPCR

DNA was isolated with the Soil DNA Purification Kit (EurX, Poland). Soil samples were passed through a 2 mm sieve and ground in a mortar. After pre-grinding, 100 mg of soil was transferred to a 2-mL tube containing glass beads and a lysis buffer and homogenized in TissueLyser LT (Qiagen, Germany). Cell lysis was carried out for 5 min at the maximum speed. Further steps of the analysis were carried out following the instructions attached to the isolation kit.

2.5.2. Load of microorganisms

Bacteria were quantified with BAC338F and BAC805R primers and the BAC516F probe (Yu et al., 2005). Fungi were determined with the Sybr Green technique with NSI1 and 58A2R primers (Martin and Rygiewicz, 2005). The Maxima SYBR Green qPCR Master Mix 2× (Thermo Fisher Scientific, USA) was used in the reaction. Antagonistic *Pseudomonas* spp. (B2BF and B2BR3 primer sets amplifying the *phlD* gene) were determined with the method described by Hu et al. (2016) 16S rDNA gene copies (*Bacillus* spp.) were enumerated with 16SBACF and 16SBACR primer sets as described by Mora et al. (2011). The reaction profile was as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 50 s. A thermal curve was developed after the reaction. The final concentration of the primers was 200 nM. The number of toxin-producing fungi of the genus *Fusarium* was determined with Tri5 forward and Tri5 reverse primer sets and the Tri5 Probe. The reaction was performed according to the protocol described by Vegi and Wolf-Hall (2013). Chitinolytic bacteria were evaluated with the Qchi-f and Qchi-r primer set described by Ramaiah et al. (2000) and Abdallah et al. (2017). The abundance of chitinase gene copies was determined using real-time PCR with 200 nM of primers under the following thermal conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s.

The quantitative analysis of N-cycle functional genes was performed based on the works of Jung et al. (2011) to estimate the abundance of *amoA* – ammonium monooxygenase, *nifH* – nitrogenase reductase, *nirS* – nitrite reductase, *norB* – nitrate reductase, *nosZ* – nitrous oxide reductase gene, and Ouyang and Norton (2020) to estimate the abundance of *ureC* (urease) gene copies (Supplement B).

A thermal curve was developed after the reaction. The appropriate amplicons of *Bacillus subtilis* (*Bacillus* spp. and *chiA* gene), *Pseudomonas putida*, *Fusarium culmorum*, and total environmental DNA (for nitrogen-cycle genes) were ligated with plasmids (TOPO™ TA Cloning™ Kit, with pCR™ 2.1-TOPO™, Thermo Fisher Scientific) and used as standards for their respective domains. The Maxima (Probe or SybrGreen) qPCR Master Mix 2× (Thermo Fisher Scientific, USA) was used, and the reaction volume was 20 μL per sample. PCR efficiency was 0.88 to 1.01 ($R^2 = 0.984$ to 1).

2.6. Statistical analysis

Data distribution was tested for normality in the Shapiro-Wilk test, followed by ANOVA – Tukey's test or Kruskal-Wallis test for non-parametric distribution. Principal component analysis (PCA) was conducted using the Pearson's correlation coefficient (the correlation matrix was generated automatically). The heatmap was made on data transformed into percentages with the function of centering the results for each of the observations. The Mankel test was performed on the correlation matrix. The agglomerative hierarchical clustering (AHC) was

performed by Bray and Curtis algorithm with Ward's clustering method. All statistical calculations were performed in XLSTAT (Addinsoft, www.xlstat.com).

3. Results

3.1. Characteristics of Tenebrio molitor meal

The nitrogen content of mealworm larvae meal exceeded 7%, and the protein content approximated 45% of dry matter content. The contents of the remaining nutrients, in particular P and K, were also very high, which indicates that mealworm larvae meal is a highly abundant source of nutrients (according to Standard PN-R 04027:1997; Table 1). The total chemical score (SC) of essential amino acids was very high, approximating 90% (sulfur-containing amino acids were limiting amino acids). The content of the remaining amino acids was also high, which suggests that mealworm larvae meal can be applied as an amino acid fertilizer (Tables 1 and Supplement C).

3.2. Plant growth observations

Mealworm larvae meal led to a significant increase in the height of wheat plants (approx. 10%, comparable to that caused by the mineral nitrogen fertilizer). In the treatment involving soil fertilization with the mealworm larvae meal, the fresh and dry weight of above-ground biomass increased highly significantly (by 50%, $p = 0.01$) compared to the unfertilized control treatment. In turn, compared with the mineral nitrogen fertilizer, the mealworm larvae meal contributed to a significant increase in the dry weight of the above-ground biomass, and to an increasing trend in the fresh weight of above-ground biomass. The fresh and dry weight of roots increased significantly (by 90% and 23%, respectively) in response to the mealworm larvae meal, whereas the mineral nitrogen fertilizer decreased the length, fresh weight, and dry weight of roots. No differences were found in leaf length or chlorophyll content (SPAD). Disease incidence decreased significantly compared to the control treatment (Table 2).

3.3. Rhizosphere chemistry and microbiology

Generally, out of the 9 chemical parameters analyzed, 5 showed statistically significant differences. The total nitrogen content was about 0.85% in the meal and nitrogen variants, while it was significantly lower (by about 35%) in the control. The content of N-NO_3 was significantly higher (more than 3 times) in the nitrogen variant than in the other 2 variants. However, in the case of the N-NH_4 content, all results were significantly different from each other ($p < 0.001$), i.e., approx. 10 in the control, approx. 40 in the meal, and almost 80 mg NH_4 kg^{-1} DM (dry matter) in the nitrogen variant. The C:N ratio was significantly higher in the control variant (approx. 13), compared with 10 and 9 determined in the mean and nitrogen variants. The content of organic carbon did not differ significantly; only a downward trend was shown after nitrogen application. There was a downward trend in the pH value between meal (5.2) and nitrogen (4.7) variants. No significant changes were observed in P and K contents, while the Mg content was significantly higher in the meal variant than in the other variants (Fig. 1).

Out of the 12 analyzed microbiological parameters, 9 differed statistically. The load of bacteria did not differ significantly among the variants; however, the fungi load was significantly higher in the meal variant than in the nitrogen variant and showed an upward trend over the control. The abundance of *Bacillus* spp. was the highest in the meal variant; it differed significantly from the control and showed an upward trend compared to the nitrogen variant. The abundance of *Pseudomonas* spp. was the highest in the control and differed significantly from the other 2 variants, being 2-fold lower in the nitrogen and almost 4-fold lower in the meal variant. A load of trichothecene-producing *Fusarium* spp. was the highest in the control and differed significantly from the meal variant, in which the TRI5 count was 4 times lower. Among the functional genes, there was an upward trend in *nifH* upon nitrogen treatment, a highly significant increase in the load of *amoA* and *nirS* after meal treatment, a highly significant increase in *norB* upon nitrogen versus control, and a significantly higher *ureC* load in meal versus control variants (Fig. 2).

Additional results showed that N-NO_3 bioavailable nutrients in the background control (soil without plants) case was significantly lower than in the meal rhizosphere. At the same time, loss of this form of nitrogen was observed in the control variant. The content of N-NH_4 in the meal was at a similar level, which indicates its slow release from organic matter and certain autoproteolytic content in this fertilizer. The nitrogen acquisition in the nitrogen variant was more dynamic. Among other parameters, an evident nutrient bioavailability was observed for Mg (Supplement A).

3.4. Overall relationship

The first and the second ordination axes in the PCA plot for biometrical observation (Fig. 3a) explained almost 100% of the variance. Based on the biplot, it was noticed that root DM, phyllosphere DM, root mass, and phyllosphere mass were associated with the meal variant, while phyllosphere height achieved higher values in the nitrogen variant. The high value of the DI (disease index) is typical of the control variant. Based on the correlation matrix, it was observed that the values of root DM, phyllosphere DM, and root M were positively correlated with each other ($R > 0.9$), and that there was an almost complete negative correlation between DI and mass of the phyllosphere.

The PCA plot for rhizosphere properties (Fig. 3b) explained almost 100% of the variance. As indicated by biplot vectors, the high values noted for TRI5, Pseudomonas spp. load, *nosZ* gene copies abundance, and C:N ratio were typical of the control variant. The high values N-NO_3 and N-NH_4 contents, bacteria load, and the abundance of *nifH* and *norB* gene copies were typical of the nitrogen variant. In turn, the high values of the following parameters: the abundance of *nirS*, *chiA*, and *amoA* gene copies; *Bacillus* spp. load, Mg content, pH, fungi load, and K content were typical of the meal variant, while the high contents of P and C org were characteristic for both control and meal variants, while TN content and the abundance of *ureC* gene copies – for nitrogen and meal variants.

Based on the correlation matrix, positive correlations of TN content with N-NH_4 content ($R = 0.865$), *Bacillus* spp. load ($R = 0.803$), and the abundance of *norB* ($R = 0.901$) and *ureC* gene copies ($R = 0.998$) were observed. In turn, negative correlations were observed with C:N ($R = -0.991$), the abundance of *Pseudomonas* spp. ($R = -0.923$), TRI5 ($R = -0.810$) and *nosZ* gene copy ($R = -0.973$). The N-NH_4 content ($R = 0.933$), C org content ($R = 0.999$), bacteria load ($R = 0.803$), and the abundance of *norB* ($R = 0.902$) and *nifH* ($R = 0.984$) gene copies were positively correlated with the N-NO_3 content. However, negative correlations with N-NO_3 were observed concerning C org ($R = -0.999$) and P ($R = -0.990$). In the case of N-NH_4, positive correlations were observed with TN ($R = 0.865$), N-NO_3 ($R = 0.933$), the abundance of *nifH* ($R = 0.982$) and *ureC* ($R = 0.830$) gene copies, while negative correlations with C:N ($R = -0.925$) and C org ($R = -0.919$). The C:N ratio was positively correlated with *Pseudomonas* spp. load ($R = 0.862$)

Table 1
Nutritional value of mealworm larvae meal[a].

Parameter	N	P	K	Na	Mg	CS (protein quality)[b]
	[mg 100 g^{-1}]					[%]
Result	7155.5	700.2	726.6	81.1	144.6	89

[a] Contents of NPK, Na, and Mg determined in previous work by Siemianowska et al. (2013).

[b] Determined based on FAO guidelines (the composition of essential amino acids in chicken egg white was used as the reference −100%) (FAO/WHO/UNU, 1985).

Table 2
Biometric parameters of spring wheat after 30 days of the experiment.

Treatment	Plant height (cm)		Mass of phyllosphere (g of fresh mass)		Phyllosphere dry matter (%)		Leaf length (cm)		Root length (cm)		Root fresh weight (g)		Root dry weight (%)		Chlorophyll (SPAD)	Disease incidence	
Control	9.07	b†	0.299	b	39.6	b	18.69	b	19.40	a	0.210	b	18.1	b	28.23	5.6	a
Nitrogen	9.93	a*	0.371	ab	30.7	b	21.91	a*	14.25	b*	0.165	b	16.0	b	31.30	4.2	ab
Mealworm larvae meal	9.81	a*	0.449	a*	58.8	a**	21.85	a*	17.89	a	0.397	a*	22.0	a*	30.03	3.2	b*

† Values marked with the same letters (a or b) do not differ significantly ($p \leq 0.05$).
* $p \leq 0.05$ – compared to control.
** $p \leq 0.01$ – compared to control.

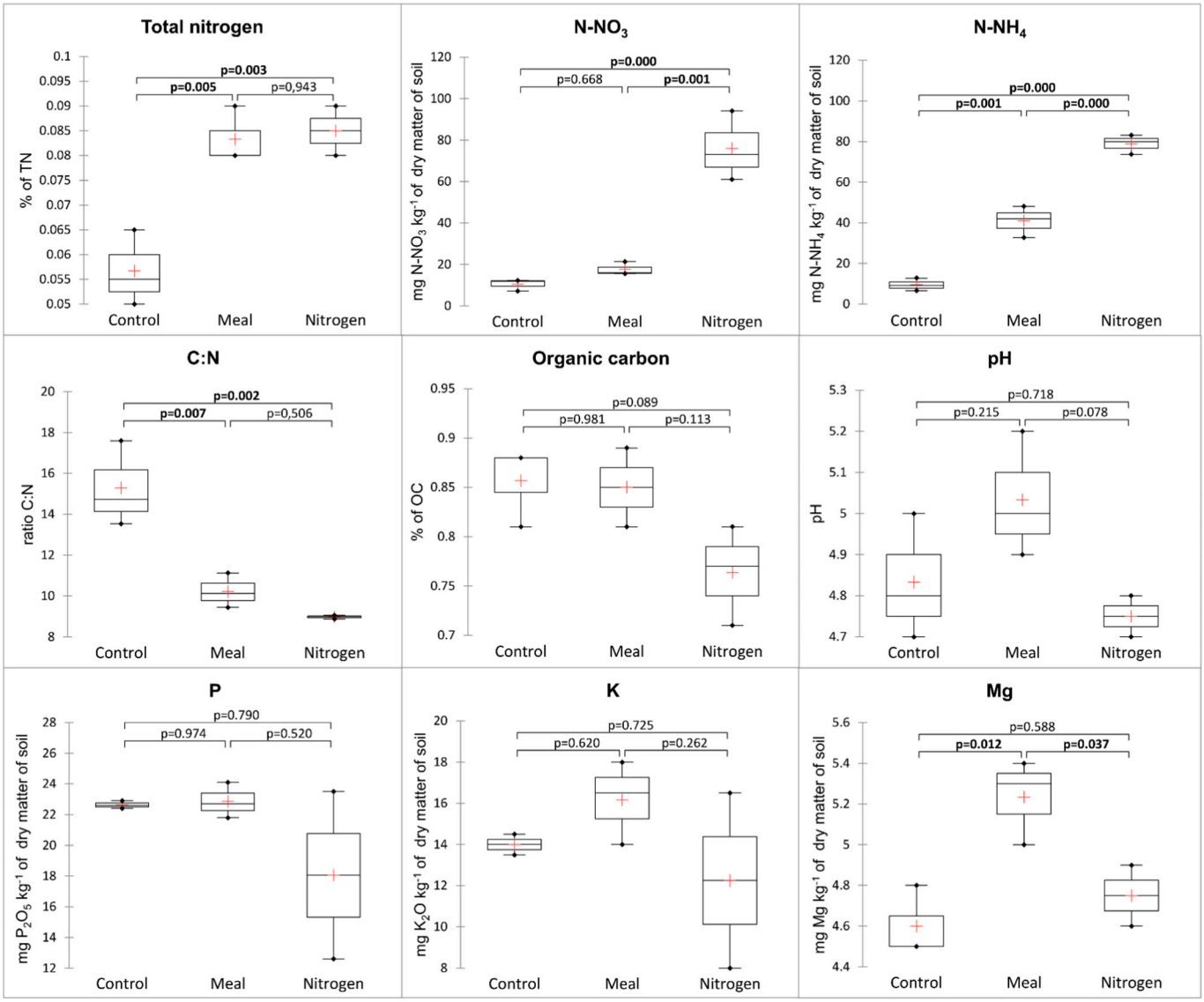

Fig. 1. Boxplots showing chemical parameters of soil after 30 days of seedling cultivation. Statistically significant results (p-value lower than 0.05) are shown in bold. The boxplot shows the 25th percentile, median (line inside box), mean (cross inside box), and 75th percentile; whiskers show the minimum and maximum values.

and the abundance of *nosZ* gene copies (R = 0.933), while negatively correlated with TN and N-NH_4 contents (described above), and the abundance of *nifH* gene copies (R = −0.836), *norB* (R = −0.952), and *ureC* (R = −0.979). In the case of C org content, no clear positive correlation was found, while its value was negatively correlated with N-NO_3 and N-NH_4 contents, bacteria load (R = −0.846), and the abundance of *nifH* (R = −0.977) and *norB* (R = −0.886) gene copies. The pH value was positively correlated with the contents of K (R = 0.985) and Mg (R = 0.868), fungi load (R = 0.990), and the abundance of *chiA* (R = 0.986), *amoA* (R = 1), and *nirS* (R = 0.871) gene copies, but negatively correlated with bacteria load (R = −0.967). Positive correlations were also observed between K content and fungi load (R = 1) and Mg, and between the abundance of *nirS* gene copies (R = 1). Moreover, negative correlations were observed between the copies of the TRI5 gene and

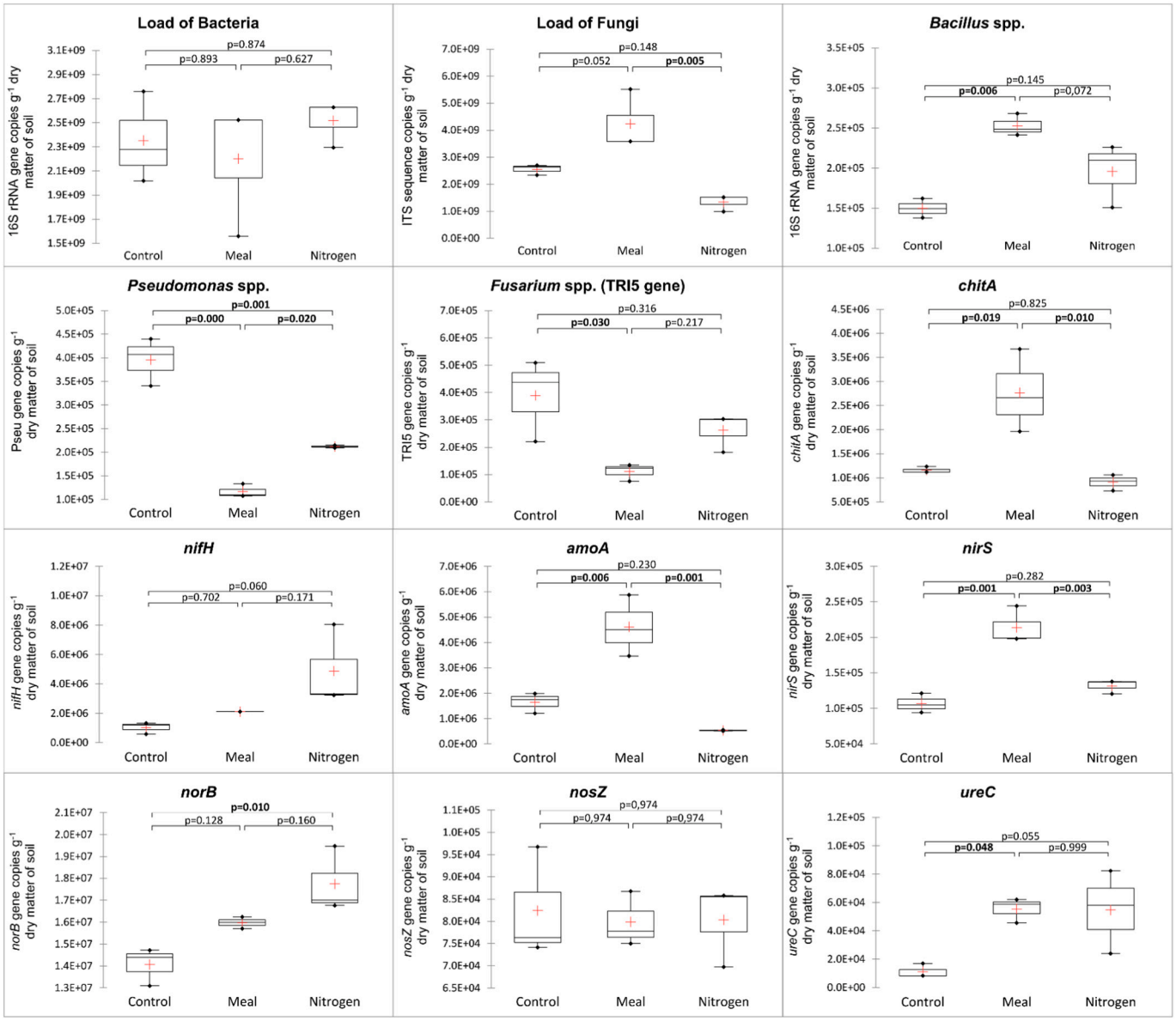

Fig. 2. Boxplots showing the abundance of selected groups of rhizospheric microorganisms and functional genes after 30 days of seedling cultivation. Statistically significant results (p-value lower than 0.05) are shown in bold. The boxplot shows the 25th percentile, median (line inside box), mean (cross inside box), and 75th percentile; whiskers show the minimum and maximum values.

Bacillus spp. load (R = −0.999) and the abundance of the *chiA* gene copies (R = −0.827).

3.5. Metabolism of rhizospheric microbiota

Based on the results of the microbial community metabolism parameters presented on the heatmap (Fig. 4), differences were observed in 16 out of the 37 analyzed parameters. However, in the case of the remaining 21 parameters, the expression of a given feature was the same (black) in each variant. The side dendrogram consisted of 5 separate branches grouping traits of similar expression depending on the variant. Based on the upper dendrogram, relatively high similarity of the expression of features was observed in the control and nitrogen variants, while the meal variant was clearly different from them. Chitinase activity was highest in the nitrogen variant, moderate in the meal variant, while no activity was found in the control. The acid phosphatase activity was strong in the nitrogen variant, while the activities of alkaline phosphatase, lipase, and esterase in the meal variant. The control variant was characterized by the highest activity of sugar metabolizing enzymes. Apart from the low esterase-lipase (C8) activity, the microbial community of the nitrogen variant showed the lowest potential for using simple compounds.

4. Discussion

The short term investigation indicated that the application of mealworm larvae meal contributed to a significant increase in soil nutrient levels such as nitrogen including ammonia, magnesium concentration, and most biometric parameters of wheat seedlings and inhibited the development of trichotecenes-producing *Fusarium* spp. The mealworm fertilizer exerted a more beneficial influence on rhizospheric microbiota than the mineral nitrogen fertilizer.

A comparison of the stability and composition of the evaluated mealworm larvae meal with literature data indicates that different types of cereal feeds lead to minor differences in the protein content of mealworm larvae, and greater variations in amino acid composition.

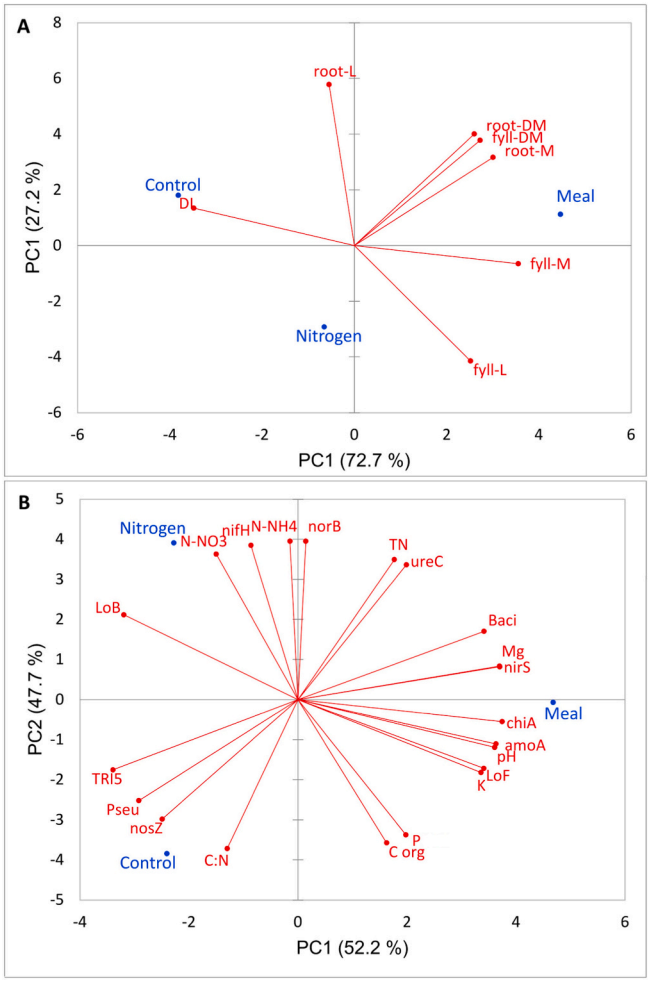

Fig. 3. Principal component analysis (PCA) – biplot of correlations with the unfertilized treatment, the treatment with ammonium nitrate, and the treatment with mealworm larvae meal; A-biometrical observation, B-chemistry, and microbial observation Abbreviations: DI – disease incidence, phyll-L – phyllosphere height, phyll-M – mass of phyllosphere, phyll-DM – phyllosphere dry weight, root-L – root length, root-M – root mass, root-DM – root dry weight, LoB – number of 16S rDNA sequences, LoF – number of ITS sequences (total fungi), Baci – number of 16S rDNA sequences (*Bacillus* spp.), Pseu – number of *phlD* sequences (*Pseudomonas* spp.), TRI5 – number of TRI5 sequences (trichothecene-producing *Fusarium* spp.); number of gene copies: *chiA* chitinase, *amoA* – ammonium monooxygense, *nifH* – nitrogenase reductase, *nirS* – nitrite reductase, *norB* – nitrate reductase, *nosZ* – nitrous oxide reductase, *ureC* – urease; C org – organic carbon.

Ravzanaadii et al. (2012) reported lower concentrations of essential amino acids in insects fed wheat bran. In their research, threonine and valine content was around 30% lower, and the total content of sulfur-containing amino acids was higher than that noted in our study. These findings suggest that mealworm larvae meal has a lower fertilizing potential in the cultivation of crops that require high levels of sulfur, such as maize, mustard, and legumes. However, it contains other amino acids, including tryptophan, and can promote the initial growth of cereals, grasses, and plants of the family Solanaceae. Tang et al. (2018) analyzed the amino acid profiles of *T. molitor* larvae following hydrolysis with the use of various techniques. The content of hydrolyzed amino acids was very low in distilled water extracts (only 2 g in 100 g of protein). The application of concentrated flavourzyme (enzymatic hydrolysis) led to a 5-fold increase in the content of the total hydrolyzed amino acids, whereas the concentrated alkaline solution increased it more than 15-fold relative to water extraction. This observation suggests that the mealworm larvae meal can be additionally processed to increase the availability of amino acids (and protein) to plants. The ability of native soil microbiota to hydrolyze proteins should also be analyzed after the application of mealworm larvae meal.

The mealworm larvae meal contributed to an increase in the counts of plant growth-promoting bacteria (PGPB), such as *Bacillus* spp., which improved nutrient availability and biometric parameters of plants, including yield. This genera of bacteria improved wheat condition, health status and grain yield (Przemieniecki et al., 2017, 2018, 2019b). In the study by Ahmad et al. (2017), mineral fertilizers impregnated with PGP bacteria (*Bacillus* sp. KAP6) were added to compost and humic acid. The applied treatment improved nitrogen and phosphorus use efficiency, and increase photosynthetic rate, plant growth and grain yield.

The chemical analysis revealed that the nitrogen value of the mealworm larvae meal and ammonium nitrate was similar and both fertilizers had similar effects to plant height. The mealworm larvae meal also increased N (mineral nitrogen) and Mg contents in the soil, contributing to an increase in root length and weight. Furthermore, the analyses of the content of individual forms of nitrogen and the C:N ratio showed dynamic mineralization processes in the meal and nitrogen variants. This is important for the rapid availability of organic nitrogen to plants in the meal variant. Ammonium nitrate has a nitrate and ammonium form, which is advantageous; however, the nitrate form can quickly migrate into the soil profile. Analyzing the ratio of total nitrogen to the forms of mineral nitrogen, mainly nitrification but also a successive release of ammonium nitrogen to the rhizosphere were observed in the meal variant. This is much safer for both the environment and plant growth. Presumably, part of the insect protein was proteolyzed, releasing a stock of amino acids, suggesting the high proteolytic potential of the microbial community. An additional aspect supporting the use of meal is the increase in soil pH compared to ammonium nitrate and soil enrichment with magnesium.

Analyzing the microbiological parameters, an increase was observed in the number of *Bacillus* spp. at the expense of *Fusarium* spp. and *Pseudomonas* spp. A predictable increase in the number of the chitin-degrading microorganisms was also observed, although the potential chitinolytic activity was higher in the nitrogen variant. On the other hand, increasing the proportion of saprotrophic fungi after complex organic matter addition is a natural process that accelerates its initial decomposition. It was also observed that the addition of ammonium nitrate increased the numbers of diazotrophs and denitrifiers (*norB*), which were strongly correlated with each other, although the number of microbiota converting nitrogen from $N\text{-}NO_2$ to NO also increased in the meal variant. Moreover, the addition of the meal increased the load of denitrifying microorganisms by increasing the concentration of $N\text{-}NH_4$ (and the $N\text{-}NH_4$: $N\text{-}NO_3$ ratio) and increasing the pH of the rhizospheric soil solution. Very important is also the unequivocal confirmation of the increase in the number of *ureC* in the meal, but also a strong correlation of ammonifying bacteria with TN content, which confirms the putative conversion of organic nitrogen into $N\text{-}NH_4$ after meal addition to the soil.

Ouyang and Norton (2020) analyzed the processes of nitrogen mineralization and the composition of the microbiome of the soil on which plants were grown without fertilization and fertilized with ammonium sulfate and compost. In their research, the enzymatic activity (as in this study) differed from the control and mineral fertilization, which also reflected the taxonomic variability of the microbiota. However, the differentiation of ureolytic and chitinolytic bacteria was modified only after the fertilization with mineral nitrogen. What is more, the load of *ureC* and *chiA* was not significantly correlated with enzymatic activity. The above conclusions made by Ouyang and Norton (2020) are similar to the observations in this study because the biochemical marker studies only partially coincided with the chemical and microbiological results of the rhizosphere. The microbial community was modificated, however, the potential activities of certain enzymes did not coincide with the load of the functional genes (e.g.,

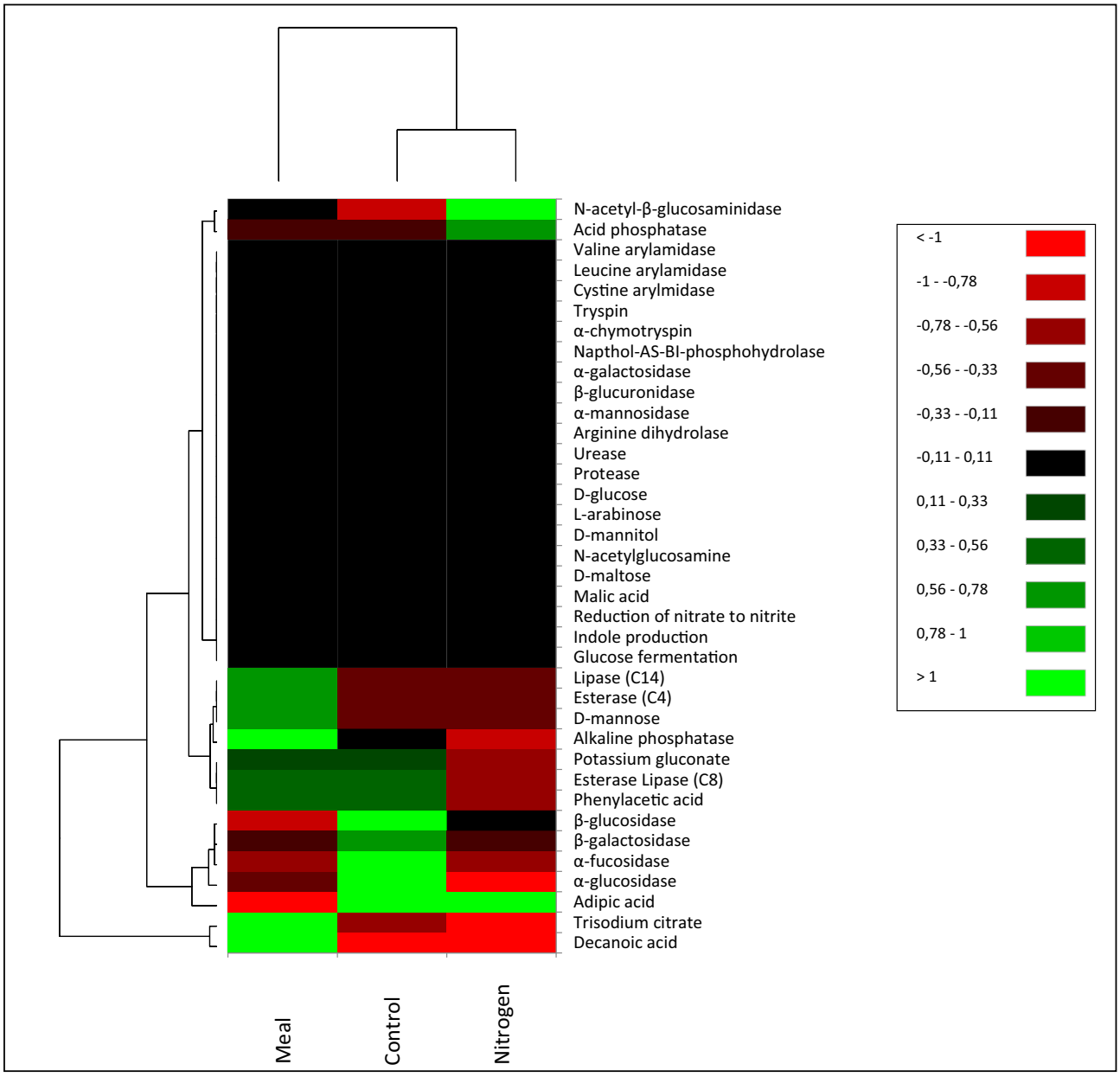

Fig. 4. Heatmap analysis of the metabolic activity of the microbial community.

chitinase activity did not coincide with the number of copies of *chiA* genes).

The regularities between the load of functional genes and nitrogen transformations in soil were described by Wang et al. (2017). They noticed that $N\text{-}NH_4$ modifications were tightly regulated by the presence of *amoA* genes. The concentration of $N\text{-}NO_3$ was regulated by *narG* and *napA* ($N\text{-}NO_2$- > $N\text{-}NO_3$), nxpA ($N\text{-}NO_3$- > $N\text{-}NO_2$), and the bacterial load. The *napA*, *narG*, *nirK*, *nirS*, *norB*, *nosZ*, and *nxrA* genes were responsible for total N regulation. The above findings of the Chinese scientists exactly match the correlations observed in this work.

Some regularities can be observed when analyzing overall relationships. The increase in *Bacillus* spp. counts in response to mealworm larvae meal was accompanied by a decrease in the load of undesirable fungi and increase in the fresh weight of the above-ground biomass. The chitinolytic bacteria and chitinolytic enzymes increased after the application of insect organic matter. Fertilization with the mealworm larvae meal positively influenced the rhizospheric microbial community because spore-forming bacteria were highly resistant to adverse environmental conditions and developed faster than fungi. *Bacillus* spp. produce not only chitinases, which inhibit the growth of pathogenic fungi, but also antifungal peptides (Mora et al., 2011; Yang et al., 2015; Przemieniecki et al., 2018). Bressan and Figueiredo (2010) applied chitinolytic bacteria of the genus *Bacillus* to maize seeds and soil to determine their antagonistic activity towards *Fusarium moniliforme*. The analyzed bacterial isolates suppressed fungal growth in maize seeds by 27–61% and reduced the incidence of plant disease in soil trials by 65–78%. In the study by Zhao et al. (2018), the bio-organic fertilizer (fermented cow manure and chicken manure compost) significantly modified the rhizospheric microbial community of watermelons, i.e., increased bacterial diversity, decreased fungal diversity, reduced *Fusarium* counts, and alleviated the severity of disease symptoms. The bio-organic fertilizer induced the highest increase in the counts of Firmicutes bacteria (mainly *Bacillus* spp.) as well as Proteobacteria. The above findings indicate that the rhizospheric bacteria of the genus *Bacillus* inhibit the growth of *Fusarium* phytopathogens. However, the reported decrease in the counts of *Pseudomonas* bacteria was not confirmed in our

study. In the present study, the mealworm larvae meal also increased fungal counts, which could be attributed decreased fungal biodiversity and the predominance of saprotrophic microorganisms. A relatively strong negative correlation between the counts of chitinase-producing bacteria and toxin-producing fungi suggests that the chitinase-producing bacteria are important. Nevertheless, increasing the load of *Bacillus* spp. in soil causes the appearance of different types of antimicrobial substances (Abdallah et al., 2017; Przemieniecki et al., 2021). The above suggests that the mealworm larvae meal was used as an additional substrate by *Bacillus* spp., which increased the size of their community and could potentially increase the concentrations of both chitinase and fungistatic compounds in the rhizosphere.

5. Conclusion

The results of this study indicate that the mealworm larvae meal offers a viable organic alternative to mineral nitrogen fertilizers as an abundant source of protein in the soil. The meal supplement undergoes normal changes in the nitrogen cycle. Importantly, mineral nitrogen is successively released into the rhizosphere, which is more beneficial than in the case of applying mineral fertilizers. Insect meal improved both the biometric parameters of wheat and the microbiological rhizosphere, which indicates its positive effect on the soil environment. The mealworm larvae meal contains chitin, protein, and fat, which can be readily utilized by PGP bacteria of the genus *Bacillus* with antagonistic properties. The increase in *Bacillus* load was correlated with load of chitinase production genes and inversely correlated with load of toxin-producing *Fusarium* spp. In order to confirm the usefulness of insect fertilizers, further research is required on the availability of nutrients, the presence of biostimulants, and nitrogen changes throughout the plant's life cycle.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2021.104037.

Declaration of competing interest

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

Acknowledgments

Project financially supported by Minister of Science and Higher Education in the range of the program entitled "Regional Initiative of Excellence" for the years 2019–2022, Project No. 010/RID/2018/19, amount of funding 12.000.000 PLN. Part of this study was financed by the University of Warmia and Mazury in Olsztyn, Faculty of Agriculture and Forestry, Department of Entomology, Phytopathology and Molecular Diagnostics nos. 20.610.015-110 and 20.610.016-110. The authors would like to thank the members of the 'Pasikonik' Student Entomology Club for technical support and assistance in laboratory analyses.

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