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Research article

Biostimulants in organic vegetable nurseries: Study case in lettuce

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Abstract: In order to create better conditions to achieve food safety and sovereignty, agroecology, as a science, looks for solutions for several steps of the technical itineraries of the crops. Crop nutrition and protection are two main crop itinerary components that have been in the center of farmers challenges and consumers concerns, and biopreparations, which have been prepared using natural substances, have been used in agroecological systems, most times based on farmers empirical knowledge. Six biopreparations—purslane vinegar, prickly pear vinegar, orange fermented fruit juice, garlic extract, nettle infusion, and horsetail decoction—were used in this study, for physicochemical analyses and field tests in two different locations (Viseu and Castelo Branco, Portugal) in nurseries of *Lactuca sativa* L. (lettuce), and aimed to validate its results and uses. The nettle infusion presented the best stimulating results for the length of aerial part and the garlic extract presented the best results for the length of root, though there weren't significant differences and effects when compared with the control. The results indicate that the biopreparations used did not exert a biostimulant action in relation to the application of water in lettuce nurseries and that more research is needed to confirm the results observed by farmers.

Keywords: vegetable growth; *Lactuca sativa*; biopreparation; agroecology; sustainability

1. Introduction

Providing food and nutrients to a continuous growing population continues to be a concern for societies [1,3], along with challenges related with the management and conservation of natural resources. Consumer knowledge and awareness of the hazards of intensive farming for the environment and human health, including the presence and spread of zoonoses, soil erosion, deforestation, loss of biodiversity, and the excessive use of chemicals, are causing these worries [2].

It is crucial to support food production with sustainable agricultural methods and cutting-edge technologies supported in scientific research, and to ensure the production of enough quality food while preserving nature.

The Food and Agriculture Organization of the United Nations (FAO) defines agroecology as a comprehensive strategy that integrates ecological and social concepts and principles into the creation and administration of sustainable agriculture. In addition to address the need for socially equitable food systems where people can have a say in what they eat, and how and where it is produced, it attempts to maximize interactions between plants, animals, humans, and the environment [4]. Longer crop rotations, organic cover crops, polycultures, green manures, integrating crop and livestock, low tillage, and integrated pest and pollinator management are all employed in agroecological systems to enhance soil health, control pests and diseases, and foster biodiversity [5].

In agroecological systems, the use of biopreparations emerges as one key element in the nutrition and protection strategies. The interest on the use of biopreparations is increasing, and the studies of different products, substances and uses are emerging in the literature [6–8].

Biopreparations are mixtures of natural products (from animals, plants, or algae) that combine different beneficial properties, such as inhibiting harmful agents, protecting against insects and diseases, promoting plant growth, triggering photosynthesis, enhancing nutrient and water uptake, activating plant's defense systems, increasing stress tolerance, and increasing yield [8–10]. These mixtures may function as biostimulants, insecticides, fungicides, acaricides, and fertilizers.

Although the usage of these biostimulants is more significant in horticulture, they are employed in several crops and industries, including ornamental horticulture, viticulture, olive culture, and fruticulture. There are currently records of use and studies in nursery crops such as tomatoes (*Solanum lycopersicum* L.), eggplants (*Solanum melongena* L.), beans (*Phaseolus vulgaris* L.), lettuce (*Lactuca sativa* L.), peppers (*Capsicum annuum* L.), garden cress (*Lepidium sativum* L.), strawberries (*Fragaria X ananassa* (Duchesne ex Weston) Duchesne ex Rozier), garlic (*Allium sativum* L.), and basil (*Ocimum basilicum* L.), as well as in outdoor crops including pears (*Pyrus communis* L.), olives (*Olea europaea* L.), and vines (*Vitis vinifera* L.) [11,12].

The objectives of this study are the evaluation of the biostimulant effect of different biopreparations on field tests in nurseries of *Lactuca sativa* L. (lettuce) and a physicochemical analysis to understand the possible relations between the chemical composition and the effects. For this study, six biopreparations were used—purslane vinegar, prickly pear vinegar, orange fermented fruit juice, garlic extract, nettle infusion, and horsetail decoction—each according to a specific recipe used by Portuguese agroecological farmers. This information was gathered from a survey of these farmers in the Center of Portugal through face-to-face interviews about the use and application of agroecological

biopreparations that were used and prepared by the farmers on their farms. The results of this research will allow for the provision of technical information to be used in agroecological production systems and the identification of hints for future research projects.

2. Materials and methods

2.1. Location of the experiment

The experiment was carried out in two locations: in the greenhouse of the Biotech Plant Lab of Beira Interior, the Soil and Fertility Lab, and the Biology Lab of the Agrarian School of Polytechnic Institute of Castelo Branco (coordinates: 39.819397º N, 7.453881º W—Figure 1A), and in greenhouse of the Agrarian School of Polytechnic Institute of Viseu (coordinates: 40°38'27.88" N; 7°54'40.15" W—Figure 1B).

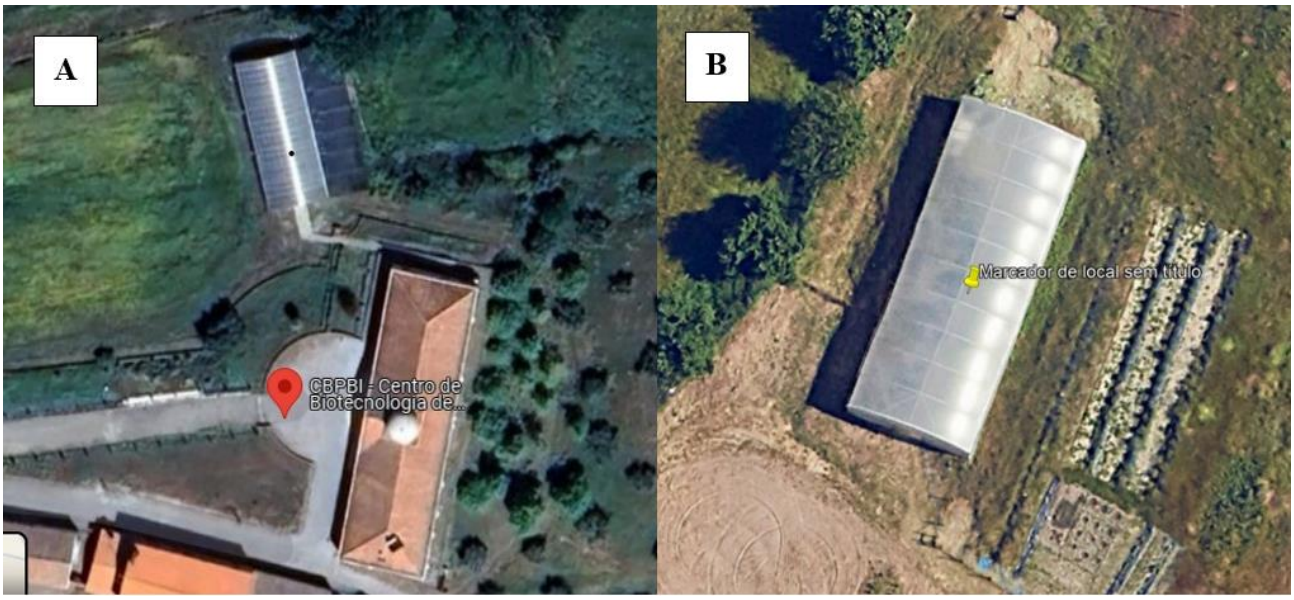

Figure 1. Localização dos ensaios in vivo do projeto: A—Castelo Branco; B—Viseu.

2.2. Biopreparations

For this study, six biopreparations (prickly pear vinegar (PPV), purslane vinegar (PV), fermented fruit juice of orange (FJO), garlic extract (GE), nettle infusion (NI), and horsetail decoction (HD)) and one control (water-CW) were used. The biopreparations were prepared at the Agrarian School of Viseu, from November 2022 to February 2023, following the recipes described within Table 1.

Table 1. Protocol for the biopreparations elaboration.

Biopreparation	Material	Preparation method	Preparation time	Dilution
Prickly pear vinegar (PPV)	1 kg Prickly pear 1 kg Brown sugar 3 L Water for each kg of leftover material	Put the prickly pear and sugar to ferment for 15 to 20 days. Strain. Add water to the remaining solid, (3L:1Kg). Let it ferment for 90 days.	15 + 90 days	1:16
Purslane Vinegar (PV)	1 kg Purslane 1 kg Brown sugar 3 L Water for each kg of leftover material	Put the purslane and sugar to ferment for 15 to 20 days. Strain. Add water to the remaining solid (3L:1Kg). Let it ferment for 90 days.	15 + 90 days	1:16
Fermented fruit juice of orange (FJO)	1 kg Oranges 1kg Brown sugar	Cut the oranges with the peel into pieces and layer them with the sugar for 7 days.	7 days	1:1000
Garlic extract (GE)	110 g unpeeled garlic 2 spoonfuls of colza or rapessed oil 1 spoonful of potassium soap 1 L water	Chop the unpeeled garlic and add it to the oil and leave to macerate for 24 hours. Filter. Add a spoonful of potassium soap and mix everything. Add 1 L of water. Filter.	24 h + 21 days in a dark and cold room	1:20
Nettle infusion (NI)	250 g dry leaves 10 L Water	Put water in a pan with the leaves, without letting it boil.	30 min.	1:20
Horsetail decoction (HD)	1 kg Horsetail 10 L Water	Place in water in a pan for 24 hours. Then boil for 20 minutes.	24 h + 29 min	1:5

For the *in vivo* field trial application, the respective dilutions were prepared and applied weekly, three times a week 375 mL per tray, at a total volume of 1.5L.

2.3. Physicochemical characterization of the biopreparations

All the biopreparations—PPV, PV, FJO, GE, NI and HD—were analyzed immediately after the production (time 0) and were performed in triplicate. The total solids content, protein content, ashes content, and micro- and macroelement contents were analyzed.

2.3.1. Proximate chemical composition

A proximate analysis of the biopreparations—PPV, PV, FJO, GE, NI and HD—was performed in triplicate following the Association of Official Agricultural Chemists (AOAC) (1997) procedures. The total solids (TS) content is a measure of the amount of material remaining after all the water has been evaporated and was determined according the AOAC Official Method 920.193 (AOAC, 1997). Either 50 mL or 10 ml of the sample (in the case of FJO sample) was evaporated at 105 °C for 2–3 h. The remaining solid after evaporation was weighed and used to calculate the TS content. The total ash content was determined according to AOAC Official Methos 942.05 (AOAC, 1997). Briefly, 10 mL of each sample was weighed and placed in a muffle furnace at 550 °C for more than 3 hours until a white to grey ash was obtained. The crude protein content was determined using the Kjeldahl method

(AOAC Official Method 973.48) (AOAC, 1997). Each kjeldahl tube was prepared with either 5 or 10 mL of sample (depending of the biopreparation), one tablet of catalyst (0.1% Se), and 12 ml of H_2SO_4, followed by digestion at 150 °C for 2–3 hours until the solution remained clear; after digestion, the digestate was neutralized by the addition of NaOH 40%, which converts the ammonium sulphate to ammonia; this was distilled off and collected in a receiving flask of excess boric acid forming ammonium borate, which was titrated with a HCl 0.1 mol/L standard solution with the use of a suitable end-point indicator to estimate the total nitrogen content of the sample. Following the determination of the total nitrogen, a factor of 6.25 was used to convert the measured nitrogen content to the crude protein content (AOAC, 1997).

2.3.2. Macro, micro and trace elements

The macroelements (calcium (Ca^{2+}), potassium (K^{+}), magnesium (Mg^{2+}), sodium (Na^{+}), and sulfur (S^{2-})) in the biopreparations were analyzed using Ion Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (EPA 6010D method), and phosphorus (P^{3-}) was determined using UV/Vis spectrophotometry by the vanadomolybdo phosphoric acid colorimetric method at 400 nm.

The microelements (copper (Cu^{2+}), iron (Fe^{3+}), manganese (Mn^{2+}), and zinc (Zn^{2+})) and the trace elements (cadmium (Cd^{2+}), lead (Pb^{2+}), and selenium (Se^{2-})) in the biopreparations were analyzed using ICP-OES (EPA 6010D method).

2.4. Installation and monitoring of the in vivo field trials

The *in vivo* field trials were prepared with the "Wonder of four seasons" lettuce variety from the company Sementes Vivas. Lettuce is one of the most produced crops in the world, with China and the United States of America as the world's largest importer and exporter in 2023, particularly for fresh consumption [13]. Lettuce is an annual plant with various plant polymorphisms and a growing cycle that can vary between 6 and 12 weeks, divided into the following: germination, emergence, leaf rosette formation, and cabbage formation [14]. The vegetative cycle also includes the following phases: spiking, flowering, and the maturation of the achenes [14].

The experiment was installed in 28 comb trays of 40 cells, filled with a mixture of a commercial biological substrate and perlite (2:1). Each cell received an organic lettuce seed. A total of 1120 seeds were sown on March 1, 2023, which equates to 160 seeds per modality (six biopreparations and control). On the experimental workbench, a thermohygrometer was used to monitor and control the temperature and humidity.

During the experiment's monitoring, spray irrigation was performed three times a week until the presence of the fifth leaf.

The plants were removed from the substrates after 72 days of the trial. Physical examinations of the plants were also conducted, which included measuring the lengths of the aerial portion of the plants, the roots of the plants, and the lengths of the fifth enlarged leaf.

2.5. Evaluations of biomass production

The lettuce seedlings were cut with a knife at the stem at the soil line. The aerial part of fifteen fresh seedlings from each trial in three repetitions, which is the same as those considered in the physical

analysis, was weighed on a precision XB 220A balance. The aerial part of fifteen fresh seedlings from each trial—PPV, PV, FJO, GE, NI, and HD—and the control with water (CW) were isolated, and the weights were recorded. Then, they were dried in an oven for 48 hours at a temperature of 60 °C. The weights were taken on the same scales and recorded.

2.6. Statistical analysis

Data from the *in vivo* assays of the different varieties were analyzed using CANOCO 5.0. Normality tests were performed using the Kolmogorov-Smirnova Test. A homogeneity test was performed using a One-Way Analysis of Variance (ANOVA) and mean comparison tests were performed to understand the possible significant differences between the means using the least significant difference (LSD) tests to measure the homogeneous variances and Tamhane for the inhomogeneous variances. For results that did not respond to normality, the Kruskal-Wallis non-parametric test was performed. All tests were performed with the Statistical Package for the Social Science (SPSS, 29.0.1.0).

3. Results

3.1. Physicochemical composition of the biopreparations

The TS, ash, and protein contents of the biopreparations at time zero are shown in Table 2.

Table 2. Chemical composition from biopreparations at time zero.

Parameter	Biopreparations					
(g/L)	PPV	PV	FJO	GE	NI	HD
TS	65.1 ± 0.2	60.3 ± 0.2	707 ± 3	6.28±0.009	3.37 ± 0.06	3.48 ± 0.07
Ash	3.21 ± 0.04	2.60 ± 0.07	2.74 ± 0.11	0.72 ± 0.011	1.24 ± 0.02	0.92 ± 0.018
Protein	0.72 ± 0.01	0.91± 0.009	1.78 ± 0.02	1.28 ± 0.02	0.8 ± 0.025	0.238 ± 0.008

TS: Total solids; PPV: Prickly Pear Vinegar; PV: Purslane vinegar; FJO: Fermented Joice of orange; GE: Garlic extract; NI: Nettle infusion; HD: Horsetail decoction.

A high content of TS was found for the FJO sample. Comparatively, the PPV and PV samples presented more TS with the lowest content of GE, NI, and HD.

The ash content is a measure of the inorganic constituents of the biological samples after ignition and complete oxidation of the organic matter. This includes minerals, mainly calcium, magnesium, sodium, and potassium; however, there can also be traces of manganese, zinc, iron, and others in smaller quantities. A higher ash content was found for the PPV, FJO, and PV samples. The protein content was higher for the FJO and GE samples.

Table 3 presents the results obtained for the macro, micro and trace elements in all the 6 biopreparations after their preparation - time zero. The PPV sample presented the highest level of K^{+}, S^{2-}, Cu^{2+}, Mn^{2+}, and Zn^{2+}. The PV sample presented the highest level of P^{3-}, Mg^{2+}, and Fe^{2+}.

Regarding the potassium and phosphorus levels, the PV and PPV biopreparations presented high values of potassium, while the highest phosphorus values were obtained for the PV, FJE, and GE biopreparations. The NI sample contained the lowest phosphorus values, which were approximately

8.5 times lower than the PV sample (Table 3).

Regarding the calcium and magnesium levels, the PPV sample contained the highest concentrations; the PV sample contained the highest concentration of magnesium (Table 3). The sulfur content varied between 19 and 60 mg/L for all the biopreparations; moreover, the sulfur content was more accentuated (60 mg/L) for the GE sample. The PV sample contained a higher iron content (32 mg/L).

Table 3. Chemical composition from biopreparations at time zero.

	Parameter (mg/L)													
	N	K^+	P^{3-}	S^{2-}	Ca^{2+}	Mg^{2+}	Na^+	Cu^{2+}	Fe^{3+}	Mn^{2+}	Zn^{2+}	Cd^{2+}	Pb^{2+}	Se^{2-}
PPV	115	1070	31	32.6	355	139	19.1	0.1	1.28	4.36	1.26	<0.001	<0.005	<0.01
PV	146	962	122	18.6	16.1	157	15	0.069	32	2.25	0.43	<0.001	0.014	<0.01
FJO	285	565	66.5	28.2	392	46.1	26.4	0.024	0.25	0.1	0.25	<0.001	<0.005	<0.01
GE	205	305	54.7	55.9	23.3	8.49	13.3	0.057	0.39	0.12	0.42	<0.001	<0.005	<0.01
NI	128	612	14.4	33.7	94.5	13.6	14.5	0.098	0.57	0.09	0.27	<0.001	0.005	<0.01
HD	38	369	22.4	36	39.7	10.2	10.1	0.04	0.14	<0.01	0.09	<0.001	<0.005	<0.01

PPV: Prickly pear vinegar; PV: Purslane vinegar; FJO: Fermented Joice of Orange; GE: Garlic extract; NI: Nettle infusion; HD: Horsetail decoction; N: nitrogen; K^+: Potassium; P^{3-}: Phosphor; S^{2-}: Sulphur; Ca^{2+}: Calcium; Mg^{2+}: Magnesium; Na^+: Sodium; Cu^{2+}: Copper; Fe^{3+}: Iron; Mn^{2+}: Manganese; Zn^{2+}: Zinc; Cd^{2+}: Cadmium; Pb^{2+}: Lead; Se^{2-}: Selenium.

3.2. In vivo field trials

Regarding the results of the *in vivo* field trial presented in Figure 2A, it can be observed that, in Viseu, the NI and HD biopreparations obtained the best results for the average length of the lettuce aerial part, while the GE biopreparation obtained the best result in Castelo Branco (Figure 2B). The PPV biopreparation, followed by PV, led to the lowest growth of aerial parts of plants in Viseu. However, in Viseu, the action of the GE biopreparation led to an increased length of the root and the expanded fifth leaf. The shortest plants were obtained by the HD biopreparation for the fifth expanded leaf, and by the PPV biopreparation for the root length. Through a statistical analysis, significant differences were observed for the root length between the PPV and GE samples; moreover, some significant differences were observed for the lengths of the aerial part and the expanded fifth leaf.

In terms of the dry weight (Figure 3), despite being the biopreparation with the highest dry biomass, the NI biopreparation did not present significant differences compared to the HD, FJO, and CW samples from Viseu (Figure 3A). In Castelo Branco (Figure 3B), it was observed that the greatest length of the aerial part was due to the action of the NI biopreparation and the smallest due to the PPV biopreparation, which contrasts with the average length of the fifth expanded leaf, in which precisely the opposite happens. Here, the differences were more significant in the biopreparations with better results for the control. Regarding root length, there were no significant differences between the different biopreparations and the control, although the longest roots were obtained in the plants treated with the FJO and GE biopreparations and the shortest with the CW. In terms of biomass, the GE and CW samples were the best. We can indicate that the biopreparations with the best results in all categories (in Viseu and Castelo Branco), except for the fresh weight of the aerial part, in Viseu, did not present significant differences compared to the CW sample.

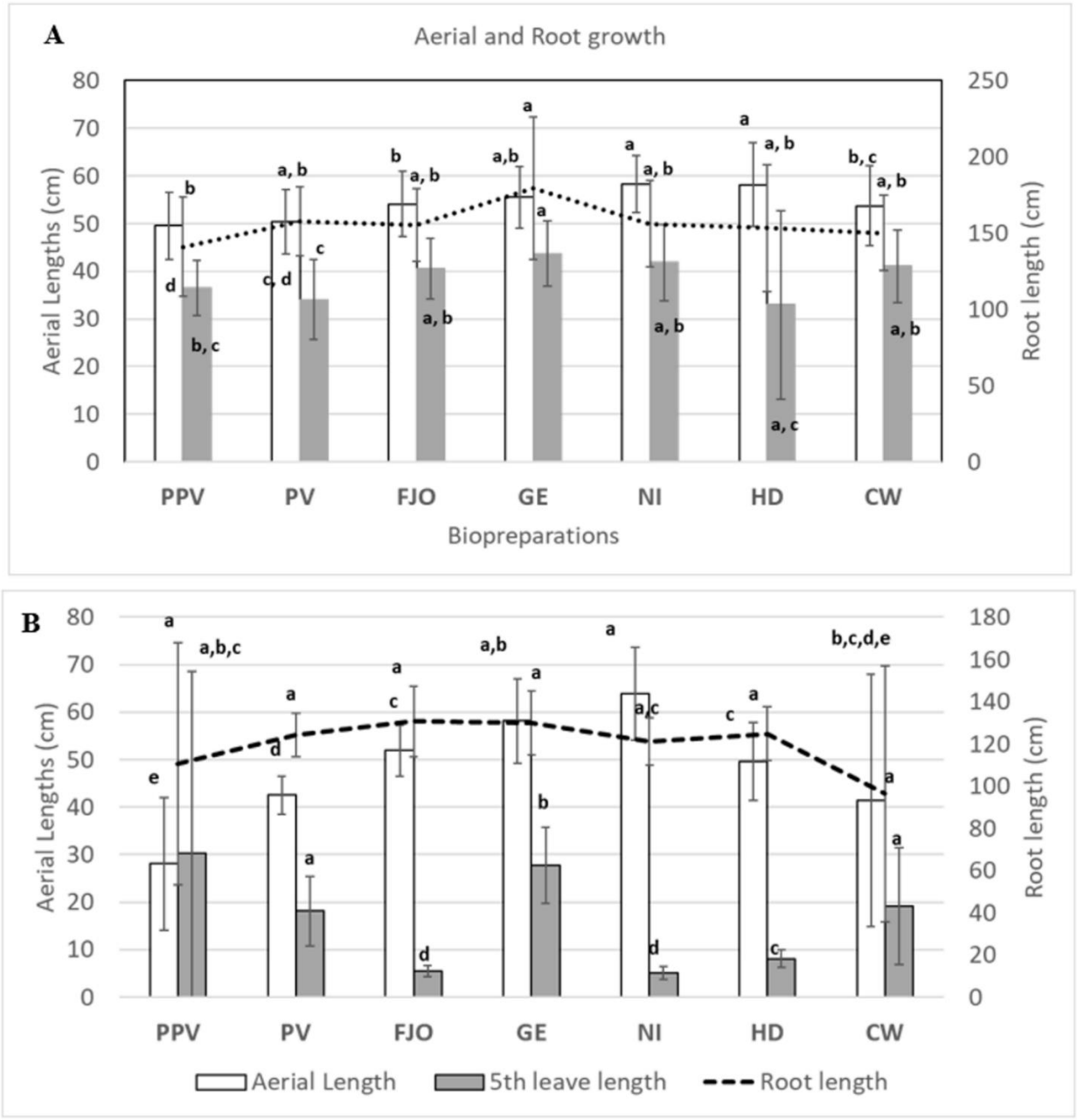

Figure 2. Lettuce's growth, in Viseu (A) and in Castelo Branco (B). *Different letters correspond to significant differences between data. PPV: Prickly pear vinegar; PV: Purslane vinegar; FJO: Fermented Joice of Orange; GE: Garlic extract; NI: Nettle infusion; HD: Horsetail decoction; CW: Control with water.

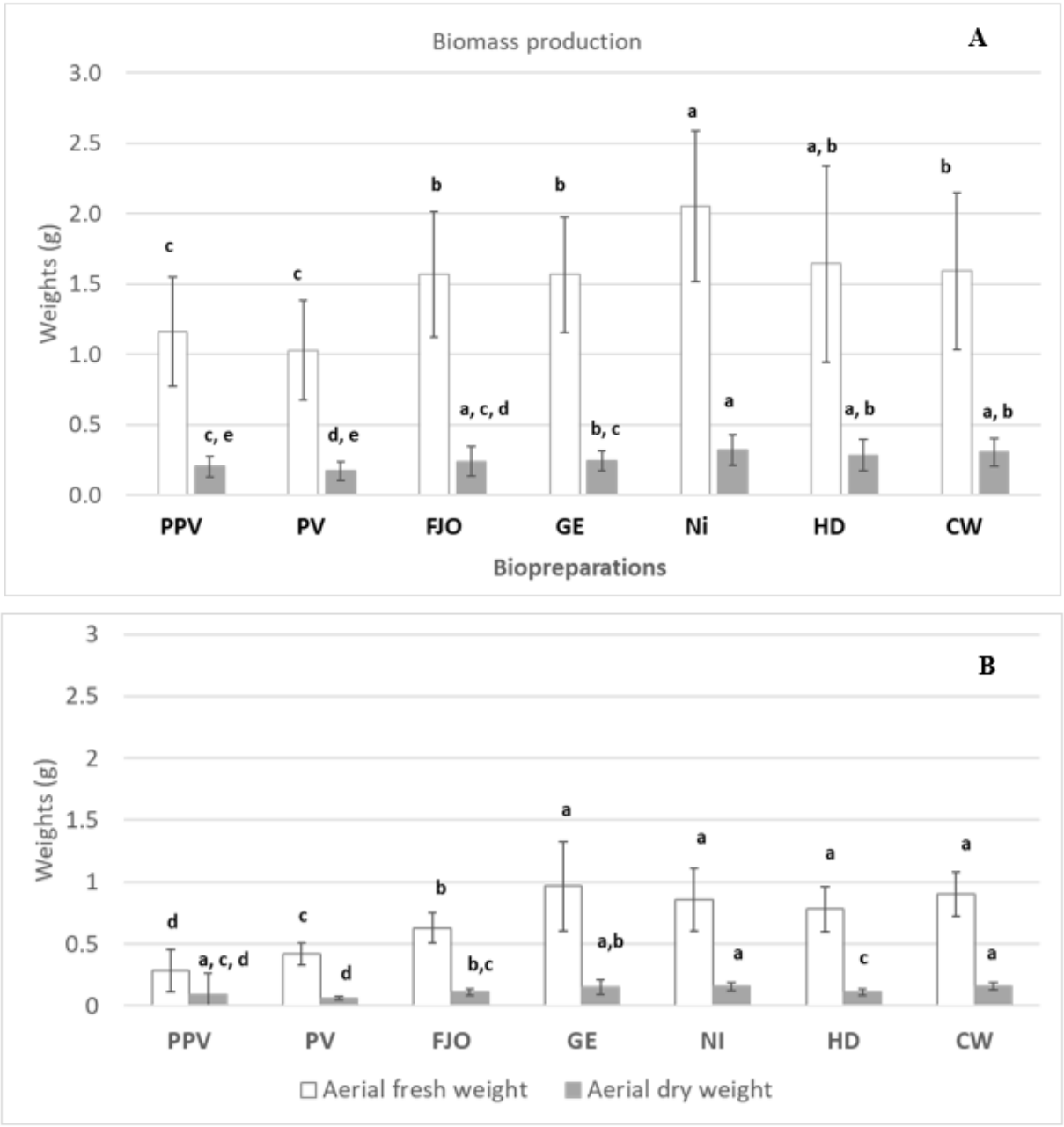

Figure 3. Lettuce's growth, in Viseu (A) and Castelo Branco (B). *Different letters correspond to significant differences between data. PPV: Prickly pear vinegar; PV: Purslane vinegar; FJO: Fermented Joice of Orange; GE: Garlic extract; NI: Nettle infusion; HD: Horsetail decoction; CW: Control with water.

Joining all results obtained for the lettuce's growth and the biomass production in each studied local, considering all biopreparations (Figure 4), we verified that the results of the lettuce's development were superior in Viseu compared to the field tests obtained from Castelo Branco. However, the percentage of water loss in the different locations was relatively similar: 50% in Viseu and 56% in Castelo Branco.

Figure 5 shows the average results obtained in all field trials at the 2 locations with regard to the lettuce growth (Figure 5A) and the biomass production (Figure 5B). Thus, we can verify that the NI biopreparation presented the best results in the aerial part length and biomass, the PPV and GE biopreparations presented the best results in the length of the fifth expanded leaf, and the GE biopreparation presented the best results in the root length. The worst results were obtained by the HD biopreparation for the fifth expanded leaf and for the root length, and by the PPV biopreparation for the shoot length. In biomass, Figure 5B shows that only the PPV and PV biopreparations presented lower results and with significant differences in relation to the control.

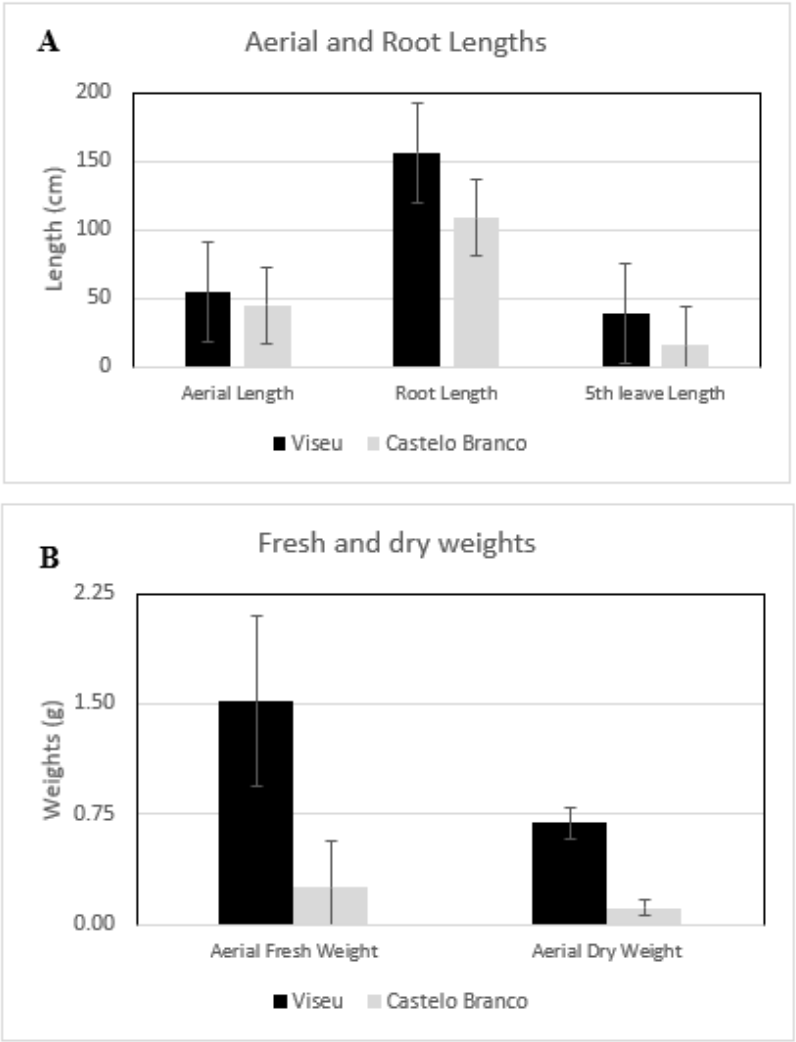

Figure 4. Average results observed for all biopreparations, for each local studied, of lettuce's growth (A) and biomass production (B).

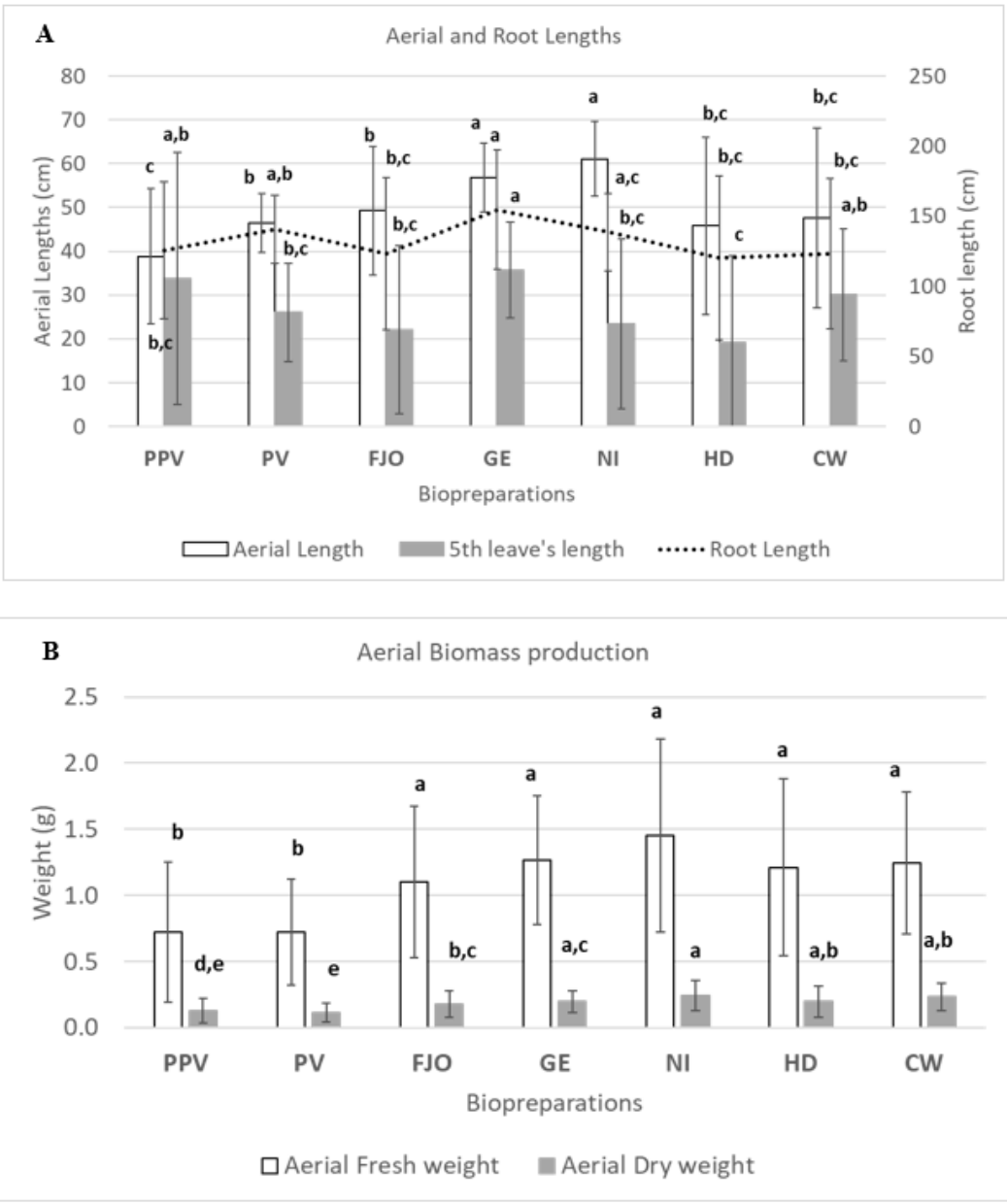

Figure 5. Mean values and respective standard deviation obtained, considering both local studied, of lettuce's growth (A) and biomass production (B). *Different letters correspond to significant differences between data. PPV: Prickly pear vinegar; PV: Purslane vinegar; FJO: Fermented Joice of Orange; GE: Garlic extract; NI: Nettle infusion; HD: Horsetail decoction; CW: Control with water.

4. Discussion

4.1. Physicochemical composition of the biopreparations

A high ash content is indicative of a high content in minerals, which can be important for plant growth. In fact, by analyzing the results obtained for the macro, microelements, and trace metals in Table 3,

the samples with the highest ash content were also those with the highest mineral content (i.e., PV, PPV, and FJO).

The development of the lettuce crop depends on critical nutrients, particularly nitrogen, which promotes vegetative growth, root system expansion, and an increase in the leaf area [14,15]. Although phosphorus is a necessary component for lettuce growth, it is particularly useful to expand the root system since this enables the plant to absorb more water and nutrients. This vitamin also affects respiration, the synthesis of energy, and cell division. Potassium promotes root system growth by increasing the tissue stiffness and plant resilience to pathogens and pests. Calcium is a necessary element since it is vital to maintain the integrity of cell membranes and regulates how other elements are absorbed (particularly nitrogen). Calcium encourages cell division and development. Sulfur is primarily present in the nitrogen-associated protein composition; it contributes to the creation of organic substances, particularly vitamins and enzymes, and some amino acids necessary for energy metabolism [15,16].

The analyses showed that all biopreparations were a good source of nitrogen, with values between 115 and 285 mg/L, except the HD biopreparation, where the nitrogen value was very low when compared with the other biopreparations (38 mg/L).

Potassium, phosphorus, calcium, and magnesium are primary macroelements essential for plant growth and a good overall state of the plant. However, this information did not coincide with the observed biostimulant effect for the PV, PPV, and FJO biopreparations.

The lettuce export values for nitrogen, potassium, and phosphorus were 368.9 mg/lettuce, 890.8 mg/lettuce, and 33.3 mg/lettuce, respectively, at harvest, which can take place between six and 12 weeks, depending on the variety, the time of year, and how the lettuce is produced [15]. The only biopreparation that managed to provide the necessary amount of phosphorus over ten weeks was the PV sample, with 34,313 mg/L of total phosphorus applied in ten weeks. No biopreparation provided the necessary amount of nitrogen and potassium in the quantities provided in the in vivo trial. In fact, in order to reach the export levels for these nutrients, all the biopreparations would have needed to be applied in greater quantities, either per irrigation or more frequently than three times a week. We judged that the FJO biopreparation would be the best to apply at a frequency around or greater than five times, although the phosphorus levels would be greatly increased, by around double, because it would be the one biopreparation that would be able to best meet the export needs, without much damage to nitrogen and potassium with this application.

Among the six biopreparations studied, the PV, PPV, and FJO biopreparations seemed to be the most suitable to use as biostimulants since they had higher contents of macronutrients. Furthermore, the results indicated that the GE, HD, and NI biopreparations could also be used as biostimulants under different conditions and cultures, as they were also rich in macro and micronutrients. In fact, considering the preparation time of each biopreparation, which were 30 min and 1 day for the NI and HD biopreparations, respectively, 3.5 months for the PV and PPV samples, and 21 days for the GE sample (information not shown), the seasonality of the plants used, and the crops to be applied, it is up to the farmer to make the best possible choice, both at an economic and practical level.

4.2. In vivo trial

The biopreparations with the best results did not present significant differences compared to the CW sample, which could mean that it may not make sense at this stage of development to use biopreparations for the development of seedlings in a nursery, as the use of water seemed to have

similar effects.

The PPV and PV biopreparations presented lower results and with significant differences in relation to the control, which may indicate that they do not exert a beneficial action in relation to the application of water in the culture development in the nursery.

5. Conclusions

This trial showed that the amount of each applied biopreparation was insufficient to achieve the amount of nitrogen, potassium, and phosphorus nutrients that the lettuce extracted during its development. The FJO biopreparation was the best applied in order to meet the needs without over-applying nitrogen or potassium, despite applying almost twice as much phosphorus with an application around five times greater than was applied.

Even though the study's findings are relevant, it's critical to continue conducting similar research to validate and organize knowledge for more sustainable agriculture. This includes conducting trials with lettuce from sowing to harvest and conducting more repetitions of all trials, including repetitions with other biopreparations and biopreparations with a single ingredient.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

All the authors declare that they have no conflict of interest.

Authors contributions

The authors confirm their contribution to the paper as follows: Study conception and design: Daniela V. T. A. Costa, Helena E. Correia, António Pinto, Dulcineia Wessel, João P. Carneiro, Carmo Horta, Kiril Bahcevandziev, Maria M. B. Vidal, Olga M. S. Filipe, Cristina Amaro da Costa; data collection: Joana Simões, Carolina Marmota, Lisa Moreira, Daniela V. T. A. Costa, Fernanda Delgado, Olga M. S. Filipe, Cristina Amaro da Costa; analysis and interpretation of results: Joana Simões, Carolina Marmota, Lisa Moreira, Daniela V. T. A. Costa, Fernanda Delgado, Olga M. S. Filipe, Cristina Amaro da Costa; draft manuscript preparation: Joana Simões, Carolina Marmota, Lisa Moreira. All authors reviewed the results and approved the final version of the manuscript.

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