

“The impact of insects larvae frass on soil fertility and plant production”

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Abstract

There is a need for alternatives or complements to synthetic fertilizers to enhance agricultural sustainability. Applying organic amendments can play a significant role in this. Insect droppings show high potential, though studies evaluating their agronomic value have only recently begun to emerge. This study compared black soldier fly (*Hermetia illucens* L.) and mealworm (*Tenebrio molitor* L.) larvae frass with another organic amendment (Nutrimais) derived from composting forestry, agro-industrial, and domestic waste. The experiment also included ammonium nitrate at two rates: the same as the organic amendments, 50 kg ha⁻¹ nitrogen (N), and half that rate and an unfertilized control. The study spanned two growth cycles of lettuce (*Lactuca sativa* L.) grown in pots, followed by unfertilized oats (*Avena sativa* L.) to assess the residual effects of the fertilizing treatments. Mealworm larvae frass mineralized rapidly, with an apparent N recovery of 37.4% over the two lettuce growth cycles, indicating its high availability to soil heterotrophic microorganisms. The average dry matter yield (DMY) of lettuce was the highest among all treatments (12.8 and 9.8 g plant⁻¹ in the first and second lettuce cycles), even compared to the FullR treatment (12.2 and 7.8 g plant⁻¹), though without significant differences. Although mealworm larvae frass exhibited a high mineralization rate, the DMY cannot be attributed solely to N supply, as plants in the FullR treatment showed better N nutritional status. Mealworm larvae frass provided strong evidence of a plant biostimulant effect, not explained by the variables measured in this study. Black soldier fly larvae frass exhibited typical behavior of a moderately reactive organic amendment, while Nutrimais showed low reactivity, with a near-neutral mineralization/immobilization balance. The results suggest mealworm larvae frass is recommended for early-maturing vegetable crops, whereas Nutrimais appears more suitable for perennial crops with low short-term nutrient requirements.

Keywords: black soldier fly frass; *Hermetia illucens*; *Tenebrio molitor*; insect droppings; manuring effect

Resumo

Há uma crescente necessidade de alternativas ou complementos aos fertilizantes sintéticos para promover a sustentabilidade agrícola. A aplicação de produtos orgânicos pode desempenhar um papel importante neste processo. Os dejetos de insetos têm mostrado um elevado potencial, embora os estudos que avaliam o seu valor agronómico tenham começado a surgir apenas recentemente. Este estudo tem como objetivo comparar o efeito dos dejetos das moscas soldado negra (*Hermetia illucens* L.) e tenébrio (*Tenebrio molitor* L.) com outro produto orgânico, o Nutrimais, resultados da compostagem de resíduos florestais, agroindustriais e domésticos. A experiência também incluiu nitrato de amónio em duas doses de aplicação: uma correspondente aos níveis de azoto (N) fornecidos pelos produtos orgânicos (50 kg ha⁻¹ N) e outra com metade dessa quantidade, bem como um tratamento controlo não fertilizado. O estudo abrangeu dois ciclos de crescimento de alface (*Lactuca sativa* L.), seguido de aveia não fertilizada (*Avena sativa* L.) para avaliar o efeito residual dos tratamentos de fertilização. O estudo incluiu dois tratamentos com fertilização mineral com N nas quantidades equivalentes e metade da quantidade de N fornecida pelos produtos orgânicos, bem como um tratamento controlo sem fertilização. Através de múltiplas comparações, procurou-se determinar como posicionar estes novos produtos orgânicos nos sistemas de recomendação de fertilização das culturas.

Palavras-chave: frass de mosca soldado negra; *Hermetia illucens*; *Tenebrio molitor*; dejetos de insetos; efeito de adubação.

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List of abbreviations

- ANR: Apparent Nitrogen Recovery
- BSFF: Black Soldier Fly Frass
- BSFly: Black Soldier Fly
- CEC: Cation Exchange Capacity
- Cont: Control
- EC: European Commission
- FAO: Food and Agriculture Organization
- FullR: Full rate
- GHG: Greenhouse Gas
- HPLC-DAD-ESI/MS: High-Performance Liquid Chromatography-Diode Array
- IPCC: Intergovernmental Panel on Climate Change
- LCWs: Lignocellulosic Wastes
- Mealw: Mealworm frass
- NPK: Nitrogen, Phosphorus, Potassium
- Nutri: Nutrimais
- UN: United Nations
- D-EIMS: Detection-Electrospray Ionization Mass Spectrometry

1. Introduction

The global population is projected to continue increasing in the coming decades. According to the latest United Nations report (United Nations, Department of Economic and Social Affairs, Population Division, 2017), estimates suggest there will be 8.5 billion people by 2030 and 9.8 billion by 2050. This upward trend in world population presents significant challenges in terms of meeting food demand and ensuring sustainable agriculture practices. Achieving a balance between environmentally friendly farming methods and high-quality yields is paramount in the 21st century (Terfa, 2021). While intensive farming may offer improved income, it tends to be unsustainable and detrimental to the environment (Pretty *et al.*, 2011). Reliance on mineral fertilizers, pesticides, and intensive tillage practices may have adverse effects on soil quality (Scotti *et al.*, 2015; Kopittke *et al.*, 2019), exacerbating climate change and rendering traditional agricultural systems susceptible to extreme weather events (Fahad *et al.*, 2019). Organic amendments have historically supplied essential nutrients to crops, but their usage declined with the mechanization of agricultural practices and the availability of affordable chemical fertilizers (Gong *et al.*, 2009). Chemical fertilizers, containing vital NPK nutrients, rapidly enhance soil fertility and support plant growth (Hakeem *et al.*, 2021). However, their long-term impacts can lead to soil pollution, reduced fertility, and imbalances in soil nutrients (Savci, 2012). Organic matter plays a vital role in soil fertility and overall soil functioning, influencing its physical, chemical, and biological properties (Huber *et al.*, 2011; Weil & Brady, 2011). Excessive application of chemical fertilizers, in turn, may lead to a reduction in soil organic matter, thereby negatively impacting general soil properties (Azizi *et al.*, 2016; Lopes *et al.*, 2021).

Intensive agricultural practices without organic amendments result in reduced soil carbon (C) content, microbial biomass, enzymatic activities, species diversity, and increased soil salinity (Bonanomi *et al.*, 2011). Establishing a soil conservation baseline entails implementing conservation agriculture techniques and enriching soils with organic matter. Common sources of organic matter for soil enrichment may include farmyard manures (Lopes *et al.*, 2021; Goss *et al.*, 2013; Rodrigues *et al.*, 2018), municipal solid waste (Nanda & Berruti, 2021 ; Arrobas *et al.*, 2022) and sewage sludge (Dhanker *et al.*, 2021; Arrobas *et al.*, 2024). The use of high-quality compost is crucial for its safe and effective utilization in soil (Arrobas *et al.*, 2024 ; Afonso *et al.*, 2021).

Scholars worldwide are currently researching sustainable waste sources and decomposition methods (Policastro & Cesaro, 2023). Insects and their excreta have shown promising potential for use in agriculture, particularly as soil amendments (Beesigamukama *et al.*, 2020). The byproduct of insect bioconversion, known as "frass", contains significant amounts of nutrients and can be marketed as fertilizing materials, offering additional income for breeding operations (Beesigamukama *et al.*, 2022). With the expanding insect industry globally, frass production is expected to increase, driven by waste recycling and circular economy policies (IPIFF, 2019; EC, 2020). Furthermore, there is an ongoing debate regarding the effectiveness of organic agriculture in ensuring food security (Pickett, 2013). Farmers have the option to adopt conservation practices, and their decisions are influenced by factors such as community pressure, financial incentives, and information availability (Liu *et al.*, 2018).

Therefore, additional research is necessary to establish the best conditions for utilizing insect frass and to scale up this industry significantly (Dzepe *et al.*, 2022). This includes studying the effects of insect byproducts on soil properties and plant growth. To address current knowledge gaps, our study began examining the effect of two commercial products resulting from black soldier fly (*Hermetia illucens*) larvae frass and mealworm (*Tenebrio molitor*) larvae frass on lettuce (*Lactuca sativa* L.) growth, plant nutritional status, and soil properties. For comparison, the study included a commercially available organic compost, which has been on the market for a long time resulting from composting forestry, agro-industrial, and domestic waste, sold as a pelletized and dehydrated material and permitted in organic farming in Portugal. Additionally, the study included two treatments with mineral N fertilization at equivalent and half rates of the N provided by organic amendments, as well as a control treatment without fertilization. In this way, through multiple comparisons, we aim to understand how to position these new organic amendments in crop recommendation systems.

2. Literature Review

Chapter I: Constraints linked to agricultural sector

2.1.1. Intensive farming

The United Nations (2019) projects that the global population could rise to 9.8 billion by 2050, which is expected to result in a nearly 70% increase in global food demand (Alexandratos & Bruinsma, 2012). To meet this demand, intensification of cultivated land use has occurred on a

global scale, propelled by both the need for increased food production and advancements in agricultural technology. However, this intensification is frequently linked to the widespread utilization of agricultural chemicals as well as an escalation in agricultural water use (Lyu *et al.*, 2022).

An example of intensive farming is cultivating crops intensively under plastic tunnels, which offers various benefits to farmers, such as enhanced microclimatic conditions and relatively low investment costs. This, in turn, can lead to a higher net income for farmers (Lamont, 2009). Despite all these benefits intensive farming is unsustainable, harmful to the environment, and poses potential human health problems (Pretty *et al.*, 2011) it relies heavily on frequent usage of mineral fertilizers, pesticides, and intensive tillage practices which has a detrimental impact on soil quality (Scotti *et al.*, 2015). Also, conventional agricultural practices prioritize short-term yield gains, leading to the overuse of fertilizers in quantities that exceed what is actually needed (Kuila & Ghosh, 2022).

Furthermore, climate change resulting from this intensification becomes more severe, it is having a profound impact on biodiversity (Fahad *et al.*, 2019) making traditional agriculture vulnerable to drought and extreme weather events, putting agricultural production at risk in many parts of the world (IPCC, 2019).

2.1.2. Excessive use of fertilizers

Since early ages, organic fertilizers have been commonly used to provide essential nutrients to crops. However, the use of organic fertilizers declined after the 1980s, because chemical fertilizers became more readily available and affordable (Gong *et al.*, 2009). Chemical fertilizers are rapid and contain essential nutrients (nitrogen, phosphorus, and potassium) that are required for plant growth and are used to enhance soil fertility (Hakeem *et al.*, 2021).

Studies have shown that the impacts of chemical fertilizers on soil may not be immediately evident because of the ability of the soil to buffer these chemicals. However, pollution, reduced soil fertility, and degradation can occur over time, causing an imbalance in soil elements (Savci, 2012). Furthermore, some of these fertilizers can cause water bloom, and accumulation of toxic substances like heavy metals in soil and vegetables, which can have harmful effects on humans and animals that consume them (Savci, 2012). The excessive use of fertilizers for example : nitrogen-based fertilization, can cause environmental degradation (Chen *et al.*, 2005). Moreover, nitrogen fertilizers are a significant contributor to global greenhouse gas emissions as they produce nitrous oxide (N₂O), a potent greenhouse gas. This led Manono (2016) to

declare that despite the fact that agriculture is not a significant source of carbon dioxide (CO₂) emissions, it is a significant source of both nitrous oxide (N₂O) and methane (CH₄) emissions. This can have a negative impact on Aquafarming and silviculture (Galloway *et al.*, 2008). To meet the increasing global demand for food, it is necessary to adopt advanced agricultural technologies such as efficient fertilization methods. Improper and excessive use of inorganic fertilizers can lead to environmental risks, making it important to adopt efficient and sustainable agricultural practices (Rahman & Zhang, 2018).

2.1.3. Depletion of organic matter in soils

Organic matter has major effects on soil fertility and its role is essential in the overall functioning of soils through their physical, chemical, and biological components (Huber *et al.*, 2011). It serves as a source of nutrients for decomposing microbes, which in turn provides minerals to plants (Abiven *et al.*, 2009).

There are several factors contributing to the depletion of organic matter in soils: i.e., excessive application of chemical fertilizers that affects soil physical properties: reduction of microbial activity, soil compaction...etc (Azizi *et al.*, 2016), and overgrazing, which is considered an important cause of a clear degradation of the rangelands in the case of steppe breeding (Belaid, 2013). Intensive agricultural practices that do not include organic amendments to restore soil organic carbon levels can have negative effects on soil chemical properties. This can cause a decrease in soil carbon content, which, in turn, can have harmful effects on soil microbial biomass, enzymatic activities, and species diversity, and can even lead to an increase in soil salinity (Bonanomi *et al.*, 2011).

It is important to prioritize the recovery and maintenance of sufficient soil organic carbon levels in order to support high and sustainable crop yields over the long term. Farmers who use this agricultural system are aware of this issue and often use organic amendments to compensate for the loss of soil's organic carbon (Scotti *et al.*, 2015).

Chapter II: Organic agriculture

2.2.1. Organic fertilizers applications to restore soil fertility

Developing countries are turning to organic farming for profit, and there is growing public awareness of the negative impacts of modern agriculture on ecological diversity, safe food, the environment, and the economy (Willer *et al.*, 2020). This has led to the emergence of sustainable agricultural management, which aims to achieve crop production without causing ecological or health damage (Andres & Bhullar, 2016).

Goss *et al.* (2013) reviewed the applications to restore soil fertility and declared that the practice of using organic amendments to enhance soil quality and fertility has a long history, dating back thousands of years. According to the same authors, the ancient Greeks and Romans applied animal manure and human sewage to soil and understood the benefits of growing wheat on fields previously cultivated with leguminous plants. Various materials such as seashells, vegetable waste, farmyard manure, and other waste products were used to improve crop growth. In addition, it has been shown that it is important to use the right combination of microbial consortia in organic farming to match the specific environment, soil conditions, and crop, as this will help maintain yields in successive years (Santos *et al.*, 2019).

Organic agriculture is a cost-effective and environmentally friendly alternative system for producing secure food using biological methods (Kuila & Ghosh, 2022) in order to ensure sustainable soil fertility and the production of safe, high-quality food over the long-term productivity (Bender & van der Heijden, 2015).

2.2.2. Common sources of organic matter

2.2.2.1. Farmyard manures

Goss *et al.* (2013) gave a full description of manure, preparation and benefits: Manure is a mixture of animal feces, urine, and bedding that is stacked and turned until it reaches a certain level of composting. It is derived from various animals, such as beef, pork, poultry, and turkey. The composition of manure varies based on its origin, the timing of excretion and mixing of urine and feces, and the duration of storage before being applied to the soil. Manure not only provides nutrients for crops but also enhances soil fertility by adding organic matter. When manure is aerobically degraded, CO₂ and organic compounds that are difficult to degrade are produced in the surface layers. Conversely, when anoxic conditions occur in the deeper layers

of manure, such as when it is not turned, volatile organic acids and CH₄ gas are produced (Kirchmann, 1989).

2.2.2.2. Municipal solid waste

Municipal solid waste is the solid waste generated in a municipality and collected from various sources, including households, offices, small-scale institutions, and commercial enterprises. (Nanda & Berruti, 2021). A significant portion of European household waste is composed of the organic fraction (EEA, 2020). One of the circular economy principles is waste management practices (EEA, 2020). Composting and anaerobic digestion are currently the most commonly used methods for biologically processing the organic municipal solid waste (Lin *et al.*, 2018). Composting is a familiar or industrial method of biological processing that takes place in the presence of oxygen. It transforms organic material into a stable fertilizer product called compost (Lasaridi *et al.*, 2018). It is important to produce high-quality compost to ensure its safe and effective use on soil because of its beneficial agronomic properties (Policastro & Cesaro, 2022). From this perspective, researchers from all over the world are continuously trying to develop and find sustainable methods of decomposing waste and determining its inherent nutritional and economic value (Singh & Kumari, 2019).

2.2.3. Farmers attitude towards organic fertilizers

The economic status of the population is heavily reliant on agricultural production. Regardless, in the pursuit of more intensive and cost-effective agriculture, the excessive use of chemical fertilizers and pesticides has become prevalent. Chemical farming can harm the environment for that reason, organic farming is considered a popular alternative to it in order to avoid its negative impacts (Patidar *et al.*, 2015). However, organic agriculture has been a subject of debate since its inception, with some arguing that it is an ineffective way to ensure food security (Pickett, 2013). By the end, farmers have the power to decide whether or not to use conservation practices or best management practices (land recovery, no tillage) (Caswell *et al.*, 2001). For that, scientists are trying to know the knowledge and attitude of farmers regarding biofertilizers and organic farming. According to Welch & Marc-Aurele (2001) research, if adopting a conservation practice would benefit nearby plots for free, farmers are less likely to do it. Late adopters feel more pressure from their community and peers, while early adopters are more motivated by money and regulations. Nowak's (2009) research confirms that farmers who adopted conservative agriculture early were mainly driven by payment, while late adopters were more influenced by their early adopting peers and neighbours. This means that social conformity is important when it comes to adopting organic agriculture. Farmers are more likely

to adopt this practice if they have access to reliable information, either directly or through their neighborhood network (Liu *et al.*, 2018). Turinawe *et al.* (2015) discovered another factor influencing farmer's decision, which is that the probability of adopting soil and water conservation technologies increases by 45% if a farmer's neighbours are also using them, all else being equal. Patel *et al.* (2017) adds that it is crucial for the government to prioritize the promotion of organic farming, and for extension agencies to provide training for their staff on organic farming techniques. This will enable them to educate farmers on the benefits and methods of organic farming. Overall, farmers worldwide are increasingly attracted to organic cultivation due to its numerous advantages over modern agricultural practices (Reddy, 2010).

Chapter III: Potential use of insect's frass as soil amendment for sustainable agriculture

2.3.1. General Information about insect frass biofertilizer

Increasing food production while preserving the environment requires a focus on developing and implementing new organic products that can enhance plant growth. By doing so, we can simultaneously promote sustainable agriculture and meet the growing demand for food (Shankar & Shikha, 2017). During the past decade, many studies have focused on the useful applications of insects and their droppings (Sarpong *et al.*, 2019). Initial large-scale field studies have provided promising prospects for application in agriculture, especially in terms of the availability of fertilizers to plants (Beesigamukama *et al.*, 2020). There is an unavoidable flux during mass rearing of insects, which can account for up to 75% of the substrate used (Diener *et al.*, 2009).

This is the main by-product of the bioconversion of waste by insects into protein, which is summarized as "excrement" and is often marketed as a fertilizer product. In general, insect excrement is referred to as "frass" in English. Insect excrement-based fertilizer would also increase the income of breeding operations through its sale as a secondary product (Beesigamukama *et al.*, 2020). This byproduct and its substantial amounts of nutrients (Lalander *et al.*, 2015) could be useful for agricultural production if converted into organic fertilizer.

With the rapid growth of the insect industry worldwide (Van Huis, 2020), many companies have started selling frass as soil conditioners and fertilizers (Halloran *et al.*, 2016). According to the IPIFF (2019), frass production in the EU is projected to reach 1.5 million tons by the

mid-2020s. As the demand for waste recycling and circular economy policies such as the EU Green Deal Policy (EC, 2020) continues to rise, the amount of frass is expected to increase further, highlighting the importance of investigating sustainable methods for its use in agriculture.

2.3.2. Types of insect frass biofertilizers

2.3.2.1. Black Soldier Fly -Hermetia illucens -

Hermetia illucens (Linnaeus, 1758) is an insect commonly known as the Black Soldier Fly, belonging to the order Diptera (Caruso *et al.*, 2014). Recently, this insect has been the subject of modern scientific research given its potential to contribute to the development of a circular economy by addressing two environmental issues : waste management and protein substitute production (Nguyen *et al.*, 2015). The black soldier fly has several advantages over many other insects. Indeed, its larvae are polyphagous and degrade a heterogeneous variety of nutrients (Barragan-Fonseca *et al.*, 2017). Today, several projects for sustainable recycling of various organic wastes, such as pig manure (Zhu *et al.*, 2017), fruits and vegetables (Klammsteiner *et al.*, 2020), palm kernel meal (Hem *et al.*, 2008), and municipal organic wastes (Diener *et al.*, 2009) have been carried out using black soldier fly. It also has the ability to solve a number of environmental problems (Nana *et al.*, 2018), such as the inhibition of housefly proliferation (Zheng *et al.*, 2013) and inactivation of *Escherichia coli* and *Salmonella* (Erickson *et al.*, 2004). During degradation, organic waste is digested, aerated, and dried, resulting in a natural product with a high pH and ammonia content (Kovacik *et al.*, 2014). This residue can be used as fertilizer for plants (Choi *et al.*, 2009). The few studies conducted thus far have demonstrated that utilizing Black soldier fly frass as a soil amendment leads to higher crop yields (Rosmiati *et al.*, 2017; Houben *et al.*, 2020, 2021), triggers plant defense mechanisms against abiotic stress factors (Poveda, 2021) and biotic stressors (Quilliam *et al.*, 2020), and enhances microbial activities in soil (Gebremikael *et al.*, 2020).

2.3.2.2. Yellow mealworm -Tenebrio molitor –

Mealworms are the offspring of *Tenebrio molitor*, commonly known as yellow mealworms. This beetle family is distributed worldwide (He *et al.*, 2020). The practice of mealworm farming has gained popularity in various parts of the world, including China, the United States, and Europe (Yang *et al.*, 2018). Recent research on agricultural residues and waste has revealed that mealworms can survive and biodegrade raw lignin-containing lignocellulosic wastes (LCWs) such as wheat straw (WS) and rice straw (Wang *et al.*, 2018). Notably, mealworms produce

frass waste. Proper utilization of this last could significantly contribute to increasing rural incomes by transforming it into an easily accessible organic fertilizer (Nasution *et al.*, 2018) to provide vital nutrients for plant growth (Poveda *et al.*, 2019). According to a study by Liu *et al.* (2003), the N-P-K balance of this fertilizer is 3.5-1.5-1.5. It has been suggested that microorganisms found in frass could potentially play a significant role in vegetative growth (Menéndez & Garcia-Fraile, 2017), and its use has been reported to increase the weight of bean (*Phaseolus vulgaris*) seeds by 18% (Liu *et al.*, 2003). Additionally, a study conducted by the same author in 2013 indicated that the application of aqueous extracts made from this excrement could enhance wheat seed germination by 4%.

2.3.3. Regulation of insects for agricultural use

Several sources of information were consulted at the national, European, and international levels to search for laws and regulations related to insect breeding substrates and their uses (EUR-Lex website, online magazine Reussir.fr, and European Commission International Insect Platform).

According to Regulation (EC) No. 1069/2009 (EC, 2009a) insects are considered farmed; therefore, their breeding substrate must meet the same requirements as those for foodstuffs for farmed animals according to regulations (EC) No. 178/2002, 178/2002, and 767 /2009 (EC, 2002, 2009b). Insect producers or products are controlled by products that are controlled by the European Regulation on Food Hygiene 854/2004 (2004) and feed hygiene 183/2005 (2005), so that HACCP testing procedures. The HACCP analysis procedures must be applied.

3. Materials and Methods

A pot experiment was conducted at the experimental farm of Instituto Politécnico de Bragança located in Northeastern Portugal (41.797254° N–6.762279° W). The experiment involved two growing cycles of lettuce (cv. Summer Wonder) and a single growing cycle of oats (*Avena sativa* L., cv. Boa Fé). Lettuce was cultivated during the first cycle, spanning from May to June, and again in the second cycle, which took place from August to October 2023. Oats were grown from October 2023 to January 2024.

The region's climate falls under the Mediterranean type, classified as Csb according to the Köppen classification, characterized by mild, dry summers (IPMA, 2024). Weather data collected during the experimental period are presented in Figure 1.

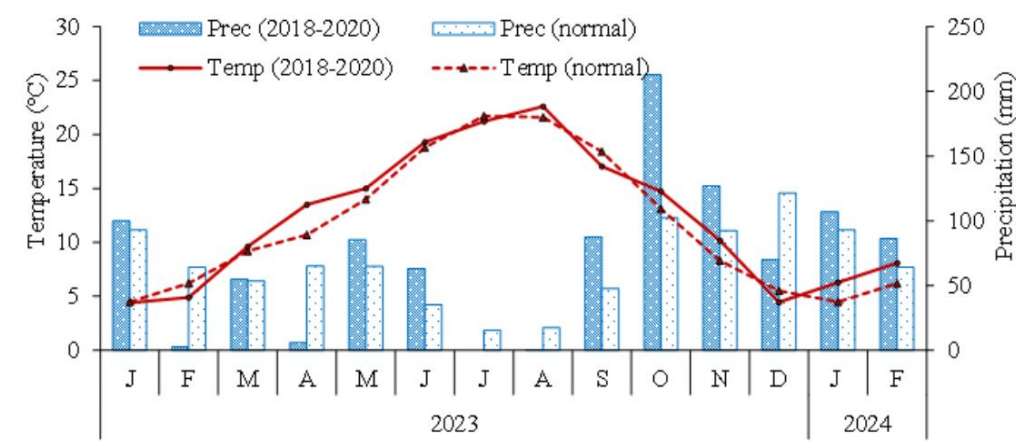


Figure 1. Climatological normals for the region (1981–2010) and monthly average air temperature and precipitation recorded during the experimental period (IPMA, 2024).

The soil used in this study was collected from the surface layer (0–0.20 m) of a fallow plot. It is classified as a Regosol of coluvial origin, presenting a sandy-clay-loam texture (soil separates of 228.7, 241.0, and 530.3 g kg⁻¹ of clay, silt, and sand, respectively). Soil organic C content was 11.0 g kg⁻¹ and the pH (H₂O) was 6.5. Extractable phosphorus (P) and potassium (K) levels were 133.5 and 191.7 mg kg⁻¹, P₂O₅ and K₂O, respectively. Exchangeable calcium (Ca), magnesium (Mg), and cation exchange capacity (CEC) were 9.6, 4.7, and 15.3 cmolc kg⁻¹, respectively.

3.1. Experimental Design and Fertilizing Materials

The first growing cycle of lettuce experiment followed a completely randomized design, comprising six treatments and six replicates. The treatments consisted of: 1) Nutrimais® (Nutri), an organic amendment derived from composting forestry, agro-industrial, and domestic waste, available in pelletized and dehydrated form, permitted for organic farming in Portugal; 2) Black soldier fly larvae frass (BSFly), a pelletized organic amendment produced from insect droppings by Entogreen®; 3) Mealworm larvae frass (Mealw), also a natural pelletized organic amendment from insect droppings and plant debris, manufactured by ThunderFoods®; 4) Ammonium Nitrate (27% N, 50% NH_4^+ , 50% NO_3^-), applied at the same N rate (FullR) as the three organic amendments; 5) Ammonium nitrate, applied at half the N rate (HalfR) of the organic amendments; and 6) a non-fertilized control (Cont). Table 1 presents the main properties of the three organic amendments used in this study.

Table 1. Selected properties (mean $\pm$ standard deviation, $n = 3$) of the organic amendments used in this study: black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw) and Nutrimais (Nutri).

Properties	BSFly	Mealw	Nutri
Moisture content	25.3 $\pm$ 0.72	7.4 $\pm$ 0.05	8.0 $\pm$ 0.19
pH (H_2O)	7.6 $\pm$ 0.21	6.5 $\pm$ 0.31	8.1 $\pm$ 0.15
El. conduct. (mS cm^{-1})	9.9 $\pm$ 0.46	7.3 $\pm$ 0.56	9.7 $\pm$ 0.45
Carbon (g kg^{-1})	499.5 $\pm$ 2.30	517.7 $\pm$ 0.64	419.0 $\pm$ 3.90
Nitrogen (g kg^{-1})	26.8 $\pm$ 0.06	31.3 $\pm$ 0.51	25.3 $\pm$ 1.00
Carbon/nitrogen ratio	18.6 $\pm$ 0.11	16.5 $\pm$ 0.26	16.5 $\pm$ 1.14
Phosphorus (g kg^{-1})	11.7 $\pm$ 0.66	10.7 $\pm$ 0.18	4.8 $\pm$ 0.13
Calcium (g kg^{-1})	3.1 $\pm$ 0.02	3.8 $\pm$ 0.07	75.9 $\pm$ 3.12
Magnesium (g kg^{-1})	4.9 $\pm$ 0.06	6.5 $\pm$ 0.28	3.2 $\pm$ 0.07
Boron (mg kg^{-1})	32.3 $\pm$ 0.35	26.0 $\pm$ 0.61	8.3 $\pm$ 0.65
Iron (mg kg^{-1})	761.9 $\pm$ 31.11	422.9 $\pm$ 12.18	5230.3 $\pm$ 212.42
Manganese (mg kg^{-1})	87.4 $\pm$ 1.50	155.7 $\pm$ 5.67	133.7 $\pm$ 2.96
Zinc (mg kg^{-1})	108.8 $\pm$ 0.94	107.5 $\pm$ 1.82	103.7 $\pm$ 2.60
Copper (mg kg^{-1})	19.6 $\pm$ 0.36	14.0 $\pm$ 0.21	36.5 $\pm$ 0.10

El. Conduct. - electrical conductivity

The treatments Nutri, BSBFly, Mealw, and FullR were applied at a rate of 50 kg N ha^{-1} . To calculate the amounts of organic and mineral products for each plot, a standard planting density of 140,000 plants ha^{-1} was used. Thus, each pot received 1/140,000 of the amendments corresponding to one hectare. Considering the moisture content and N concentration of the organic amendments, they were applied at rates of 19.93, 15.33, and 22.72 g pot^{-1} for BSBFly,

Mealw, and Nutri. Ammonium nitrate was applied at rates of 1.59 g pot⁻¹ for FullR and 0.79 g pot⁻¹ for HalfR treatments. The application of ammonium nitrate at full and half rates was split into two applications: at transplanting and in the middle of the growing season. Each pot received 3 kg of dried (40 °C) and sieved (2 mm) soil, 50 g of perlite to ensure aeration, and the corresponding quantity of amendments and fertilizer. The first growing cycle ran from May 3rd to June 15th.

After completing the first lettuce cycle, the experiment was divided into two, forming two groups of pots with three replicates each. In one group, the fertilization from the first cycle was repeated to assess the continued effect of the fertilizer treatments on lettuce growth and nutrition. In the other group, no fertilizers were applied. Since organic amendments release nutrients gradually through the mineralization process, the aim in this second group of pots was to evaluate the residual effect of fertilization from the first cycle. This second lettuce growth cycle ran from August 25th to October 2nd, 2023.

After completing the second lettuce growth cycle, oats, a species with high N requirements capable of thriving during the autumn-winter period, were sown. Oats were planted in both groups of pots, with 20 seeds per pot, later adjusted to 15 plants per pot after germination. The oats were not fertilized and were intended to evaluate the residual effect of the fertilization applied to the lettuce. The oat cropping cycle began on October 13th, 2023, and concluded on January 18th, 2024, when all plants exhibited chlorosis and stunted growth, at the stem elongation phase (with node 2 at least 2 cm above node 1) (Meier, 2018). This approach aimed to gather detailed information on the short and long-term effects of organic fertilization. Mineral fertilization was included in the experiment as a positive control to assess the nutrient use efficiency of organic amendments compared to the more standardized use of mineral fertilizers.

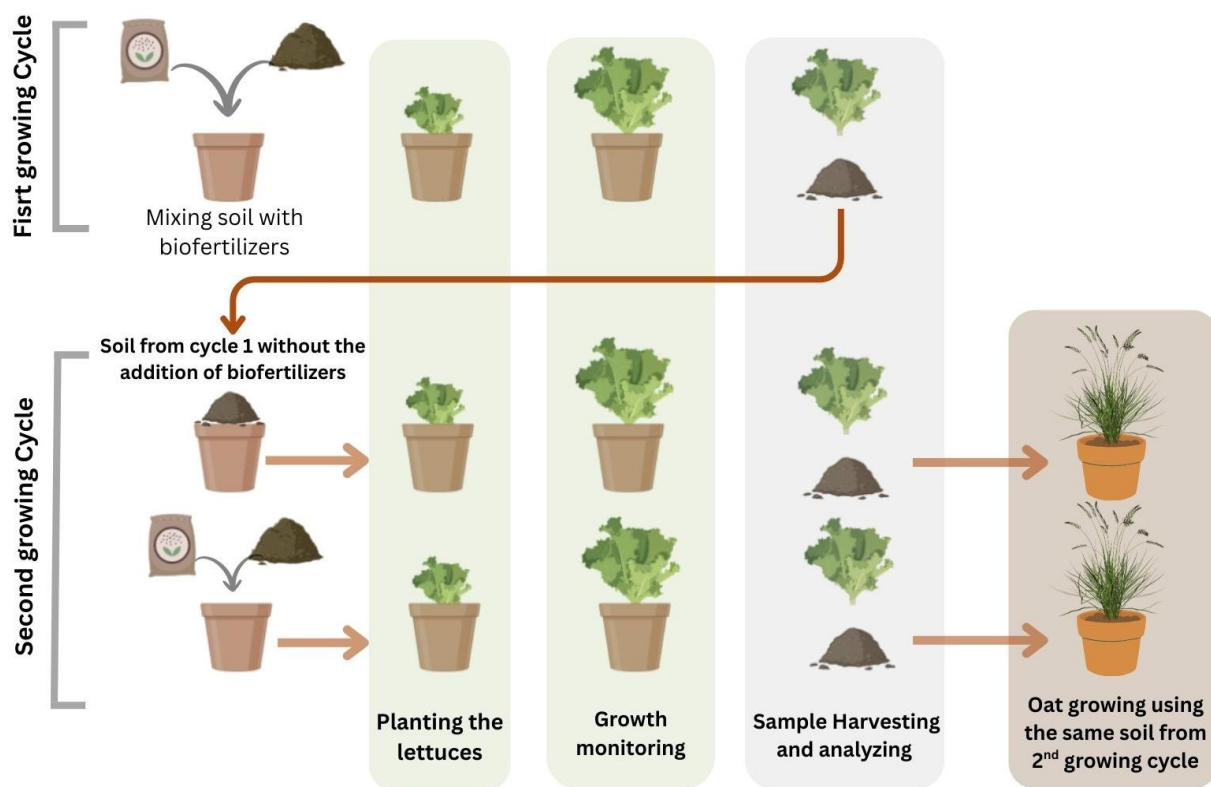


Figure 1. Schematic representation of the experimental design

3.2. Conducting the Experiment

Seedlings at phenological growth stage 13, 3rd true leaf unfolded (Meier, 2018) were used, having been previously grown in commercial organic substrate. The pots were placed outdoors on a wooden platform with lateral protection to prevent direct sunlight and minimize pot heating. Regularly, the pots were rotated to ensure equivalent radiation exposure conditions. Weeds were promptly removed after germination.

The pots were watered as needed, ensuring that the lettuce plants remained well hydrated. In this type of experiment, the same amount of water is not applied to all pots because different fertilizations induce varying growth and transpiration rates, necessitating constant adjustment of water quantities per pot. Plates were placed under the pots to prevent nutrient leaching.



Figure 2. Visual comparison of the first cycle of lettuce at the end of the experiment

3.3. Sampling and Laboratory Analyses

The lettuces and oats were harvested by cutting them at ground level and then divided into several segments to facilitate the drying process. Once cut, the plants were oven-dried at 70 °C until reaching constant weight, after which they were ground through a 1 mm mesh. Subsequently, tissue samples were digested using nitric acid. Elemental tissue analysis was conducted using Kjeldahl method for N, colorimetry for boron (B) and P, flame emission spectrometry for K, and atomic absorption spectrophotometry for Ca, Mg, copper (Cu), iron (Fe), zinc (Zn), and manganese (Mn). For a detailed description of these methodologies, the reader is referred to Temminghoff and Houba (Temminghoff & Houba, 2004). Nitrate concentration in lettuce tissues were determined by UV-vis spectrophotometry in a water extract (dry matter:water, 1:50 m/v) (Baird *et al.*, 2017).

After the conclusion of the second lettuce growing cycle, soil samples were collected from the pots immediately before they were used for the oat experiment. The soil samples were oven-dried at 40 °C, sieved again through a 2 mm mesh, and analyzed for various parameters including organic C (determined using the wet digestion Walkley-Black method), soil pH (measured in water and KCl solutions at a soil-to-solution ratio of 1:2.5 using potentiometry), exchangeable bases (extracted with ammonium acetate at pH 7.0 and determined by atomic absorption spectrometry), exchangeable acidity (extracted with KCl and quantified through titration with sodium hydroxide), P and K (analyzed using the Egnér–Riehm method), and B

(extracted with hot water and determined using the azomethine-H method). For detailed information on these analytical procedures, please refer to Van Reeuwijk (Van, 2002). The other micronutrients (Cu, Fe, Zn, and Mn) were extracted using diethylenetriaminepentaacetic acid (DTPA) buffered at pH 7.3 and determined by atomic absorption spectrometry, following the procedures of FAO. (<https://www.fao.org/global-soil-partnership/glosolan-old/soil-analysis/standard-operating-procedures/en/>). The analysis conducted on the initial soil samples (table 1) also followed these same procedures. Additionally, soil separates were determined in these initial soil samples using the Robison pipette method.

3.4. Data Analysis

The data were subjected to analysis of variance (one-way ANOVA) after being tested for normality and variance homogeneity using the Shapiro-Wilk and Levene's tests, respectively. Whenever the ANOVA revealed significant differences among treatments, means were separated using the Tukey HSD multiple comparison test ($\alpha = 0.05$). Apparent N Recovery (ANR) was estimated by the equation: $\text{ANR (\%)} = 100 \times (\text{N recovered by plants in fertilized treatments} - \text{N recovered by plants in the control}) / \text{N applied as fertilizer}$.

4. Results

4.1. Lettuce Dry Matter Yield

Lettuce dry matter yield (DMY) varied significantly between treatments in the first lettuce growing cycle (Figure 2). Mealw, FullR, and HalfR gave significantly higher values than the other treatments, while Nutri and Cont displayed significantly lower values. It should also be noted that the highest average value was obtained in the Mealw treatment ($12.8 \text{ g plant}^{-1}$), even higher than the value recorded in the FullR treatment ($12.2 \text{ g plant}^{-1}$). DMY in the BSFLy treatment was lower than in the Mealw, FullR, and HalfR treatments, but higher than in the Cont and Nutri treatments. It is also worth noting that the average DMY in the Nutri treatment (5.1 g plant^{-1}) was lower than in the Cont treatment (6.7 g plant^{-1}).

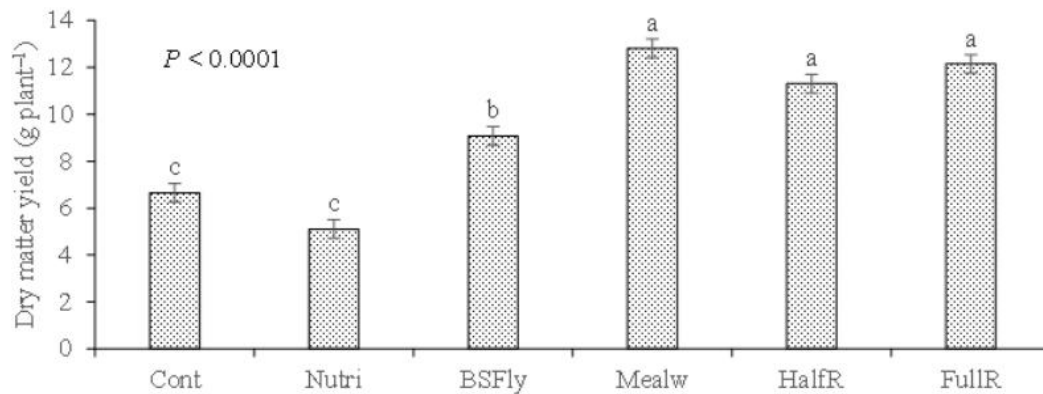


Figure 3. Dry matter yield of lettuce across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the first growing cycle. Means with the same letter are not significantly different (Tukey's HSD test, $\alpha = 0.05$). Vertical bars represent standard errors.

The pots subjected to the second rate of organic amendments and fertilizers (Figure 3a) exhibited results consistent with those observed during the initial growing cycle. However, the mean value of the Mealw treatment (9.8 g plant⁻¹) stood out, being significantly higher than all others except for the FullR treatment (7.8 g plant⁻¹). The BSFly treatment exhibited a lower mean value (5.7 g plant⁻¹), although not significantly different from that of the HalfR treatment (6.9 g plant⁻¹). The Nutri treatment maintained a very low mean value (2.7 g plant⁻¹), comparable to the Cont treatment (3.1 g plant⁻¹), and lower than that of the BSFly treatment.

In the pots that did not receive the second dose of organic amendments and fertilizers, where plant growth depended on the residual fertility from the treatments of the first lettuce cycle, the BSFly treatment stood out (5.8 g plant⁻¹), with no significant differences compared to the FullR treatment (5.7 g plant⁻¹), but significant differences were observed with all other treatments (Figure 3b). Nutri (4.3 g plant⁻¹) and Mealw (4.3 g plant⁻¹) displayed slightly higher mean values, but without significant differences compared to the Cont treatment (3.2 g plant⁻¹).

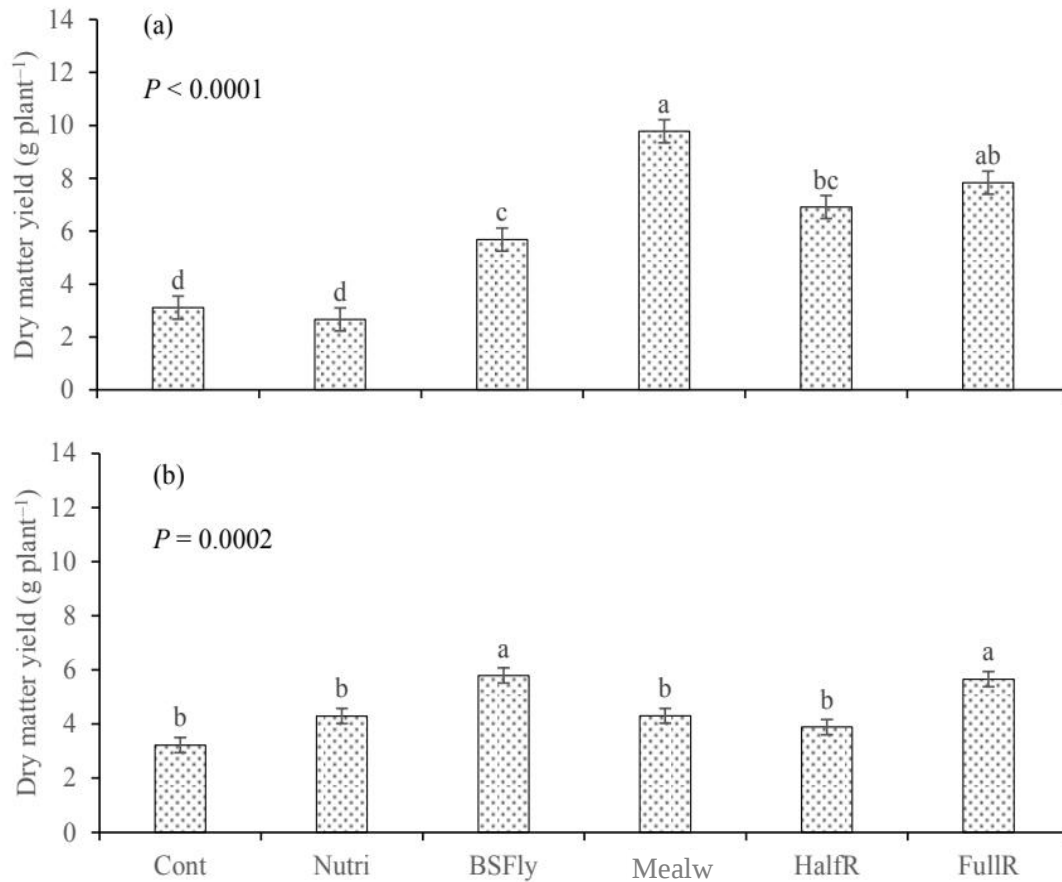


Figure 4. Dry matter yield of lettuce across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the second growing cycle, in pots that received (a) and did not receive (b) the second dose of organic amendments and fertilizers. Means with the same letter are not significantly different (Tukey's HSD test, $\alpha = 0.05$). Vertical bars represent standard errors.

4.2. Plant Nitrogen Nutritional Status

The N concentration in the tissues of lettuces grown during the first growing cycle followed a slightly different pattern from DMY (Figure 4). The highest values were observed in the FullR treatment (33.0 g plant⁻¹), followed by HalfR (26.5 g kg⁻¹), and Mealw (19.6 g kg⁻¹). The mean values observed in the BSFly (15.2 g kg⁻¹) and Nutri (14.4 g kg⁻¹) treatments did not differ significantly from those observed in the Cont treatment (13.6 g kg⁻¹).

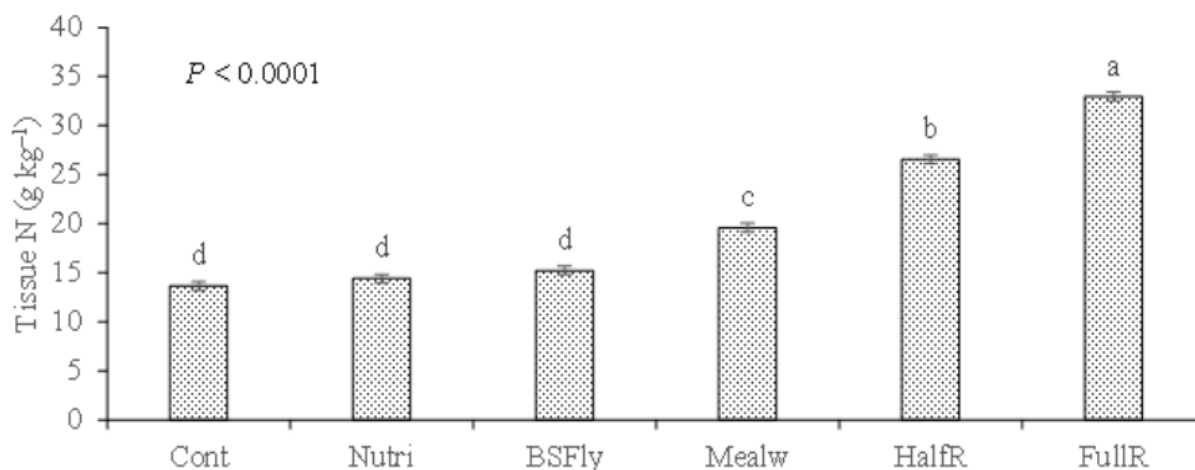


Figure 5. Nitrogen (N) concentration in lettuce tissues across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the first growing cycle. Means with the same letter are not significantly different (Tukey's HSD test, $\alpha = 0.05$). Vertical bars represent standard errors.

The response pattern observed in the first growing cycle of lettuce was repeated in the second cycle in pots that received the second rate of fertilizers (Figure 5a). Treatments receiving mineral fertilizer resulted in higher N concentrations in the tissues compared to other treatments. In pots not fertilized for the second growing cycle of lettuce (Figure 5b), the significant effect of the FullR treatment over others was maintained, but no significant differences were recorded among the others.

The nitrate concentration in the tissues did not vary as markedly with the fertilizer treatments as the N concentration in the tissues (Figure 6). In the first growth cycle of lettuce, significant differences occurred only between the FullR treatment (10917 mg kg⁻¹) and the Cont treatment (8975 mg kg⁻¹). No significant differences were observed between the other fertilized treatments and the control treatment.

In the second growing cycle of lettuce, in pots that received the second rate of fertilizers, the Mealw and BSFly treatments resulted in nitrate tissue values equivalent to those of treatments with mineral fertilizer and significantly higher than those of the Nutri and Cont treatments (Figure 7a). Once again, Nutri resulted in a value equivalent to that of the control. In the second growing cycle of lettuce, in pots that did not receive a second dose of fertilizers, a greater gradient between treatments was observed in tissue nitrate levels (Figure 7b). The mean values

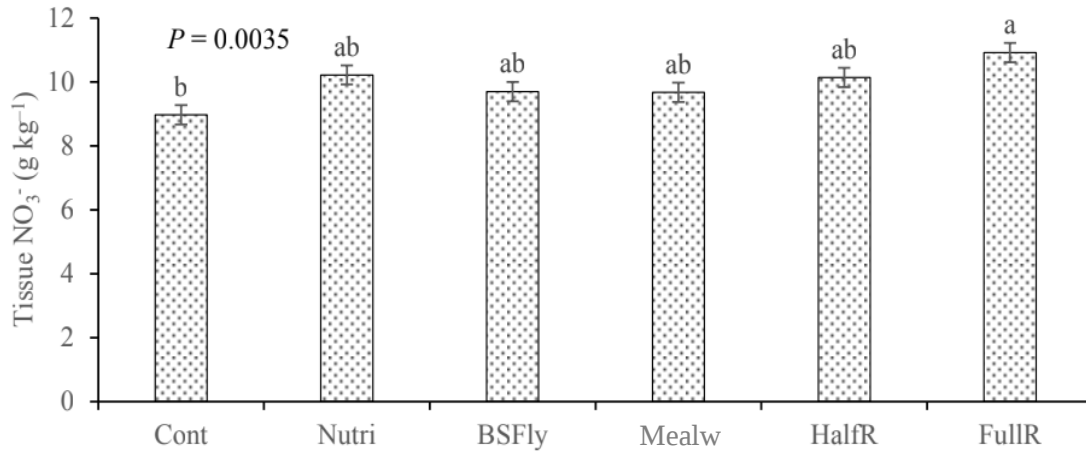


Figure 7. Nitrate (NO₃⁻) concentration in lettuce tissues across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the first growing cycle. Means with the same letter are not significantly different (Tukey's HSD test, $\alpha = 0.05$). Vertical bars represent standard errors.

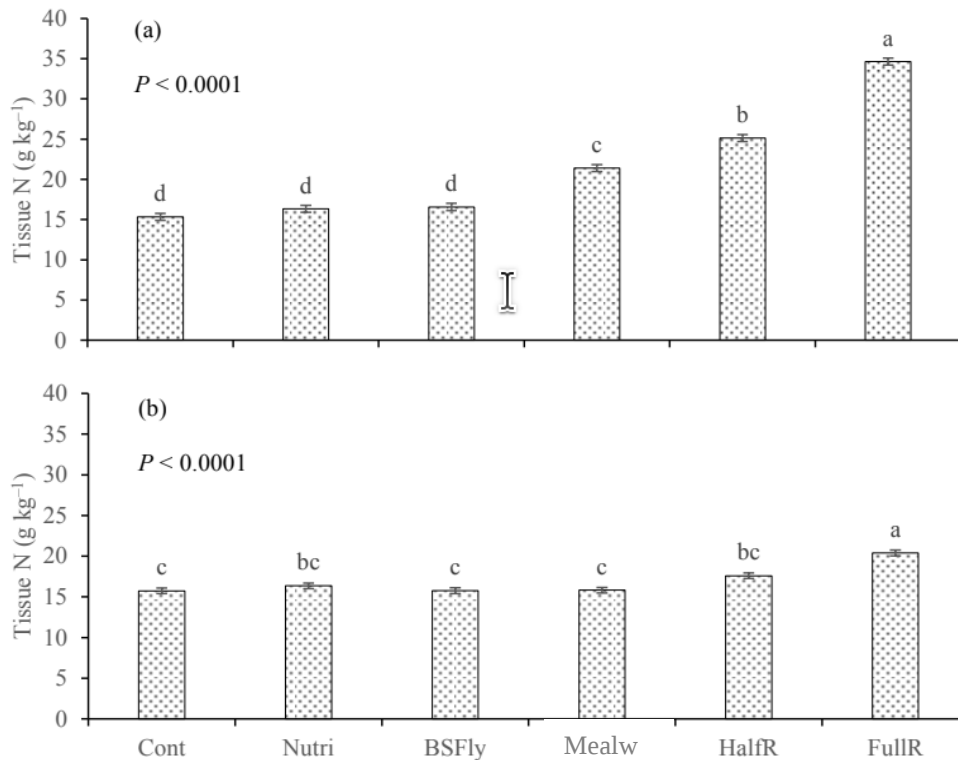


Figure 6. Nitrogen (N) concentration in lettuce tissues across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the second growing cycle, in pots that received (a) and did not receive (b) the second dose of organic amendments and fertilizers. Means with the same letter are not significantly different (Tukey's HSD test, $\alpha = 0.05$). Vertical bars represent standard errors.

of the FullR and Mealw treatments were significantly higher than those of the control.

The ANR in the first growing cycle of lettuce was very high in the HalfR treatment (96.6%), followed by the FullR treatment (71.9%) (Table 2). Among organic amendments, the mean value of Mealw (37.3%) was higher than that of BSFly (11.1%), being negative in the Nutri treatment (-4.0%). In pots where the second dose of fertilizer was not applied, the values increased comparatively to those of the first growing cycle in all treatments. The mean value exceeded 100% (105.9%) in the HalfR treatment and was slightly above 0% (0.6%) in the Nutri treatment. In pots that received the second dose of fertilizer, organic amendments maintained ANR values similar to those of the first lettuce cycle, while they decreased in treatments with mineral N application. Considering both growing cycles and pots that received both rates of fertilizers, the ANR values followed the decreasing order HalfR (78.3%), FullR (62%), Mealw (37.4%), BSFly (10.9%), and Nutri (-2.5%).

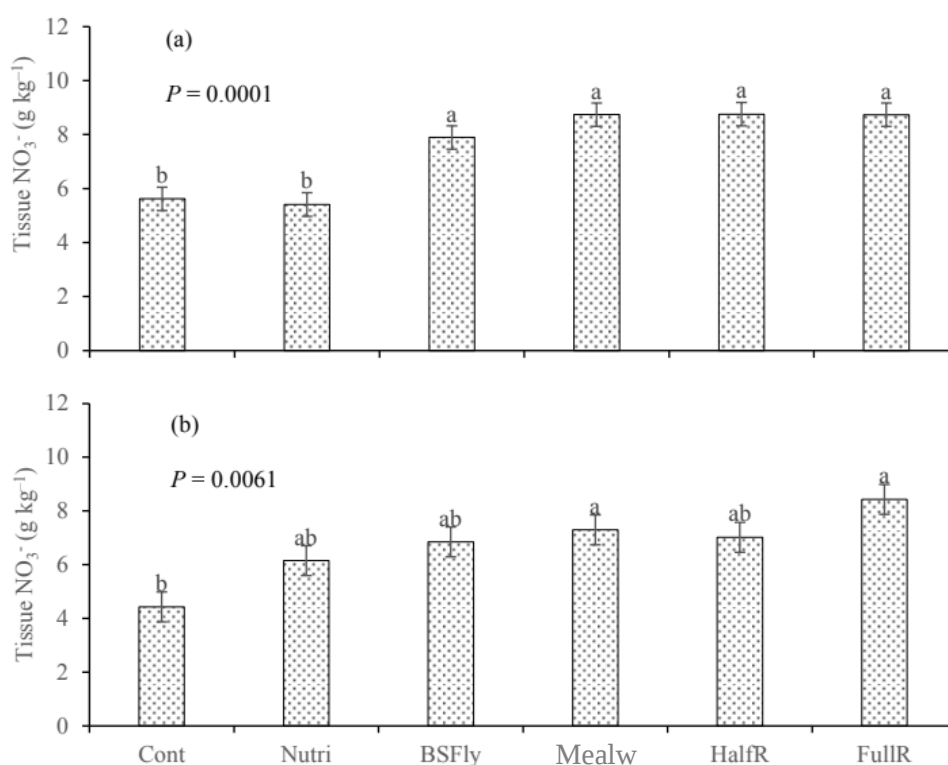


Figure 8. Nitrate (NO_3^-) concentration in lettuce tissues across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the second growing cycle, in pots that received (a) and did not receive (b) the second dose of organic amendments and fertilizers. Means with the same letter are not significantly different (Tukey's HSD test, $\alpha = 0.05$). Vertical bars represent standard errors.

Table 2. Apparent nitrogen (N) recovery by lettuces across fertilization treatments [Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the first and second growing cycle (GC) in pots that received (with) and did not receive (without) the second dose of organic amendments and fertilizers.

Apparent N Recovery (%)				
Treatment	1st GC	2nd CG (without)	2nd CG (with)	Total (1st + 2nd with)
Nutri	-4.0	0.6	-1.0	-2.5
BSFly	11.1	20.5	10.8	10.9
Mealw	37.3	41.3	37.6	37.4
HalfR	96.6	105.9	59.0	78.3
FullR	71.9	87.2	52.0	62.0

Apparent N recovery (%) = $100 \times [(N \text{ recovered in the fertilized treatments} - N \text{ recovered in the control}) / N \text{ applied as a fertilizer}]$.

4.3. Concentration of other Macro and Micronutrients in the tissues

The concentration of P in lettuce tissues varied significantly between treatments in the first growing cycle, with the highest average value found in the Mealw treatment (3.7 g kg^{-1}), followed by BSBFly (3.2 g kg^{-1}) (Table 3). The average values of the other treatments did not vary significantly. The Mealw treatment also exhibited the highest average concentration of K in lettuce tissues (56.4 g kg^{-1}), which was significantly higher than that of the Cont treatment (43.8 g kg^{-1}). The Nutri treatment showed significantly higher Ca levels in tissues (8.3 g kg^{-1}) than those of FullR (6.7 g kg^{-1}). FullR showed the highest values of Mg (2.9 g kg^{-1}), while BSBFly (2.3 g kg^{-1}) and Cont (2.3 g kg^{-1}) showed the lowest ones. Tissue B concentration did not vary between treatments. The Fe levels in tissues appeared lower in treatments associated with higher DMY, a trend apparently followed by Cu values. The Mn and Zn values seem to show a tendency to be higher in treatments with mineral fertilization.

The concentration of P in lettuce tissues in the second growth cycle, and in pots that received the second dose of fertilizer, did not vary significantly between treatments (Table 4). K values were lower in the FullR treatment compared to treatments with organic amendments and control. Ca values in the tissues were again high in the Nutri treatment, this time accompanied by those of the control treatment. Mg, B, Mn, and Zn levels in lettuce tissues did not vary significantly with the treatments. Fe levels in the tissues showed the same trend of being lower in the more productive treatments, and Cu levels did not show consistency with the values

observed in the first growing cycle of lettuce.

Table 3. Concentration of macro and micronutrients in lettuce tissues across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the first growing cycle.

Treatment	P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
	g kg ⁻¹				mg kg ⁻¹				
Cont	2.6 c	43.8 b	6.9 ab	2.3 b	35.5 a	2161.9 a	88.4 bc	76.1 ab	32.7 a
Nutri	2.7 c	53.5 ab	8.3 a	2.7 ab	32.8 a	2062.8 a	73.8 c	58.4 b	29.4 ab
BSFly	3.2 b	54.2 ab	7.2 ab	2.3 b	34.3 a	1889.1 a	86.0 c	67.7 ab	33.8 a
Mealw	3.7 a	56.4 a	7.3 ab	2.4 ab	34.6 a	1407.5 b	82.1 c	71.9 ab	28.3 ab
HalfR	2.6 c	44.1 ab	7.2 ab	2.5 ab	33.9 a	1133.2 b	103.2 ab	80.0 ab	25.6 b
FullR	2.6 c	45.0 ab	6.7 b	2.9 a	31.3 a	1115.1 b	107.0 a	87.7 a	23.9 b
Prob	<0.0001	0.0077	0.0453	0.0115	0.1314	<0.0001	<0.0001	0.0118	0.0002

In columns, means followed by the same letter are not significantly different by Tukeys' HSD test ($\alpha=0.05$).

Table 4. Concentration of macro and micronutrients in lettuce tissues across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the second growing cycle in post that received the second dose of organic amendments and fertilizers.

Treatment	P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
	g kg ⁻¹				mg kg ⁻¹				
Cont	1.3 a	49.5 a	8.9 a	3.8 a	27.8 ab	1332.5 ab	70.5 a	57.2 a	11.0 ab
Nutri	1.0 a	50.7 a	9.5 a	4.1 a	26.1 a	1487.8 a	58.8 a	70.4 a	14.8 a
BSFly	1.1 a	49.9 a	7.5 a	3.2 a	27.9 a	660.2 ab	62.1 a	79.1 a	10.0 b
Mealw	1.0 a	50.2 a	7.2 b	3.5 a	27.7 a	816.9 ab	84.1 a	68.9 a	11.4 ab
HalfR	0.9 a	41.4 ab	7.5 b	4.0 a	24.2 a	733.4 ab	80.6 a	76.8 a	12.5 ab
FullR	1.3 a	39.6 b	6.7 b	3.8 a	22.8 a	570.9 b	87.6 a	79.3 a	12.2 ab
Prob	0.3630	0.0300	<0.0001	0.2630	0.0554	0.0171	0.1772	0.4862	0.0476

In columns, means followed by the same letter are not significantly different by Tukey's HSD test ($\alpha=0.05$).

In the pots that did not receive the second dose of fertilizers, no significant differences occurred in the concentration of macronutrients in lettuce tissues (Table 5). In this group of pots, significant differences in tissue B levels appeared for the first time, with the lowest mean values observed in the FullR treatment. No significant differences were observed among treatments for metallic micronutrients.

Table 3. Concentration of macro and micronutrients in lettuce tissues across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in the second growing cycle in pots that did not receive the second dose of organic amendments and fertilizers.

Treatment	P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
	g kg ⁻¹				mgkg ⁻¹				
Cont	1.2 a	47.8 a	9.1 a	3.9 a	27.2 ab	1117.0 a	69.0 a	63.1 a	11.6 a
Nutri	1.1 a	47.4 a	7.9 a	3.3 a	27.6 a	1058.4 a	53.5 a	58.0 a	10.8 a
BSFly	1.2 a	42.2 a	7.3 a	3.3 a	27.2 a	691.2 a	54.5 a	54.3 a	10.0 a
Mealw	0.9 a	38.9 a	8.3 a	3.5 a	28.3 ab	983.7 a	74.3 a	54.7 a	13.3 a
HalfR	1.1 a	48.2 a	9.1 a	4.0 a	28.7 a	986.0 a	78.0 a	75.0 a	11.4 a
FullR	1.1 a	44.2 a	7.0 a	3.4 a	23.8 b	661.5 a	87.2 a	71.9 a	10.6 a
Prob	0.8482	0.1440	0.0686	0.0604	0.0119	0.2245	0.0443	0.4587	0.3428

In columns, means followed by the same letter are not significantly different by Tukey's HSD test ($\alpha=0.05$).

4.4. Residual Effect of Treatments on Inorganic Nitrogen in Soil and Oat Crop

The nitrate, ammonia, and total inorganic N levels in the soil at the end of the second growing cycle of lettuce were the highest in the FullR treatment, with significant differences compared to all other treatments in ammonia and total inorganic N levels (Table 6). In pots that did not receive the second dose of fertilizer, elevated values were also observed in some treatments with organic amendments, such as Mealw and Nutri. The control values were typically the lowest.

After the second lettuce cycle, oats were grown in pots without receiving further fertilization. In pots that received the second dose of fertilizer, DMY was highest in the BSBFly treatment (3.1 g pot⁻¹), followed by Mealw (2.8 g pot⁻¹) and FullR (2.6 g pot⁻¹) (Table 7). The Cont (1.2 g pot⁻¹)

¹) and Nutri (1.4 g pot⁻¹) treatments recorded the lowest values. The trend in DMY results in pots that did not receive the second dose persisted, with higher values in the BSFly treatment (2.0 g pot⁻¹) and significantly different from the Cont (1.3 g pot⁻¹) and Nutri (1.3 g pot⁻¹) treatments. No significant differences were observed in N concentration in the tissues, whether in pots that received or did not receive the second dose of fertilizer. However, the amount of N taken up by the plant followed the trend of DMY, with the highest mean values observed in the BSFly treatment, although no significant differences between treatments were observed in pots that did not receive the second dose of fertilizer.

Table 4. Nitrogen (N) in the form of nitrate (NO₃⁻) and ammonium (NH₄⁺) in the soil at the end of the second lettuce growing cycle across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in pots that received and did not receive the second dose of organic amendments and fertilizers

	Two Fertilizer Applications			Fertilizers Applied Once		
	NO ₃ ⁻ -N	NH ₄ ⁺ -N mg kg ⁻¹	Inorg -N	NO ₃ ⁻ -N	NH ₄ ⁺ -N mg kg ⁻¹	Inorg -N
Cont	2.2 b	0.3 b	2.5 b	2.3 bc	0.5 c	2.8 b
Nutri	3.9 ab	1.5 b	5.4 b	3.8 ab	1.6 bc	5.4 ab
BSFly	1.7 b	1.8 b	3.4 b	1.4 c	2.9 ab	4.3 b
Mealw	4.1 ab	3.4 b	7.4 b	4.3 a	3.8 a	8.1 a
HalfR	3.4 ab	3.0 b	6.4 b	2.6 abc	1.2 bc	3.8 ab
FullR	6.4 a	10.1 a	16.5 a	3.0 abc	2.7 ab	5.7 ab
Prob	0.0039	0.0001	<0.0001	0.0035	0.0013	0.0011

In columns, means followed by the same letter are not significantly different by Tukey's HSD test ($\alpha=0.05$).

Table 5. Dry matter yield (DMY), tissue N and N recovery of oats across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in pots that received and did not receive the second dose of organic amendments and fertilizers.

	Two Fertilizer Applications			Fertilizers Applied Once		
	DMY g pot ⁻¹	Tissue N g kg ⁻¹	N Recovery mg pot ⁻¹	DMY g pot ⁻¹	Tissue N g kg ⁻¹	N Recovery mg pot ⁻¹
Cont	1.2 c	17.0 a	21.4 c	1.3 b	18.6 a	26.0 a
Nutri	1.4 c	16.1 a	22.2 c	1.3 b	17.3 a	22.0 a
BSFly	3.1 a	15.7 a	48.1 a	2.0 a	17.2 a	34.3 a
Mealw	2.8 ab	16.5 a	46.2 ab	1.8 ab	15.5 a	28.3 a
HalfR	2.2 b	15.9 a	35.1 b	1.4 ab	17.7 a	25.2 a
FullR	2.6 ab	14.2 a	37.3 ab	1.5 ab	16.8 a	25.0 a
Prob	<0.0001	0.0571	<0.0001	0.0318	0.1232	0.3163

In columns, means followed by the same letter are not significantly different by Tukey's HSD test ($\alpha=0.05$).

4.5. Soil Properties

Soil organic C in the pots that received the second rate of fertilizers varied significantly between treatments (Table 8). The higher average values were found in the Mealw treatment (11.5 g kg^{-1}), while the lower ones observed in the Cont treatment (8.8 g kg^{-1}). Soil pH did not vary significantly between treatments. Extractable P was particularly high in the BSFly and Mealw treatments and low in the HalfR and FullR treatments. Soil K levels were also particularly low in the HalfR and FullR treatments. Exchangeable Ca did not vary significantly between treatments, nor did the CEC, although significant differences were observed among treatments for soil Mg levels, which were particularly high in the BSFly treatment and low in the FullR treatment. Cation micronutrients did not vary significantly between treatments.

Table 6. Soil properties at the end of the second growing cycle of lettuce across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in pots that received the second dose of organic amendments and fertilizers.

Parameter	Cont	Nutr	BSFly	Mealw	HalfR	FullR	Prob
Organic C (g kg^{-1})	8.8 b	9.9 ab	11.4 a	11.5 a	8.4 b	10.6 ab	0.0337
pH (H_2O)	6.6 a	6.7 a	6.5 a	6.7 a	6.7 a	6.5 a	0.1076
Extract P (mg kg^{-1})	124.5 bc	148.8 bc	216.6 a	193.4 a	89.8 c	97.4 c	<0.0001
Extract K (mg kg^{-1})	128.3 ab	123.7 b	151.7 a	110.7 b	83.3 c	75.3 c	<0.0001
Exchang Ca ($\text{cmol}^+ \text{kg}^{-1}$)	16.0 a	17.0 a	16.8 a	15.9 a	16.4 a	14.8 a	0.2424
Exchang Mg ($\text{cmol}^+ \text{kg}^{-1}$)	5.3 bc	5.3 bc	6.4 a	5.8 ab	5.3 bc	4.8 c	0.0023
CEC (cmolc kg^{-1})	21.9 a	23.2 a	24.0 a	22.4 a	22.4 a	20.1 a	0.0858
Extract Fe (mg kg^{-1})	123.0 a	106.4 a	107.1 a	100.3 a	108.2 a	107.7 a	0.1963
Extract Zn (mg kg^{-1})	4.1 a	4.6 a	5.1 a	5.3 a	4.0 a	4.2 a	0.2432
Extract Cu (mg kg^{-1})	71.0 a	67.7 a	69.5 a	67.0 a	73.2 a	75.3 a	0.8506
Extract Mn (mg kg^{-1})	176.3 a	144.2 a	153.6 a	146.0 a	159.5 a	154.0 a	0.0781

In rows, means followed by the same letter are not significantly different by the Tukey HSD test ($\alpha=0.05$).

Table 7. Soil properties at the end of second growing cycle of lettuce across fertilization treatments [control (Cont), Nutrimais (Nutri), black soldier fly larvae frass (BSFly), mealworm larvae frass (Mealw), half the rate ammonium nitrate (HalfR), full rate of ammonium nitrate (FullR)] in pots that did not receive the second dose of organic amendments and fertilizers.

Parameter	Cont	Nutri	BSFly	Mealw	HalfR	FullR	Prob
Organic C (g kg ⁻¹)	8.2 c	11.2 a	11.3 a	10.5 ab	8.6 bc	9.4 abc	0.0014
pH (H ₂ O)	6.5 a	6.7 a	6.7 a	6.7 a	6.5 a	6.5 a	0.0531
Extract P (mg kg ⁻¹)	96.7 b	153.9 a	166.2 a	163.4 a	90.1 b	90.3 b	<0.0001
Extract K (mg kg ⁻¹)	104.0 b	128.0 a	115.3 b	115.3 b	91.7 c	82.0 c	<0.0001
Exchang Ca (cmol ⁺ kg ⁻¹)	15.8 a	17.1 a	16.1 a	15.9 a	14.8 a	15.4 a	0.1119
Exchang Mg (cmol ⁺ kg ⁻¹)	5.3 ab	5.3 ab	5.8 a	5.5 ab	5.0 b	4.9 b	0.0147
CEC (cmolc kg ⁻¹)	21.7 a	23.2 a	22.6 a	22.0 a	20.5 a	20.9 a	0.1178
Extract Fe (mg kg ⁻¹)	117.8 a	118.0 a	109.0 a	94.3 a	108.7 a	104.7 a	0.2596
Extract Zn (mg kg ⁻¹)	5.0 a	5.1 a	5.0 a	4.9 a	3.9 a	4.1 a	0.3151
Extract Cu (mg kg ⁻¹)	71.2 a	73.0 a	76.0 a	69.5 a	69.0 a	70.4 a	0.8828
Extract Mn (mg kg ⁻¹)	175.5 a	162.0 a	160.4 a	137.8 a	160.6 a	151.0 a	0.2789

In rows, means followed by the same letter are not significantly different by the Tukey HSD test ($\alpha=0.05$).

In pots that did not receive the second dose of fertilizer, soil organic C varied similarly between treatments as in pots that received the second dose, although with generally lower values, even in treatments that did not receive organic amendments (Table 9). Soil pH also did not vary significantly between treatments. P and K values followed the trend observed in pots that received the second dose of fertilizers, although P levels were generally lower now. Exchangeable Ca and Mg, as well as CEC, showed a very similar pattern to what was observed in pots that received the second dose of fertilizer. Among cationic micronutrients, no significant differences occurred between treatments.

5. Discussion

5.1. Mealworm Larvae Frass

Mealworm larvae frass (Mealw) resulted in the highest average DMY among all treatments in the first lettuce growing cycle, significantly surpassing the BSBFly, Nutri, and Cont treatments (Figure 2). This trend of high values persisted in the second lettuce cycle upon reapplication of

fertilizers, with DMY significantly exceeding that of the other treatments, except for the FullR treatment (Figure 3a). N concentration in the tissues was notably higher in the Mealw treatment compared to treatments with other organic amendments. Often considered the most crucial nutrient in crop fertilization programs, N has an undeniable effect on crop growth (Lopes *et al.*, 2021; Yahbi *et al.*, 2022; Aghabeygi & Donmez, 2024), and this study is no exception.

The ANR in the Mealw treatment was very high for an organic amendment, reaching 37.4% over the two growing cycles that received fertilization. This result indicates a high mineralization rate of the organic substrate. Houben *et al.* (2008) reported a significant effect of mealworm larvae frass application on ryegrass grown in pots, also attributing the result to the rapid appearance of nutrients in the soil in readily available forms for plants. The mineralization rate of organic substrates in the soil largely depends on their N content and/or the C/N ratio. The balance between biological immobilization and mineralization is typically found for N values between 15 and 20 g kg⁻¹ and C/N ratios between 20 and 30 (Weil & Brady, 2017; Myrold & Bottomley, 2008). The N content and C/N ratio of mealworm larvae frass were 31.3 g kg⁻¹ and 16.5, respectively. Although these values may suggest net mineralization in the short term, they would be unlikely to predict such high mineralization rates, given results reported in previous studies using other organic amendments (Rodrigues *et al.*, 2018 ; Arrobas *et al.*, 2022, Cao *et al.*, 2020 ; Xie *et al.*, 2022), especially considering the short growing cycle of lettuce.

However, the availability of N alone cannot account for the good performance of lettuces subjected to the Mealw treatment. Lettuces that received ammonium nitrate, particularly those in the FullR treatment, exhibited higher N concentrations in tissues compared to those in the Mealw treatment. Moreover, the N concentration in the tissues of the Mealw treatment is still below the sufficiency range for lettuce (Bryson *et al.*, 2014), indicating that the FullR treatment did not supply excess N. The ANR in treatments with mineral N reached notably high percentages, especially when compared to typical field trial values, which usually fall below 50% (Xie *et al.*, 2022; Havlin *et al.*, 2017; Rodrigues *et al.*, 2021). This also suggests that excess N was not used. The N rate used in the FullR treatment, amounting to 50 kg ha⁻¹, is not excessive when considering the common N requirements for lettuce (Almeida, 2006). Lettuces in the Mealw treatment exhibited high tissue P levels, particularly during the first growing cycle. This result suggests that P might have played a significant role in the DMY of the lettuce, considering the well-documented importance of P in crop fertilization across various regions worldwide (Li *et al.*, 2016; Tian *et al.*, 2020). Additionally, with organic amendments, the

simultaneous availability of multiple nutrients can create positive synergies in root system development, thereby benefiting plant growth (Du *et al.*, 2022). However, some of the more productive treatments, FullR and HalfR, showed lower tissue P concentrations, diminishing the relevance of P in lettuce growth. Given the concentrations of other nutrients in the tissues, there seems to be no justification to assert that the role of any specific nutrient was significant in explaining the high DMY in the Mealw treatment. Therefore, other factors unrelated to tissue nutrient concentrations must be identified to explain the DMY results. Organic amendments can positively affect plant growth in several ways, particularly by improving the physical, chemical, and biological properties of the soil (Arrobas *et al.*, 2022; Su *et al.*, 2021; Hua *et al.*, 2020). Some studies have demonstrated that the positive effects on plants achieved by applying organic amendments cannot be replicated by using only mineral fertilizers. This beneficial action of organic amendments is sometimes referred to as the "manuring effect", a term used to describe measurable growth benefits that may be difficult to attribute to a specific cause (Weil, 2017; Rodrigues *et al.*, 2018; Dimande *et al.*, 2023). By testing various organic materials produced by mealworm larvae, Poveda *et al.* (2019) suggested that some microorganisms present in the excreta act as plant growth promoters, which explains the benefits they observed in the growth of chard (*Beta vulgaris* var. cicla) and bean (*Phaseolus vulgaris*) seedlings. The authors also found that each type of excrement had a different potential to promote plant growth and stress resistance, depending on the diets of the insects. In another study, although positive effects on lettuce growth were recorded when using mealworm frass, Fuertes-Mendizábal *et al.* (2023) found that the use of the product at a dose above 2.5% of the peat substrate resulted in a negative effect on lettuce plant growth, particularly on root development, indicating that the applied dose may be relevant in the management of this organic amendment. In the study presented here, the Mealw treatment appears to have a positive effect on plant growth that extends beyond its nutrient content and its impact on the nutritional status of the plants. The result suggests a clear manuring or biostimulant effect. Considering the use of soil as substrate and a fertilizer rate similar to field cultivation of lettuce, no negative effects were observed.

5.2. Black Soldier Fly Frass

Black soldier fly frass initially presented a composition not very different from that of mealworm larvae frass (Table 1). The composition of the black soldier fly frass used in this study is also comparable to organic materials of the same origin reported in other studies (Beesigamukama *et al.*, 2022). However, the lettuce DMY was significantly lower than that of the Mealw treatment in the cycles where fertilizers were applied. The N concentration in the

tissues was also lower (Figures 4 and 5), as well as the ANR (Table 2), which averaged 10.9% over the two growing cycles where fertilizers were applied. The N content (26.8 g kg^{-1}) and the C/N ratio (18.6 g kg^{-1}) of this material also indicate a high likelihood of short term net mineralization, since the C/N ratio value that leads to a balance between mineralization and biological immobilization ranges from 20 to 30, as previously mentioned (Weil & Brady, 2017; Myrold & Bottomley, 2008). The BSFly treatment increased lettuce DMY compared to the control treatment. Menino et al. 2021, using a black soldier fly frass organic amendment, also observed an increase in ryegrass production in a greenhouse experiment compared to the control treatment. Similar results were found by Chavez *et al.* 2024 in a pot experiment with arugula (*Eruca sativa* L.) and lettuce. However, in practice, the results of the BSFly treatment were not significantly different from those reported worldwide with other organic amendments (Rodrigues *et al.*, 2018; Cao *et al.*, 2020; Xie *et al.*, 2022). Carroll *et al.* (2023), for instance, using black soldier fly frass, reported cereal seedling growth at a level equivalent to that observed with pelletized chicken manure. The productivity loss in the BSFly treatment compared to the FullR treatments was likely due to the reduced availability of N in the soil, as the nutrient levels in the tissues were much lower in the BSFly treatment and below the lower limit of the sufficiency range for lettuce (Bryson *et al.*, 2014). N availability in the soil is crucial for cell growth and expansion, particularly for the formation of proteins and other organic molecules (Hawkesford *et al.*, 2023). Unlike the Mealw treatment, the BSFly treatment did not appear to have any evident effect beyond what is typically observed with high-quality organic amendments.

5.3. Nutrimais

The lettuce DMY in the Nutri treatment remained consistently at the level of the Cont treatment in both lettuce cycles, regardless of whether fertilization was repeated or not. The N levels in the tissues were always low, and not significantly different from those in the Cont treatment. On the other hand, the nitrate levels in the tissues, another indicator of N availability in the soil (Arrobas *et al.*, 2024; Conversa *et al.*, 2021; Luetic *et al.*, 2023), tended to be lower than those in the other fertilized treatments. There did not appear to be significant differences between the Nutri treatment and the other treatments regarding the concentration of nutrients other than N in the tissues. Thus, the lack of available N for the plants was likely the main cause of the low productivity of the lettuces subjected to this treatment, considering that the N concentration in the tissues was well below the lower limit of the sufficiency range as reported by Bryson *et al.*

(2024). The ANR by lettuces subjected to the Nutri treatment was globally negative, including when considering the two cropping cycles that received fertilization (Table 2). This indicates that biological immobilization occurred, meaning that the lettuces in this treatment had less available N than those in the Cont treatment. Biological immobilization resulting from the application of an organic amendment tends to occur when the amendment has a very low N concentration or a very high C/N ratio (Weil & Brady, 2007; Myrold & Bottomley, 2008). Under these conditions, energy is available for the heterotrophic communities in the soil (reflected in the C of the substrate), but there is little N (essential for the formation of amino acids and proteins). This leads to competition between heterotrophic microorganisms and plants for the available mineral N in the (Weil & Brady, 2007; Myrold & Bottomley, 2008). Although this organic substrate initially had a composition not very different from the others (C/N ratio, 16.5), it proved to be unreactive in the soil, not releasing nutrients to the plants. Previous studies using this organic amendment have already indicated its low reactivity (Arrobas *et al.*, 2022; Arrobas *et al.*, 2023). Perhaps because it is a highly dehydrated and pelletized material, it becomes biologically inactive when applied to the soil. This aspect, combined with the short duration of the lettuce growing cycle, resulted in a situation where its effect on crop productivity was practically negligible. Objectively, it seems to be an organic amendment unsuitable for early maturing horticultural crops that require a rapid supply of nutrients.

5.4. Residual Effect of Fertilization and Soil Properties

The effect of lettuce fertilization on unfertilized oats highlighted the role of the BSFLy treatment, which showed higher average values for DMY and N recovery both when fertilizers were applied in both lettuce growing cycles and when applied in only one of them (Table 7). This may indicate that, although Mealw had a higher short-term effect, over time, BSFLy also released its nutrients, increasing consistency in the medium term. This result emphasizes the general knowledge about organic amendments and the care that should be taken in their management. Although N content and the C/N ratio have been used as the main indicators of the balance in the mineralization/immobilization process and nutrient release for crops (Weil & Brady, 2007; Myrold & Bottomley, 2008), the process is highly unpredictable. Therefore, it is important to have the best information about organic amendments for more effective management in agricultural fields (Xie *et al.*, 2022; Dimande *et al.*, 2023).

The effect of treatments on soil properties, considering the situations of two or just one application, demonstrated a tendency for an increase in soil organic matter in the Nutri, BSFLy,

and Mealw treatments compared to the unfertilized control. However, the FullR treatment consistently exhibited higher average values compared to the control, although without significant differences. This study's limited duration prevents providing fully consolidated results on soil organic matter dynamics. Nevertheless, it seemed evident that the application of organic amendments increased soil organic matter content through direct C input, a phenomenon frequently observed following such applications (Lopes *et al.*, 2021; Arrobas *et al.*, 2022; Su *et al.*, 2021; Menino *et al.*, 2021). However, mineral fertilization can also sometimes increase soil organic matter content (Arrobas *et al.*, 2023; Silva *et al.*, 2021). Increased primary productivity results in more organic substrate being deposited into the soil through roots and associated mycorrhizal fungi (Godblod *et al.*, 2006; Hogberg *et al.*, 2020). In this study, comparing organic amendments is complex because some (e.g., Mealw) may have undergone more mineralization, potentially reducing soil organic C content. On the other hand, the stimulation of lettuce growth was greater, contributing to higher C deposition in the soil through root system development. With the Nutri treatment, for example, the opposite may have occurred, with more original C remaining in the soil and a lesser contribution from the lettuce root system.

Due to the short duration of the experiments, only a few other soil properties varied significantly with the treatments. Extractable P and K values were the most dynamic variables, reflecting higher values in treatments with organic amendments, where significant amounts of nutrients were applied to the soil, and lower values in mineral treatments, where nutrients were not applied and there may have been greater nutrient removal by lettuce. Similar findings were observed in studies by Menino *et al.* (2021) utilizing black soldier fly frass, demonstrating an elevation in soil nutrient levels corresponding to the main components of the organic amendment.

6. Conclusions

Mealworm larvae frass mineralized very rapidly, releasing N to lettuce at a much faster rate than its C/N ratio would predict. This suggests that the action of mealworm larvae produces an organic substrate that is very easily degraded by soil heterotrophic microorganisms. Consequently, it becomes a highly valuable organic amendment for application in early maturing annual crops that require a rapid release of nutrients. However, the high N release does not fully explain its effect on DMY, which tended to be even higher than that of the treatment that received the highest rate of mineral fertilizer (FullR) and resulted in plants with higher tissue N concentrations. A manuring effect was observed that cannot be achieved solely with mineral fertilizer. The results suggest the possibility of compounds within the mealworm larvae frass composition exerting a plant biostimulant effect, capable of enhancing productivity beyond nutrient release and its impact on soil properties. In future research, efforts should be made to assess the persistence of the biostimulant effect and elucidate its underlying mechanisms. Black soldier fly frass exhibited typical behaviour of a conventional high-quality organic fertilizer, with just over 10% N release during the short lettuce growing cycle. It did not appear as reactive in the soil as mealworm larvae frass, nor did it demonstrate any apparent manuring or plant biostimulant effects. Despite its low C/N ratio, Nutrimais showed little reactivity, with a nearly negligible N mineralization/immobilization balance during the short lettuce growing cycle. This result suggests that this fertilizer should preferably be used in perennial crops that do not rely on immediate nutrient release following the application of the organic amendment.

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