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Lettuce response to the application of two commercial leonardites and their effect on soil properties in a growing medium with nitrogen as the main limiting factor

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ABSTRACT

The aim of the study was to compare the effect of two leonardites (Humitec® and Humic Gold®) and an organic compost (Nutrimais®) with an untreated control on lettuce. The pot experiment was carried out in NE Portugal, in autumn 2019 and spring 2020. Humitec, Humic Gold and Nutrimais were applied alone or as a supplement to nitrogen (N), phosphorus (P) and potassium (K). The experiment was arranged as a three-factor experimental design (organic amendment, NPK addition and growing season). The growing season significantly influenced lettuce dry matter yield (DMY), the spring cycle being the highest yielding (10.6 to 6.5 g plant^{-1}). NPK fertilization also significantly increased lettuce DMY (11.2 to 5.9 g plant^{-1}). The organic amendment significantly influenced the concentration of some nutrients in plant tissues and some soil properties, but it did not influence lettuce DMY. In respect of growing season, Nutrimais increased lettuce DMY in the first one. In relation to the absence or otherwise of NPK fertilization, Nutrimais increased lettuce DMY in the pots not receiving NPK. Leonardites did not influence soil properties, tissue nutrient concentration or lettuce DMY. The positive effect on the lettuce DMY of organic compost was probably due to the supply of N, apparently the plants' most significant limiting factor. The non-positive results of leonardites were due to the fact that they neither provided N, nor enhanced its bioavailability from the soil. Thus, NPK fertilizer and Nutrimais, a compost of low C/N ratio, providing some N had the greatest effect on lettuce DMY.

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Fulvic acids; humic acids; humic substances; *Lactuca sativa* L; organic compost; plant biostimulants

Introduction

The world population will continue to increase in the coming decades (FAO (Food and Agriculture Organization of the United Nations) 2021). To meet the increased demand for food, on a planet where arable land should not be allowed to expand into natural ecosystems, cropping intensification must continue (Weil and Brady 2017). Continuous cultivation, however, is a major cause of declining soil fertility, mainly due to crop nutrient removal and nutrient loss through erosion, leaching and greenhouse gas emissions (Bashagaluke et al. 2018). Nowadays it is believed that ecological intensification is the way forward, trying to keep in mind that crop productivity

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must be maintained whilst reducing off-farm production factors, mainly those that jeopardize the sustainability of agro-systems (Tilman et al. 2011; Tittonell 2014). In this respect, crop fertilization, and in particular the use of N and P fertilizers, has been receiving considerable attention. N fertilization is of particular concern due to the risk of greenhouse gas emissions into the atmosphere, in particular N oxides (Coyne 2008; Pelster et al. 2011), nitrate leaching to aquifers and groundwater (Yang et al. 2018; Poikane et al. 2019), and the excessive accumulation on nitrates in edible vegetable such as lettuce (Zandvakili et al. 2019; Tabaglio et al. 2020). The use of phosphate fertilizers is also facing problems of sustainability since phosphate rocks, from which phosphate fertilizers are manufactured, are finite resources which will disappear before the end of the century (Gilbert 2009; Hawkesford et al. 2012).

As a result of this, the use of plant biostimulants in agriculture has been gaining increasing interest over recent years. In the form of plant biostimulants, it is possible to use several substances to enhance plant growth, such as algae extracts, protein hydrolysates, humic and fulvic acids, inorganic compounds and beneficial microorganisms (Du Jardin 2015; Du Jardin, Xu, and Geelen 2020; Rouphael and Colla 2020). The market for plant biostimulants is expected to grow at a rate of 12.3% per year and reach a value of 5.5 million dollars in 2027 (Carmody et al. 2020). Plant biostimulants are substances that can benefit plant growth not so much by providing nutrients, but by improving soil nutrient bioavailability, the efficiency of their use by plants, or by increasing the plant's tolerance to abiotic and biotic stresses (Shukla et al. 2019; Patel et al. 2020).

Humic substances (HS) represent an important group of plant biostimulants (Canellas et al. 2015; Du Jardin 2015, Du Jardin, Xu, and Geelen 2020). They are part of soil organic matter and are recognized for positively influencing physical, chemical and biological soil properties (Du Jardin 2015; Weil and Brady 2017). HS are mostly used in soil applications, as fertigation or in hydroponic systems. HS are obtained from different raw materials, such as peat, compost, vermicompost or leonardite, the latter a coal-like substance with a high content of humic acids (Canellas et al. 2015). It has been reported that the use of HS can increase crop growth and yield, mainly because of their positive effect on plant nutrient use efficiency (Kaya et al. 2020; Purwanto et al. 2021).

HS positively influence metabolic and signaling pathways involved in plant development (Kołodziej, Sugier, and Bielińska 2013). Nardi et al. (2002) reported evidence that the low molecular fraction is the main candidate to determine the positive effects of HS on plant growth, as it can easily reach the plasmalemma of higher plant cells. Conselvan et al. (2017) found an increase in glutamine synthetase and glutamate synthetase activity and total protein content in 11-day-old maize seedlings treated with HS. Pizzeghello et al. (2020) also reported increased activity of plant development-related enzymes (invertase, peroxidase and esterase), and N (nitrate reductase and glutamine synthetase) and S (O-acetylserine sulfhydrylase) assimilation into amino acids. Barone et al. (2019) found that root morphology traits, such as total root length and number of root tips, of sugar beet seedlings grown in Hoagland's solution were significantly increased in plants treated with a leonardite formulation. They also reported that some genes involved in the hormonal response were significantly upregulated by leonardite treatment. Ertani et al. (2019) reported increased values of RuBisCO, SPAD (Soil Plant Analysis Development) and leaf sugar accumulation of maize plants treated with a commercial leonardite-humate due to enhanced photosynthesis.

Although several other authors have found a positive effect on plant growth and yield with the use of HS (Rose et al. 2014; Sugier, Kołodziej, and Bielińska 2013; Medina Litardo et al. 2022), there is a possibility that HS have a negative impact on plant growth and development (Atiyeh et al. 2002). This negative response may be related to inordinate interactions with essential proteins or due to the presence of entrapped small soil phenols exhibiting plant phytotoxicity (Lee et al. 2019). Thus, it seems to be of great importance to continue to study the response of plants to the application of HS, especially from commercial products, to also establish under which conditions farmers can better take advantage of their use (Arrobas et al. 2022a).

Lettuce is a horticultural crop of great importance worldwide. In Portugal, in 2021, 2690 ha were cultivated with a total production of 75,370 t (FAO (Food and Agriculture Organization of the United Nations) 2023). Lettuce is often cultivated in very intensive farming systems with several annual cycles over the same growing medium. There is also a long tradition of applying organic amendments to horticultural systems as a way of increasing their resilience to cultural intensification (Almeida 2006). Thus, the aim of this study is to compare the effect of two commercial leonardites with a conventional organic amendment resulting from composting forestry, agro-industrial and domestic waste and a non-amended control, in an experimental design in which organic materials were tested both alone and as a supplement to mineral NPK fertilization over two successive growing cycles of potted lettuce. As a working hypothesis, it was stated that commercial leonardites can improve soil fertility, plant nutritional status and/or lettuce DMY, and deserve to be included in lettuce fertilization plans as recommended by the vendor.

Materials and methods

Experimental conditions

The pot experiment was undertaken in Bragança, NE Portugal, a region benefiting from a warm-summer Mediterranean climate (Csb), characterized by dry and warm summers and rainy winters (IPMA (Instituto Português do Mar e da Atmosfera) 2021). The pot experiment was carried out under indoor conditions, in a greenhouse cultivated for commercial purposes. The cover of the greenhouse consisted of a double-wall polycarbonate panel. Aeration and heat dissipation in summer relied on lateral and zenith openings and a reflective screen which automatically slid across when the temperature reached 28 °C. No other environmental variables were measured.

In this experiment, pots of 3 kg of dried and sieved (2 mm mesh) soil were used. The soil was sampled from the arable layer (0-0.20 m) of a fallow plot. The soil is a Regosol of colluvial origin, sandy–clay–loam textured. Soil separates and other soil properties are presented in Table 1.

Experimental design and treatment characterization

The experiment was arranged as a three-factor experimental design. The factors included in the trial were: organic amendment, with four treatments [compost Nutrimais® (Nu), leonardite Humitec® (Ht), leonardite Humic gold® (Hg) and an unfertilized control (Co)]; NPK addition, with two treatments [supplementation with N, P and K (Yes) and control (No)]; and growing

Table 1. Selected properties of the soil ($n = 3$) used in the pot experiment.

Soil properties		Soil properties (cont.)	
[a]Clay (g kg^{-1})	242 ± 18.7	[e]Exchang. K^+ (cmol$^+$ kg^{-1})	0.5 ± 0.12
[a]Silt (g kg^{-1})	217 ± 21.1	[e]Exchang. Na^+ (cmol$^+$ kg^{-1})	1.6 ± 0.13
[a]Sand (g kg^{-1})	542 ± 17.8	[f]Exchang. acidity (cmol$^+$ kg^{-1})	0.0 ± 0.00
[b]Organic carbon (g kg^{-1})	11.7 ± 1.10	[g]CEC (cmol$^+$ kg^{-1})	17.9 ± 0.71
[c]pH (H_2O)	6.8 ± 0.09	[h]Extract. boron (mg kg^{-1})	0.3 ± 0.04
[c]pH (KCl)	5.9 ± 0.07	[i]Extract. copper (mg kg^{-1})	41.1 ± 3.34
[d]Extract P (P_2O_5) (mg kg^{-1})	85.7 ± 19.7	[i]Extract. zinc (mg kg^{-1})	3.5 ± 0.45
[d]Extract. K (K_2O) (mg kg^{-1})	94.0 ± 29.4	[i]Extract. iron (mg kg^{-1})	85.5 ± 2.09
[e]Exchang. Ca^{++} (cmol$^+$ kg^{-1})	11.6 ± 0.62	[i]Extract. manganese (mg kg^{-1})	115.7 ± 9.08
[e]Exchang. Mg^{++} (cmol$^+$ kg^{-1})	4.2 ± 0.34	[j]Electrical conductivity (dS m^{-1})	0.4 ± 0.04

[a]Robinson pipette method; [b]Walkley-Black, wet digestion; [c]Potentiometry; [d]Ammonium lactate; [e]Ammonium acetate, pH 7; [f]Potassium chloride; [g]Cation exchange capacity); [h]Hot water, azomethine-H; [i]Ammonium acetate and EDTA (ethylenediamine-tetraacetic acid); [j]Soil/water, 1:5.

season, with two treatments [lettuce autumn (1st) and spring (2nd) growing cycles]. All treatments were included in triplicate (three replicates).

Nutrimais® is a dehydrated and pelletized product, resulting from composting forestry, agro-industrial and domestic waste, whose main properties are 10.5% humidity, 52.5% organic matter, 29.2% total C, 2.41% total N, 0.65% P, 1.50% K and a $pH_{(H2O)}$ of 8.68. Humitec® contains 23.2% organic C, 30% humic acids, 10% fulvic acids, 2% organic N, 5% sulfur trioxide (SO_3) and 24% silicone dioxide (SiO_2). Humic gold® contains 56% humic acids, 16% fulvic acids and 6.64% K.

The amount of organic amendment applied per pot was estimated taking into account what each individual lettuce would receive in a cultivated field at a planting density of 140,000 plants ha^{-1}. For experimental purposes, an amount corresponding to the upper limit of the range in which manufacturers recommend the application of products was used. This experimental option was based on the fact that it is of great importance to know if the doses recommended by the manufacturers, mainly the highest ones, are effective in the growth of lettuce. Thus, 71.4 g pot^{-1} (10 Mg ha^{-1} of field grown lettuce) of Nutrimais, 3.37 g pot^{-1} (500 kg ha^{-1}) of Humitec, and 0.36 g pot^{-1} (50 kg ha^{-1}) of Humic gold were applied per growing season. NPK addition was performed at a rate of 70 kg ha^{-1} (N, P_2O_5, K_2O) per growing season. Thus each pot received 0.5 g of N, P_2O_5 and K_2O, applied as ammonium nitrate 20.5% N (2.44 g pot^{-1}), superphosphate 18% P_2O_5 (2.78 g pot^{-1}), and potassium chloride 60% K_2O (0.83 g pot^{-1}). The autumn (1st growing cycle) in Bragança (41° 48′ 20′' N) is characterized by a period in which solar radiation, temperature and photoperiod are reduced throughout the crop cycle, while in the spring (2nd growing cycle) the opposite occurs.

Pot experiment installation and management

The pots were filled with 3 kg of dried and sieved soil mixed with 50 g of perlite, to increase aeration and drainage, and the amendments and fertilizers reported in the experimental design.

In the first growing season, lettuces of the cultivar Summer Wonder (Maravilha de Verão, in Portuguese) were sown on September 10, 2019 in 8 cm^3 micropots with a commercial peat-based substrate, and transplanted into pots on October 4, at the phenological growth stage '2nd true leaf unfolded' (Meier 2018). The cut was performed on December 16, 2019, when plants stopped growing, due to reduced light available and low temperatures. In the second growing cycle, lettuces of the same cultivar were sown on March 3, 2020 and transplanted at a similar phenological state on March 30, 2020. The cut was made on June 8, 2020 when the phenological growth stage 'typical size, form and firmness of heads' was reached (growth stage 49, Meier 2018).

After planting, the pots were kept free of weeds by pulling them out by hand and watered when necessary, applying individual doses of 100 ml of water. The daily amount of water used varied according to growth stage, lettuce size and air temperature.

Sampling and laboratory analyses

The lettuces were cut at ground level. Thereafter, they were crushed into small pieces, oven-dried at 65 °C until they reached a constant weight, ground (1 mm mesh) and analyzed for elemental composition. Tissue N concentration was determined in a Kjeltec Auto 8400 analyzer, after sample digestion with sulfuric acid and selenium as a catalyst. For the determination of B, the samples were incinerated in calcium oxide and the ash diluted with sulfuric acid. In the extract, B was determined by colorimetry by the azomethine-H method. For the other nutrients, the samples were digested in nitric acid in a microwave. P was determined by colorimetry, using the blue ammonium molybdate method with ascorbic acid as a reducing agent. The cations (K, Ca, Mg, Cu, Fe, Zn and Mn) were determined by atomic absorption spectrophotometry. For more details on these analytical procedures, the reader is referred to Temminghoff and Houba (2004).

After completing the two lettuce growing cycles, carried out in the same soil, some of its properties were analyzed. First, the soil samples were mixed and oven-dried at 40 °C. Thereafter, the samples were analyzed for pH (H_2O and KCl) (soil: solution, 1:2.5), cation-exchange capacity (ammonium acetate, pH 7.0), organic C (wet digestion, Walkley-Black method) and extractable P and K (Egner-Riehm method). Soil B was extracted by hot-water and determined by the method of azomethine-H. For more details on these analytical procedures, the reader is referred to Van Reeuwijk (2002). The availability of other micronutrients (Cu, Fe, Zn, and Mn) in the soil was determined by atomic absorption spectrometry after extraction with ammonium acetate and EDTA, according to the method described by Lakanen and Erviö (1971).

Data analysis

Data was analyzed for normality and homogeneity of variance using the Shapiro-Wilk and Bartlett's test, respectively. The analysis of variance was performed according to the experimental design firstly as a three-way ANOVA (Field 2017), using the Statistical Package for the Social Sciences (SPSS) version 25 (IBM Corporation, New York, NY, USA). For a more detailed observation of the results, the analysis of variance was also performed separately by season and by NPK addition as a two-way ANOVA (a pot experiment can be arranged in all these different forms). When significant differences were found between experimental treatments, the means were separated by the Tukey HSD test ($\alpha = 0.05$) (only necessary for the factor Organic amendment with four levels).

Results

When the analysis of variance of lettuce DMY included the three factors under investigation (organic amendment, NPK addition and growing season), significant interaction was found between all of the combinations of the three factors. This means that the response to organic amendments depended not only on NPK addition but also on the growing season. For a detailed observation of the results, an interaction term for the three variables is presented as supplemental material in Table S1. The mean values of DMY, arranged per experimental factor are presented in Figure 1. Figure 1a shows the results of the joint analysis of the three variables (three-way ANOVA). Figure 1b and c show the results separated by growing season (two-way ANOVA). In Figure 1d and e the results are presented separately by organic amendments either used alone or supplemented with NPK (two-way ANOVA).

Although significant interaction was found and the response to a given variable was dependent on the others, the strong influence of the NPK addition and the growing season on lettuce DMY is clear from Figure 1a, in contrast to the application of the organic amendments, whose average dry matter yields varied little from each other. When the data was separated by growing season (Figure 1b and c), the strong effect not only of the NPK addition but also of the growing season (comparing Figure 1b and c) stands out again. This analysis also shows that, in the first growing season, organic amendments had a significant effect on DMY, with Nutrimais showing the highest average value. Separating by only organic amendment or by NPK addition, there was found to be a strong effect on the results of the growing season and NPK addition. Moreover, without NPK addition, the organic amendments had a significant effect on the results, with Nutrimais presenting again the higher average value.

In the analysis of the concentration of nutrients in plant tissues, there was often found to be significant interaction between two and sometimes three factors under investigation. For a more detailed analysis of the interaction between the three factors, an interaction term is presented as supplementary material (Table S2). To facilitate an overview of the results, the average values, as the individual effect of each of the treatments, are presented in Table 2.

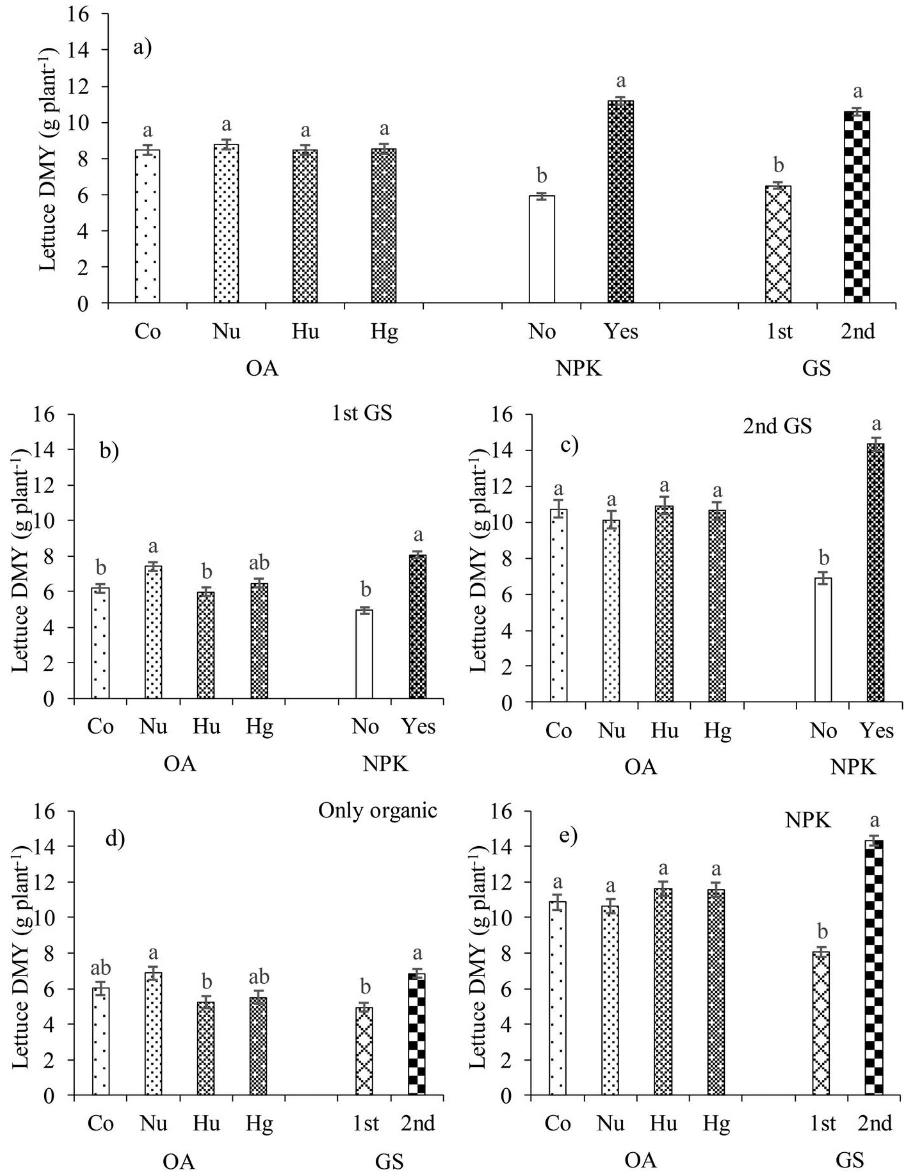

Figure 1. Lettuce dry matter yield (DMY) as a function of organic amendment (OA) (Co, control; Nu, Nutrimais; Hu, Humitec; Hg, Humic gold), nitrogen, phosphorus and potassium (NPK) addition (only organic, and NPK addition) and growing season (GS) (1^{st} and 2^{nd}): a) joint analysis of the three factors; b, c) separated analysis by GS; d, e) separated analysis by only organic or NPK addition. Within each experimental factor, means followed by the same letter are not significantly different ($\alpha < 0.05$). Error bars are the standard errors.

Regarding tissue N concentration, significant interaction between all possible combinations of the factors studied was found (Table S2). Even so, the values were markedly higher in pots receiving NPK in comparison to those receiving only organic amendments (Table 2). In the first growing season, the average tissue N concentration was higher than in the second. In the case of organic amendments, Nutrimais displayed mean values clearly higher than those of the other treatments. However, the combination of factors that gave rise to the highest mean tissue N concentration was Nutrimais, NPK addition and the second growing season (36.3 g kg^{-1}) (Table S2),

Table 2. Tissue nutrient concentration as a function of organic amendment, nitrogen, phosphorus and potassium (NPK) addition (No, Yes) and growing season (GS) (1st and 2nd).

	N	P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
Treatments	g kg^{-1}					mg kg^{-1}				
Organic amendment										
Control	22.1 b	2.7 b	65.3 a	3.1 ab	2.6 a	25.0 a	309.8 b	87.7 a	86.8 a	16.4 a
Nutrimais	26.1 a	3.0 a	69.2 a	3.1 ab	2.6 a	26.1 a	393.8 ab	55.3 b	61.4 b	14.7 a
Humitec	21.9 b	2.6 b	61.5 a	3.0 b	2.4 a	24.4 a	412.4 ab	91.7 a	57.8 b	13.6 a
Humic gold	22.6 b	2.9 ab	65.0 a	3.3 a	2.5 a	25.3 a	482.7 a	93.4 a	66.3 b	16.0 a
Standard error	0.37	0.09	2.43	0.10	0.08	0.64	37.65	5.15	3.23	0.63
NPK addition										
No	16.4 b	2.9 a	59.0 b	3.0 b	2.1 b	26.6 a	463.9 a	56.8 b	58.2 b	14.7 a
Yes	29.9 a	2.8 a	71.5 a	3.3 a	3.0 a	23.9 b	335.4 b	107.2 a	78.0 a	15.6 a
Standard error	0.26	0.06	2.72	0.07	0.06	0.46	26.62	3.64	2.28	0.45
Growing season										
1st	24.4 a	3.2 a	82.0 a	3.2 a	2.8 a	24.5 b	500.3 a	70.8 b	109.4 a	19.7 a
2nd	21.9 b	2.5 b	48.5 b	3.0 b	2.3 b	26.0 a	299.1 b	93.3 a	26.7 b	10.6 b
Standard error	0.26	0.06	2.72	0.07	0.06	0.46	26.62	3.64	2.28	0.45

Note: Within each experimental factor, means followed by the same letter are not significantly different ($\alpha < 0.05$).

Table 3. Nutrient recovery in aboveground biomass as a function of organic amendment, nitrogen, phosphorus and potassium (NPK) addition (No, Yes) and growing season (GS) (1st and 2nd).

	N	P	K	Ca	Mg	B	Fe	Mn	Zn	Cu
Treatments	mg plant^{-1}					μg plan^{-1}				
Organic amendment										
Control	195 b	22 b	513 b	26 ab	23 a	198 b	2273 b	872 a	620 a	123 a
Nutrimais	250 a	27 a	616 a	27 ab	23 a	237 a	3049 ab	496 b	515 b	128 a
Humitec	205 b	21 b	494 b	25 b	22 a	199 b	2766 ab	903 a	442 b	110 a
Humic gold	218 b	24 b	549 ab	29 a	23 a	216 ab	3549 a	935 a	510 b	127 a
Standard error	6.10	0.90	22.88	0.83	0.71	8.37	226.55	37.91	27.09	4.95
NPK addition										
No	95 b	16 b	329 b	17 b	12 b	156 b	2603 b	323 b	299 b	81 b
Yes	339 a	31 a	757 a	37 a	33 a	269 a	3215 a	1271 a	744 a	163 a
Standard error	4.31	0.63	16.18	0.59	0.50	5.92	160.19	26.81	19.16	3.50
Growing season										
1st	166 b	20 b	537 a	21 b	18 b	157 b	3161 a	481 b	724 a	126 a
2nd	267 a	27 a	549 a	33 a	27 a	269 a	2657 b	1123 a	319 b	117 b
Standard error	4.31	0.63	16.18	0.59	0.50	5.92	160.19	26.81	219.16	3.50

Note: Within each experimental factor, means followed by the same letter are not significantly different ($\alpha < 0.05$).

which highlights the greater effect of either organic and mineral fertilization in comparison to the growing season.

For the other nutrients, significant interaction between some factors was also found, but not in a similar way for all nutrients (Table S2). The organic amendments seem to have influenced significantly the concentration of P in plant tissues, with the mean values of Nutrimais being higher than those of the other treatments (Table 2). Tissue Ca levels were higher in the Humic gold than in the Humitec treatment. Fe levels were higher in lettuce treated with Humic gold than in the control. The concentration of Mn was lower in Nutrimais than in the other treatments. Zn average values were the highest in the control. The concentration of K, Mg, B and Cu in plant tissues were not significantly influenced by the organic amendments. NPK addition increased the average tissue concentrations of K, Ca, Mn and Zn and reduced the average values of B and Fe. Average tissue concentrations of P, K, Ca, Mg, Fe, Zn and Cu were higher in the first growing season, while B and Mn concentrations were higher in the second growing season.

Nutrient recovery is the result of the multiplicative effect of DMY by the concentration of nutrients in plant tissues, thus being able to accentuate the differences of the effect of treatments in comparison to the separate analysis of the concentration of the nutrients. Mean values of nutrient recovery in aboveground lettuce biomass are shown in Table 3. In the case of N recovery, significant interaction was found for all factor combinations, as was observed for tissue N concentration. For the other nutrients, the recording of interaction between two and sometimes three of the factors under study was also carried out. Thus, to allow a detailed view of the interactions between variables, an interaction term was prepared and is presented as supplemental material in Table S3.

The effect of NPK addition in increasing N recovery was clear (Table 3). In the second growing season of lettuce, more N was also recovered than in the first, due to the increase in DMY (Figure 1), although tissue N concentration was lower (Table 2). The Nutrimais treatment stood out from the others, with higher average values due to the combined effect of higher DMY and tissue N concentration. The combination of factors that gave rise to the highest average value of N recovery was Nutrimais, with NPK addition in the second growing season (Table S3).

For the other nutrients, significant interaction between the factors under study persisted. However, in a separate analysis of the factors, it was also evident that the P recovered in the tissues followed the same pattern as N, with higher values in the Nutrimais treatment, NPK addition and in the second cycle of lettuce. As for N, the highest average value resulted from the combination Nutrimais, NPK addition and the second growing season.

Regarding K recovery, the Nutrimais treatment showed significantly higher average values than Humitec, Humic gold and control. Higher values were also observed with NPK addition, but differences between the two lettuce cycles were not found (Table 3). The combination of factors that gave rise to the highest average value of K recovery was also Nutrimais, NPK addition and second growing season (Table S3).

Recovered Ca and Mg maintained significantly higher records in the NPK treatment and in the second growing season of lettuce (Table 3). The effect of organic amendments was less evident. The recovered B was higher in the treatments with NPK addition and in the second lettuce cycle, as observed for N, P, Ca and Mn. The Nutrimais treatment showed higher recovered B values than the other treatments. Tissue recovery of Mn and Zn increased significantly with NPK addition while Fe recovery varied in the opposite direction. Regarding the growing season, recovered Mn was significantly higher in the second while Fe, Zn and Cu were higher in the first growing season. The effect of organic amendments was complex and varied with each element.

Average values of soil organic C increased with the application of Nutrimais, as well as soil pH and extractable P (Table 4). The combination of Nutrimais and NPK addition gave the highest average value of organic C (Table S4) in spite of NPK addition having had a global negative impact on soil organic C (Table 4). NPK addition decreased soil pH in general terms and increased soil P. Extractable Mn was significantly lower in the control than in the other treatments and NPK addition decreased the average values of Cu. The bases of exchangeable complex

Table 4. Soil properties as a function of organic amendment and nitrogen, phosphorus and potassium (NPK) addition.

Treatments	Organic carbon g kg^{-1}	pH (H_2O)	Extractable P (P_2O_5) mg kg^{-1}	B	Fe	Zn	Cu	Mn	Exchangeable Ca^{++} cmol$_c$ kg^{-1}	Mg^{++}	K^+	Na^+	CEC
Organic amendment													
Control	14.6 b	6.3 b	167.9 b	0.6 b	184.4 a	6.5 a	82.3 a	239.8 b	12.6 b	4.3 b	0.4 b	1.4 b	18.7 b
Nutrimais	16.5 a	6.7 a	275.1 a	1.1 a	200.5 a	6.6 a	80.3 a	255.8 a	12.9 ab	4.5 a	1.6 a	2.0 a	21.1 a
Humitec	14.3 bc	6.2 b	171.8 b	0.3 b	184.7 a	5.7 a	81.4 a	251.7 a	13.2 a	4.4 ab	0.3 b	1.4 b	19.4 b
Humic gold	13.2 c	6.3 b	179.2 b	0.3 b	193.4 a	6.0 a	82.6 a	259.1 a	13.1 ab	4.5 ab	0.4 b	1.4 b	19.2 b
Standard error	0.31	0.03	6.77	0.10	4.47	0.27	0.76	2.57	0.14	0.05	0.03	0.02	0.19
NPK addition													
No	15.1 a	6.7 a	161.9 b	0.6 a	190.3 a	6.0 a	82.8 a	251.3 a	12.7 b	4.3 b	0.7 a	1.5 a	19.2 b
Yes	14.2 b	6.1 b	235.0 a	0.6 a	191.2 a	6.4 a	80.5 b	251.9 a	13.2 a	4.5 a	0.7 a	1.5 a	20.0 a
Standard error	0.22	0.02	4.79	0.07	3.16	0.19	0.54	1.82	0.10	0.04	0.02	0.01	0.14

Note: Within each experimental factor, means followed by the same letter are not significantly different ($\alpha < 0.05$).

varied significantly with the organic amendments. Nutrimais displayed the higher values of exchangeable complex mainly due to the contribution of K^+ and Na^+. Although some effect on exchangeable complex has found due to NPK addition, the overall effect was less than that of organic amendments (Table S4).

Discussion

The results of DMY showed significant interaction between the factors under study. That is, the response to the application of organic amendments depended on the addition of NPK and on the growing season. The high availability of nutrients in the short term due to NPK applications will have prevailed over the slower and smaller availability of nutrients, which normally occurs after the application of organic amendments (Beegle, Kelling, and Scmitt 2008; Rodrigues, Ladeira, and Arrobas 2018). The different ecological conditions under which the two cycles of lettuce occurred influenced the DMY and may have led to a dilution/concentration effect of nutrients (Afonso, Arrobas, and Rodrigues 2021; Arrobas et al. 2022b) which, associated with the reduced effect of the organic amendments, may have favored the appearance of significant interaction. For instance, the highest DMY (15.06 g pot^{-1}) was found under the combination of Humitec, NPK addition and the second growing season (Table S1), although the lowest average value (3.70 g pot^{-1}) was also associated with Humitec, in the combination without NPK addition and the first lettuce cycle. This reflects the reduced influence of Humitec on the results compared to the NPK addition and growing season.

The average tissue N concentration in the set of pots without NPK addition was 16.4 g kg^{-1}, rising to 29.9 g kg^{-1} in the set of pots that received NPK. Although tissue concentrations of some other nutrients also increased with the addition of NPK (i.e. K, Ca, Mg), the lesser magnitude of the differences seems to make it clear that N supply was the most important factor determining the increase of DMY. This finding becomes even more evident when nutrient recovery is analyzed. In agreement, N is considered one of the most limiting ecological factors for plant productivity in agro-systems, the crop response to N applications being one of the most consistent results found in worldwide research on crop fertilization (i.e. Arrobas et al. 2019; Haberman et al. 2019; Morais et al. 2020; Rodrigues et al. 2020a; Lopes et al. 2021a).

P was also applied in the treatments of NPK addition, but its effect on tissue nutrient concentration appears to have been quite modest compared to N. Although soil P availability is limiting for crop growth and yield in several parts of the world (Tian et al. 2020; Zavaschi et al. 2020; Purwanto et al. 2021; Vistoso, Iraira, and Sandaña 2021), this pattern is not as regular as in the case of N. In soils of similar properties to that used in this study, it has been common to find no response of plants to the application of P as a fertilizer (Arrobas et al. 2017; Ferreira et al. 2018a; Rodrigues et al. 2020b). Thus, in this study, P would not have had a comparable effect to N on DMY.

The application of NPK increased the concentration of K in plant tissues, although the differences between treated and untreated plants have been smaller than in the case of N. K is classified as a macronutrient because plants take up large quantities of K during their life cycle (Weil and Brady 2017). K response studies showing a positive effect on crop yield and/or quality are relatively frequent (Pettigrew 2008; Zhao et al. 2014). However, the response of plants to the use of K is less frequent than to the application of N and P. In soils of similar properties to that used in this study, a weak or nil response of plants to the application of K has been often recorded (Ferreira et al. 2018b; Rodrigues et al. 2020b). Furthermore, analyzing the results of 2,100 K response trials, Khan, Mulvaney, and Ellsworth (2014) concluded that plants rarely respond to K application. Although tissue levels of K were significantly increased in the NPK-treated pots, they were within the established sufficiency range for this species (Bryson et al. 2014) and therefore the effect of K on DMY must have been reduced.

Soil pH decreased with the application of NPK fertilizer. This fertilizer contains part of the N in the ammoniacal form (50%). Nitrification is globally an acidifying process (Weil and Brady 2017). Perhaps this was the cause leading to the decrease of soil pH. Tissue Mn and Zn concentrations increased with the addition of NPK. In acidic soils, metal cations such as Mn, Zn, Fe and Cu tend to solubilize (Broadley et al. 2012), and it was probably the drop in pH that led to increased uptake of Mn and Zn by the plants. In any case, either the drop in pH or the increase in the concentration of Mn and Zn in tissues would not have had a relevant effect on the plants. The soil pH in the pots treated with NPK was still within the range considered adequate for lettuce (Almeida 2006) and the levels of Mn and Zn were in the range of adequate concentrations (Bryson et al. 2014).

The NPK application reduced the content of soil organic C. The addition of mineral N to the soil has often been associated with a stimulus in the mineralization of organic matter by activating the biological activity of the soil. The effect is known as 'priming effect' or 'added N interaction' (Schnier 1994) and has often been observed in recent N fertilization trials (Rodrigues et al. 2015; Lopes et al. 2021a). Given the beneficial effects on soil properties that result from the application of manure, compost or other organic materials (Su et al. 2021; Arrobas et al. 2022b), these practices are always recommended as an alternative or complement to mineral fertilization.

The growing season also had a marked effect on DMY. The first cycle took place in autumn between October 4 to December 16, 2019, which in the region corresponds to low temperatures, reduced light intensity and short photoperiod. These conditions are not the most suitable for the development of lettuce, since they limit maximum photosynthetic rate and available photosynthates (Almeida 2006; Lopes et al. 2021b). The second cycle took place in the spring between March 30 and June 8, 2020. In this second cycle, the plants reached a typical size for commercialization, presenting generically higher DMY than in the first growing cycle. Plants from the second cycle may also have benefited from the residual effect of fertilization from the first cycle, taking into account the deficient growth of lettuce. In the case of organic amendments, in particular Nutrimais, the plants of the second cycle may have benefited from the slow process of nutrient release that occurs from the mineralization of organic substrates (Beegle, Kelling, and Scmitt 2008; Rodrigues, Ladeira, and Arrobas 2018). Thus, even though the tissue concentration of most nutrients was lower in the second cycle of lettuce, due to a dilution effect (Afonso, Arrobas, and Rodrigues 2021; Arrobas et al. 2022b), nutrient recovery was higher in the second cycle (Table 3), due to the higher DMY (Figure 1).

The increase of DMY in the Nutrimais treatment was clear only when the results of pots without NPK addition were analyzed. Nutrimais was associated with an increase in tissue N concentration and, although less evident, also with P. Bearing in mind that N was the element that most increased when the treatments with and without NPK were compared (highlighted above all by the nutrient recovery), it was certainly the increase in N availability that caused the increase of DMY in the Nutrimais treatment. Organic amendments release N to the plants depending on their C/N ratio or N content. Nutrimais has 2.41% N and a C/N ratio of 11.9, values normally associated with net N mineralization in the short term, since the balance between biological immobilization and mineralization is found for N values in the organic substrate between 1.5 and 2.0%, and a C/N ratio between 20 to 30 (Beegle, Kelling, and Scmitt 2008; Weil and Brady 2017). Nutrimais increased also soil organic C, due to the combination of a direct contribution of the added organic substrate and the slow process of mineralization, as reported in other studies (Su et al. 2021; Arrobas et al. 2022b). Nutrimais increased soil pH. Organic matter mineralization releases CO_2 into the soil, producing carbonic acid (H_2CO_3) which can reduce soil pH (Weil and Brady 2017). However, H_2CO_3 is a weak acid with little effect on soil acidification (Pines et al. 2016). Furthermore, the pH of Nutrimais (8.7) was higher than the pH of the soil (6.8), probably due to its high Ca content, this effect prevailing over the acidifying effect of the mineralization of organic matter. The application of Nutrimais increased CEC. When pH increases, more functional groups undergo dissociation of H^+ ions, leaving behind an increasing number of negatively charged sites (Weil and Brady 2017). Nutrimais reduced the concentration of Mn in plant tissues. This result might have been due to the combined effect of increase in pH and aeration, the main factors responsible for Mn insolubilization, making it less available to plants (Broadley et al. 2012; Afonso, Arrobas, and Rodrigues 2020; Arrobas et al. 2022b). These results confirm the benefits to overall soil fertility of using organic amendments.

Leonardites did not show a positive effect on lettuce yield, tissue nutrient concentration or soil properties. Leonardites are marketed for their high content of humic and fulvic acids. Products rich in humic and fulvic acids are currently included in the vast group of plant biostimulants, substances that can benefit plants not so much for their nutrient content as for their effect in protecting against environmental stresses and improving nutrient use efficiency (Conselvan et al. 2017; Du Jardin, Xu, and Geelen 2020; Nasiroleslami et al. 2021). In some previous studies it was shown that the beneficial effect of the use of leonardite was due to better nutrition of P, as humic acids are capable of competing with P to be bound to soil adsorption complexes (Purwanto et al. 2021). According to Canellas et al. (2015) enhanced plant growth in response to HS addition appears to be related to the interactions of HS with plant membrane transporters responsible for nutrient uptake and membrane-associated signal transduction cascades which regulate growth and development. In agreement, some kind of positive effect on plant growth has been previously reported from the use of HS (Salin, Karadogan, and Tonguc 2013; Barone et al. 2019; Ertani et al. 2019; Medina Litardo et al. 2022). Cao et al. (2022) reported also a decrease in NH_3 and N_2O emissions from slurry application with the use of leonardite, the result of a combined effect of increasing N immobilization and reducing N denitrification. Negative effect of HS on plant growth have also been reported (Atiyeh et al. 2002). The negative action of HS would be associated with inordinate interactions with essential proteins (Lee et al. 2019). Another structural feature in terms of this toxicity is the presence of entrapped small soil phenols exhibiting a strong phytotoxicity affecting glycolysis and the pentose phosphate pathway in plants (Lee et al. 2019). According to Du Jardin (2015), the presence of HS in the soil is the result of the interaction between organic matter, microbes and plant roots. Any attempt to use HS for promoting plant growth and crop yield needs to optimize these interactions, and this is the reason why the application of HS – soluble humic and fulvic acids fractions – often shows inconsistent results in plant growth. In this study, the control of environmental variables and minimization of biotic and abiotic stresses, in which only N appeared as a limiting factor, would have led to reduced effects of the application of leonardites. On the other hand, previous studies have emphasized the importance of the dosage used in the biostimulating effect of HS (Nardi et al. 2002; Pizzeghello et al. 2020). We are not aware of the studies carried out by the company that sells these products to establish the doses they are recommending to producers, but under the conditions of this study they were not effective in promoting lettuce growth. Nevertheless, field studies are needed for a more realistic assessment of the potential interest of these materials as plant biostimulants.

Conclusions

The growing season greatly influenced lettuce dry matter yield, as well as the application of mineral NPK fertilizer. Based on lettuce dry matter yield and tissue nutrient concentration, it was possible to highlight N as the main limiting factor in the growing medium. Nutrimais, a compost of low C/N ratio, providing some N also had a positive effect on lettuce dry matter yield, but only when no mineral fertilizer was applied. In addition, Nutrimais increased soil organic C, a direct effect of the addition of organic substrate and its partial mineralization, and soil pH, due to the alkaline nature of the compost (pH, 8.68). Leonardites did not show significant differences in comparison to the control in soil properties, elemental tissue composition and lettuce dry matter yield. This result is attributed to the high control of environmental variables and to the fact that leonardites did not contribute to the supply of N to the plants, which was identified in this study as the main limiting factor for their growth.

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No potential conflict of interest was reported by the authors.

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