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Application of a Protein Hydrolysate-Based Biostimulant Obtained from Slaughterhouse Sludge on Pepper Crops

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Abstract: Currently, biostimulants obtained from protein hydrolysates are considered essential agronomic tools to promote sustainable agriculture without the use of chemical fertilizers. This study aimed to investigate the effectiveness of a biostimulant obtained from slaughterhouse sludge via enzymatic hydrolysis processes on green pepper crops in a greenhouse. The biostimulant was administered through both root and foliar applications at two different doses (0.7 and 1.4 g L^{-1}), with a total of four applications made over the 140-day experimental period. Throughout the crop growth period, various parameters were assessed, including plant height, the number of flowers and fruits, macro- and micronutrient content, and photosynthetic pigments in the leaves. Additionally, the nutritional content and vitamin C levels in the harvested fruits were determined. The results obtained indicated higher values of these parameters in the pepper plants when the biostimulant was applied at a higher dose and through root application. These higher values are likely a consequence of the increased plant absorption of the low-molecular-weight amino acids and nutrients derived from the biostimulant.

Keywords: biostimulant; plant mineral nutrition; photosynthetic pigments; fruit quality; vitamin C; production

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1. Introduction

In the coming years, the demand for food is expected to increase significantly due to the continuous growth of the world population [1,2].

To address the growing demand for food, chemical fertilizers play an essential role in conventional agriculture as they improve crop yields, enabling profitable agriculture on soils with low chemical fertility [3]. However, the application of chemical fertilizers is considered an inefficient strategy due to several limitations and associated problems. These issues arise from both agricultural practices and techniques, as well as the quantities applied. When chemical fertilizers are solubilized in the soil, only part of the nutrients is consumed by plants, and the rest is lost to the environment through leaching, volatilization, degradation, or immobilization, causing environmental, economic, and health problems [3–5].

Consequently, one of the most important contemporary challenges for agriculture center around producing enough food to meet the needs of the world's growing population while simultaneously reducing the damage caused in food production [1,5].

Currently, biostimulants are being proposed as promising, safe, effective, and meaningful alternatives to tackle sustainability challenges in agriculture while also ensuring high yields and quality in agricultural products [5–7].

The most recent piece of European Union legislation, Regulation (EU, 2019/1009), defines a biostimulant as a product capable of promoting nutrient utilization efficiency, abiotic stress tolerance, the morphological and nutritional characteristics of a plant, and soil or rhizosphere qualities [8].

Biostimulants obtained via enzymatic hydrolysis are a category of biostimulants composed of mixtures of polypeptides, oligopeptides, and amino acids manufactured from organic residues with high protein contents through the use of one or more enzymes [8]. The use of these biostimulants in various crops has shown a positive effect by increasing the productivity and quality of harvested fruits or grains [5,7,9,10].

According to Ahmad et al. [11], the positive effects of biostimulants composed of a mixture of amino acids and peptides are attributed to their role in the biosynthesis of non-protein nitrogenous compounds (such as purines and pyrimidine bases, coenzymes, vitamins, and pigments). This impact influences the mineral nutrition of plants and, consequently, their growth and development.

Animal waste from slaughterhouses is characterized by a high protein content [12]. Several authors have obtained various biostimulants with high protein contents through enzymatic hydrolysis processes involving slaughterhouse sludge and applied them in agriculture. In a study conducted by Pérez-Aguilar et al. [13], a biostimulant was obtained from poultry slaughterhouse sludge and applied to Chinese cabbage and lettuce seeds, resulting in a significant increase in seed germination. Ávila-Pozo et al. [5] obtained a biostimulant from slaughterhouse sludge via enzymatic hydrolysis and applied it to a tomato crop through foliar and root routes. The findings of this study showed significant enhancements in plant morphological parameters, as well as improvements in crop production and nutritional quality. Therefore, the use of this type of biostimulant, i.e., those with high peptide (mainly of low-molecular-weight peptides) and macro- and micronutrients contents, could be a good alternative to the use of chemical fertilizers in crops.

Green pepper (*Capsicum anuum*, L.) is a crop of significant economic importance, particularly in the Mediterranean region, thanks to its highly nutritious fruits rich in antioxidant compounds like phenolic agents, vitamins C and E, and carotenoids [14,15]. These compounds hold considerable value for human health, as they play a pivotal role in preventing specific diseases, including cancer, cardiovascular diseases, and cerebrovascular diseases, when consumed regularly in adequate amounts [16,17].

Although the response of tomato crops to this type of biostimulant (i.e., a biostimulant obtained from slaughterhouse sludge via enzymatic hydrolysis) has been documented [5], there is currently no evidence of its use in pepper crops. We hypothesize that the application of this type of biostimulant can improve the morphological and photosynthetic parameters of the pepper plant and, consequently, the harvest and nutritional quality of the fruit.

The aim of this study was to investigate the response of this type of biostimulant in a pepper crop when applied through root and foliar applications. Hence, this study seeks to enhance our understanding of the use and effectiveness of this biostimulant on pepper crops, focusing on determining the optimal dosage and application method. This aspect of our study represents a significant, novel contribution to the existing literature because there is currently a lack of studies on the utilization of biostimulants derived from slaughterhouse sludge through enzymatic processes in green pepper crops.

2. Materials and Methods

2.1. Biostimulant Characteristics

The experimental biostimulant was obtained from slaughterhouse sludge supplied by the "Mataderos del Sur" company (Salteras, Seville, Spain).

Slaughterhouse sludge derives from organic waste (stomach remains, feces, etc.) and blood. These residues are filtered through a self-cleaning rotating sieve (screen) in order to eliminate larger solid residues. Subsequently, the waste is passed through a DAF (dissolved air flotation) to eliminate grease and suspended solids. Next, the waste obtained is introduced into a biological reactor in order to carry out biological purification, obtaining a biomass that is the slaughterhouse sludge, from which the aforementioned biostimulant can be developed. The chemical composition of this slaughterhouse sludge is shown in Table 1.

Table 1. Chemical characteristics (mean ± standard error, n = 3) of the slaughterhouse sludge.

Chemical Composition	
Organic matter (g kg^{-1})	745 ± 22
N (g kg^{-1})	57.7 ± 7.7
P (g kg^{-1})	19.4 ± 2.4
K (g kg^{-1})	4.8 ± 1.0
S (g kg^{-1})	17.8 ± 1.6
Ca (g kg^{-1})	47.1 ± 6.3
Mg (g kg^{-1})	4.9 ± 0.9
Fe (g kg^{-1})	6.3 ± 1.5
Cu (mg kg^{-1})	77.5 ± 7.3
Mn (mg kg^{-1})	93.0 ± 10.2
Zn (mg kg^{-1})	377 ± 46
Pb (mg kg^{-1})	18.6 ± 1.4
Ni (mg kg^{-1})	9.1 ± 1.3
Mo (mg kg^{-1})	1.6 ± 0.2
Cd (mg kg^{-1})	≤0.1 ± 0.01
Cr (mg kg^{-1})	≤0.1 ± 0.01

Following the criteria of Rodríguez-Morgado et al. [18], the slaughterhouse sludge was autoclaved to eliminate pathogens and facilitate the degradation of the high-molecular-weight proteins present in the sludge.

Subsequently, the sludge was concentrated using a rotary evaporator until a dry matter content of approximately 15% was achieved, resulting in a concentrated sludge that was easy to handle.

The enzymatic hydrolysis process, enzymes used, temperature conditions, pH, reaction time, enzyme concentration, and substrate concentration are described in Ávila-Pozo et al. [5].

Once the biostimulant was obtained in a soluble form, it underwent chemical characterization (Table 2). The methodology used for the chemical characterization of each parameter is detailed in Rodríguez-Morgado et al. [18].

Table 2. Chemical characteristics mean ± standard error, n = 3) of the experimental biostimulant.

Chemical Composition	
Dry matter (%)	14.8 ± 1.7
Organic matter (g kg^{-1})	664 ± 49
N (g kg^{-1})	4.8 ± 1.7
P (g kg^{-1})	7.5 ± 1.5
K (g kg^{-1})	10.4 ± 1.6
S (g kg^{-1})	10.9 ± 3.1
Ca (g kg^{-1})	8.9 ± 1.7
Mg (g kg^{-1})	1.1 ± 0.3
Fe (g kg^{-1})	2.9 ± 1.1
Cu (mg kg^{-1})	28.4 ± 1.5
Mn (mg kg^{-1})	35.4 ± 8.6
Zn (mg kg^{-1})	172 ± 21
Pb (mg kg^{-1})	5.1 ± 1.1
Ni (mg kg^{-1})	4.3 ± 1.6
Mo (mg kg^{-1})	1.4 ± 0.6
Cd (mg kg^{-1})	≤0.1 ± 0.01
Cr (mg kg^{-1})	≤0.1 ± 0.01
Protein molecular weight distribution (Da)	

Table 2. *Cont.*

Chemical Composition	
>10,000	39.6 ± 2.3
10,000–5000	3.8 ± 1.4
5000–3000	1.9 ± 0.5
3000–1000	6.2 ± 1.6
1000–300	8.8 ± 1.9
<300	39.7 ± 2.1

2.2. Experimental Procedure

The experiment was conducted in a greenhouse at the University of Seville under controlled humidity (80 ± 2.3%) and temperature (25 ± 1.8 °C) conditions, and natural light was used. Regarding the natural light cycle in the greenhouse, the hours of light corresponded to the hours of natural light corresponding to the hours of light in the study area during the experimental period.

Sweet pepper (*Capsicum annuum* L. cv. *italiano*) seedlings were purchased from a commercial nursery and transplanted into 25 L pots (33.5 × 33.5 × 33.5 cm) filled with a universal substrate (Blumenerde, Gramoflor). This substrate consisted of a mixture of *Sphagnum* peat, wood fiber, and perlite. The physicochemical properties of the substrate are detailed in Table 3. We avoided drainage during irrigation, and we estimated the admissible irrigation volume in the undrained pots using control plants. Watering was performed three times a week with drinking water.

Table 3. Chemical composition of substrate physicochemical properties.

pH ($CaCl_2$) = 5.4–6.2
Electric conductivity = 80 mS cm^{-1}
N (mg L^{-1}) = 210
P (mg L^{-1}) = 150
K (mg L^{-1}) = 270

Before applying any biostimulant, the plants were given a 30-day period for proper adaptation to the pots. After this adaptation period, two doses of the experimental biostimulant (0 and 1.4 g L^{-1}) were administered. These doses were randomly selected, but their choice was influenced by previous positive results obtained in a tomato crop [5].

The biostimulants were applied to the substrate either through the root or foliar route every 20 days. This 20-day interval was chosen based on findings from Tejada et al. [9], who observed that biostimulants obtained via enzymatic hydrolysis had a short persistence in the soil due to rapid assimilation of amino acids and low-molecular-weight peptides by soil microorganisms.

Therefore, the biostimulant was applied at 20, 40, 60, and 80 days after the indicated adaptation time. Consequently, the total doses of the applied biostimulant were 2.8 and 5.6 g L^{-1}. Details of the fertilizer treatments used in this experiment are provided in Table 4.

Table 4. Detailed scheme of the fertilizer treatments used in the experiment.

1. Biostimulant applied via the roots
CA treatment: Control. The pepper plants were not fertilized with the biostimulant
A1 treatment: The pepper plants were treated with the biostimulant at a dose of 0.7 g L^{-1}
A2 treatment: The pepper plants were treated with the biostimulant at a dose of 1.4 g L^{-1}
2. Foliar application of the biostimulant
CB treatment: Control. The pepper plants were not fertilized with the biostimulant
B1 treatment: The pepper plants were treated with the biostimulant at a dose of 0.7 g L^{-1}
B2 treatment: The pepper plants were treated with the biostimulant at a dose of 1.4 g L^{-1}

For each fertilizer treatment described above, a total of 80 pepper plants were used, and the crops were allowed to grow for 140 days.

Throughout the growth period, the pepper plants were watered every 4–5 days depending on the substrate's moisture level.

These operational conditions (substrate type, greenhouse conditions, application dose, foliar application, and direct application to the substrate) were selected in a manner similar to the study conducted by Ávila-Pozo et al. [5]. This choice aimed to verify and differentiate the biostimulant's behavior across various horticultural crops.

During the 140-day growth period for each fertilizer treatment, the following parameters were determined: plant height, the number of flowers per plant, and the number of fruits per plant.

To assess the nutritional status of the crops, 50 leaves were collected from each fertilizer treatment 100 days after the root and foliar application of the biostimulant. These leaves, according to Hochmuth et al. [19], were the recently matured ones.

After washing, drying, and crushing the leaves according to the procedure described by Madejón et al. [20], Kjeldahl-N was determined using the method described by Herse [21] for fresh matter and other macro- and micronutrients (P, K, S, Ca, Mg, Fe, Mn, Zn, and Cu) in the extracts via inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7500c ICP-MS, Technologies, Tokyo, Japan).

Photosynthetic pigments were extracted from 0.5 g of fresh leaves in acetone (80%). The extracts were centrifuged at 3000 rpm for 5 min, and the chlorophyll a (at a wavelength of 662 nm), chlorophyll b (at a wavelength of 646 nm), and total carotenoid (at a wavelength of 470 nm) contents were determined using a UV-visible spectrophotometer (Libra S22, Harvard Bioscience Inc., Cambridge, MA, USA). The amounts of these pigments were calculated according to the formulas of Lichtenthaler and Wellburn [22].

Throughout the experimental period and for each fertilizer treatment, different fruits were collected. Thus, the number of fruits and the mean weight of the fruits were determined.

The harvested fruits were freeze-dried and crushed before chemical analysis. The methodology used in the determination of macro- and micronutrients in fruits resembled that described for leaves. Furthermore, the vitamin C content in the aqueous extracts of fresh pepper fruits was assessed using the methodology outlined by Parađiković et al. [18]. To do so, 5 g of fruit were homogenized in 100 mL of distilled water for 30 min. After filtration and centrifugation, the supernatant was used to determine vitamin C levels by following the method described by Benderitter et al. [23].

2.3. Statistical Analysis

To identify significant differences among the parameters determined in each fertilizer treatment, we conducted an analysis of variance (ANOVA) with Tukey's post hoc tests, setting the significance level at $p < 0.05$. Data analysis was performed using the statistical software package Statgraphics Plus 2.1.

3. Results

The plant height, number of fruits per plant, and number of flowers per plant exhibited significant increases ($p < 0.05$) when the biostimulant was applied through both the root and foliar applications (Table 5). Notably, the highest values for these parameters were observed when the biostimulant was consistently applied to the substrate at a dose of 1.4 g L^{-1}.

Table 5. Plant height, number of flowers per plant, and number of fruits per plant (mean ± standard error) for each fertilizer treatment. Rows followed by the same letter(s) are not significantly different ($p < 0.05$). Nd: Not determined.

Crop Tyme (Days)	Plant Height (cm)					
	CA treatment	**A1 treatment**	**A2 treatment**	**CB treatment**	**B1 treatment**	**B2 treatment**
35	39.2 ± 1.3 a	41.8 ± 1.8 a	42.5 ± 1.5 a	40.1 ± 1.2 a	42.3 ± 2.0 a	44.0 ± 1.7 a
55	53.8 ± 3.5 a	62.7 ± 3.3 b	65.9 ± 2.3 b	52.9 ± 3.1 a	60.3 ± 2.6 b	61.8 ± 2.1 b
75	58.7 ± 3.1 a	71.4 ± 3.1 b	75.2 ± 3.5 b	60.1 ± 4.3 b	66.2 ± 3.0 b	66.8 ± 3.2 b
95	63.8 ±3.9 a	75.6 ± 2.9 b	79.4 ± 3.7 b	64.2 ± 3.8 b	69.3 ± 3.5 b	72.8 ± 2.9 b
140	64.9 ± 2.2 a	76.9 ± 3.6 b	81.2 ± 3.1 c	65.3 ± 3.0 b	70.4 ± 3.6 b	73.7 ± 3.6 b
	Number of flowers per plant					
35	1.4 ± 0.2 a	1.5 ± 0.2	1.5 ± 0.1 a	1.3 ± 0.2 a	1.5 ± 0.2 a	1.5 ± 0.2 a
55	3.7 ± 1.1 a	6.4 ± 1.7	8.7 ± 2.1	3.4 ± 1.3 a	5.3 ± 1.3 b	7.9 ± 1.6 b
75	Nd	Nd	Nd	Nd	Nd	Nd
95	3.3 ± 1.0 a	3.9 ± 1.3 a	4.3 ± 1.3	2.9 ± 1.1 a	3.4 ± 1.0 a	3.8 ± 1.2 a
140	1.1 ± 0.2 a	1.3 ± 0.3 a	1.3 ± 0.4 a	1.0 ± 0.2 a	14 ± 0.3 a	1.5 ± 0.2 a
	Number of fruits per plant					
35	-	-	-	-	-	-
55	4.1 ± 0.8 a	5.7 ± 1.1 b	6.4 ± 1.0 c	3.8 ± 0.6 a	5.0 ± 1.0 b	5.5 ± 0.9 b
75	3.8 ± 0.8 a	6.0 ± 0.9 b	6.9 ± 0.6 c	3.6± 0.3 a	5.5 ± 0.9 b	6.0 ± 1.0 b
95	2.1 ± 0.3 a	3.4 ± 0.7 b	4.5 ± 0.8 c	1.9 ± 0.8 a	2.7 ± 0.4 b	3.9 ± 0.7 c
140	1.7 ± 0.2 a	1.7 ± 0.3 a	1.9 ± 0.3 a	1.3 ± 0.2 a	1.6 ± 0.4 a	1.2 ± 0.3 a

In comparison with the control treatment, the macro- and micronutrient contents in the leaves were significantly higher in the plants treated with the biostimulant (Table 6). The results indicated significant differences ($p < 0.05$) based on the method and dose of biostimulant application, with the highest values observed when the highest experimental dose was applied through the roots compared to the foliar application. Specifically, for the macronutrients analyzed, treatment A2 led to a significant increase ($p < 0.05$) of 30.6% in N, 21.6% in P, 13% in K, 34.5% in S, and 40.9% in Ca compared to treatment B2. Among the micronutrients analyzed, treatment A2 displayed a significant increase ($p < 0.04$) of 13.8% in Fe, 13.7% in Zn, and 13.6% in Cu compared to treatment B2.

Table 6. Pepper leaf mineral nutrient content (mean ± standard error) (on a dry matter basis) for each fertilizer treatment. Rows followed by the same letter(s) are not significantly different ($p < 0.05$).

Parameter (Unit)	CA Treatment	A1 Treatment	A2 Treatment	CB Treatment	B1 Treatment	B2 Treatment
N [↑] (%)	1.9 ± 0.3 a	2.8b ± 0.5 b	3.6 ± 0.3 c	1.7 ± 0.2 a	2.6 ± 0.4 b	3.0 ± 0.4 b
P (%)	0.45 ± 0.11 a	0.66 ± 0.19 b	0.74 ± 0.15 c	0.47 ± 0.12 a	0.58 ± 0.11 b	0.64 ± 0.13 b
K (%)	5.6 ± 1.3 a	6.7 ± 1.2 b	7.7 ± 1.4 c	5.5 ± 1.0 a	6.7 ± 1.1 b	6.9 ± 1.0 b
S (%)	0.29 ± 0.07 a	0.41 ± 0.10 b	0.55 ± 0.08 c	0.30 ± 0.09 a	0.36 ± 0.13 ab	0.41 ± 0.11 b
Ca (%)	2.0 ± 0.3 a	5.1 ± 0.4 b	6.6 ± 0.7 c	2.1 ± 0.3 a	3.9 ± 0.9 b	4.8 ± 0.7 b
Mg (%)	0.48 ± 0.11 a	0.52 ± 0.10 b	0.54 ± 0.08 b	0.46 ± 0.11 a	0.49 ± 0.14 b	0.47 ± 0.12 b
Fe (mg kg^{-1})	102.5 ± 7.6 a	134.7 ± 8.6 b	148.9 ± 7.9 c	103.2 ± 9.7 a	114.7 ± 10.2 b	128.3 ± 9.9 b
Mn (mg kg^{-1})	100.2 ± 8.4 a	127 ± 7.5 b	132.2 ± 8.3 b	98.6 ± 8.1 a	112.1 ± 10.6 b	129.1 ± 13.5 b
Zn (mg kg^{-1})	92.9 ± 7.6 a	128.3 ± 5.9 b	139.5 ± 6.9 c	94.3 ± 7.6 a	116.2 ± 9.7 b	129.1 ± 11.6 b
Cu (mg kg^{-1})	6.2 ± 1.1 a	7.9 ± 1.3 b	8.8 ± 1.6 c	6.0 ± 1.0 a	6.9 ± 1.4 b	7.6 ± 1.6 b

[↑] Fresh matter.

Leaf pigment contents followed a similar trend to the results described above (Table 7). The chlorophyll a, chlorophyll b, and total carotenoid contents were lower in the control treatment than in the treatments where the biostimulant was applied. Among the pepper plants treated with the biostimulant, the highest values for these photosynthetic pigments were observed in treatment A2, followed by treatments B2, A1, and B1. This suggests that the content of the photosynthetic pigments analyzed depended on both the method of biostimulant application and the applied dose.

Table 7. Effect of biostimulant on pigments (fresh weight) in pepper leaves for each fertilizer treatment. Columns followed by the same letter(s) are not significantly different ($p < 0.05$). FW: fresh weight.

Treatments	Chlorophyll *a* (g kg^{-1}, FW)	Chlorophyll *b* (g kg^{-1}, FW)	Total Carotenoids (g kg^{-1}, FW)
CA treatment	1.2 ± 0.2 a	0.63 ± 0.09 a	0.32 ± 0.06 a
A1 treatment	1.6 ± 0.2 b	0.77 ± 0.12 b	0.50 ± 0.08 b
A2 treatment	2.1 ± 0.2 c	0.88 ± 0.13 b	0.60 ± 0.11 c
CB treatment	1.1 ± 0.2 a	0.62 ± 0.11 a	0.31 ± 0.08 a
B1 treatment	1.6 ± 0.2 b	0.78 ± 0.10 b	0.49 ± 0.10 b
B2 treatment	1.9 ± 0.3 b	0.84 ± 0.12 b	0.54 ± 0.10 b

Regarding the fruits harvested throughout the experiment, the results also indicated that the application of the biostimulant and the application rate significantly influenced the number of fruits harvested and the average weight of these fruits (Table 8). The highest values for both parameters analyzed were obtained for treatment A2, followed by treatments B2, A1, B1, and the control treatment.

Table 8. Fruit number and mean weight of fruits for each fertilizer treatment. Columns followed by the same letter(s) are not significantly different ($p < 0.05$).

Treatments	Fruit Number (n. plant^{-1})	Average Fruit Weight (g)
CA treatment	12.5 ± 1.2 a	259.3 ± 10.2 a
A1 treatment	15.0 ± 1.5 b	295.7 ± 9.9 b
A2 treatment	18.8 ± 1.2 c	324.7 ± 11.3 c
CB treatment	12.1 ± 1.3 a	258.4 ± 10.1 a
B1 treatment	14.3 ± 1.7 b	289.6 ± 11.4 b
B2 treatment	16.7 ± 1.5 b	312.6c ± 10.0 bc

Furthermore, in comparison to the control treatment, the macro- and micronutrient contents in the fruit were significantly ($p < 0.05$) lower in the treatments where the biostimulant was applied (Table 9). The highest levels of macro- and micronutrients were observed in plants treated with the highest dose of biostimulant in the roots, followed by treatments B2, A1, and B1.

Table 9. Chemical analysis (mean ± standard error) (fres wt.) in peppers harvested for each fertilizer treatment. Rows followed by the same letter(s) are not significantly different ($p < 0.05$).

Parameter (Unit)	CA Treatment	A1 Treatment	A2 Treatment	CB Treatment	B1 Treatment	B2 Treatment
N [↑] (%)	1.6 ± 0.2 a	2.2 ± 0.4 b	2.8 ± 0.3 c	1.4 ± 0.2 a	1.9 ± 0.3 b	2.4 ± 0.3 b
P (%)	0.29 ± 0.07 a	0.35 ± 0.11 b	0.40 ± 0.10 c	0.30 ± 0.13 a	0.34 ± 0.15 ab	0.3 ± 0.12 b
K (%)	2.4 ± 0.8 a	3.5 ± 1.0 b	4.3 ± 1.2 c	2.5 ± 0.6 a	3.2 ± 1.3 b	3.8 ± 1.3 b
S (%)	0.20 ± 0.04 a	0.25 ± 0.07 b	0.28 ± 0.03 b	0.20 ± 0.05 a	0.25 ± 0.06 b	0.27 ± 0.04 b
Ca (%)	0.12 ± 0.06 a	0.19 ± 0.03 a	0.22 ± 0.05 b	0.12 ± 0.06 a	0.18 ± 0.03 a	0.20 ± 0.06 b
Mg (%)	0.11 ± 0.03 a	0.18 ± 0.05 ab	0.25 ± 0.07 b	0.13 ± 0.02 a	0.17 ± 0.04 ab	0.22 ± 0.05 b
Fe (mg kg^{-1})	37.6 ± 2.3 a	45.2 ± 3.0 b	53.6 ± 3.8 c	37.1 ± 1.6 a	43.4 ± 2.7 b	49.8 ± 3.0 b
Mn (mg kg^{-1})	10.4 ± 1.5 a	13.8 ± 1.1 b	15.2 ± 1.4 c	10.1 ± 1.2 a	12.9 ± 1.6 b	14.6 ± 1.3 bc
Zn (mg kg^{-1})	18.7 ± 1.6 a	21.4 ± 1.9 b	24.7 ± 2.1 c	19.3 ± 1.5 a	20.3 ± 1.3 b	22.9 ± 2.0 bc
Cu (mg kg^{-1})	4.0 ± 1.3 a	5.6 ± 1.5 b	6.6 ± 1.1 c	4.0 ± 1.1 a	5.2 ± 1.2 b	6.0 ± 1.7 b

[↑] Fresh matter.

The vitamin C contents in pepper fruits also showed a similar trend to the analyzed parameters (Table 10), with higher values observed in the treatments where the biostimulant was applied. Once again, the highest values were observed in the treatment where the biostimulant was applied at the highest dose through the roots (treatment A2). This vitamin C content was 14.6% higher than in treatment B2, 18.8% higher than in treatment A1, and 22.9% higher than in treatment B1.

Table 10. Effect of biostimulant on vitamin C (fresh weight) in pepper fruits for each fertilizer treatment. Rows followed by the same letter(s) are not significantly different ($p < 0.05$). FW: fresh weight.

Parameter (Unit)	CA Treatment	A1 Treatment	A2 Treatment	CB Treatment	B1 Treatment	B2 Treatment
Vitamin C (g kg^{-1}, FW)	3.1 ± 0.2 a	3.9 ± 0.42 a	4.8 ± 0.7 a	3.0 ± 0.3 a	3.07 ± 0.3 a	4.1 ± 0.4 a

4. Discussion

The results obtained in this study suggest that the biostimulant, which was rich in low-molecular-weight peptides and organic matter and obtained via the enzymatic hydrolysis of slaughterhouse sludge, played a role in stimulating the mineral nutrition of pepper crops. As a result, it led to enhancements in fruit quality and yield.

These findings are consistent with those reported by Ávila-Pozo et al. [5] when they studied the efficacy of this biostimulant when applied to tomato crops. They observed a positive effect on the nutrition, growth, and fruit quality of the tomato plants upon the application of this biostimulant.

Several studies have detailed the positive effect of biostimulants consisting of low-molecular-weight peptides and organic matter on crop growth and physiology. For instance, Colla et al. [24] examined the efficacy of biostimulants that were rich in protein hydrolysates and obtained from plants and seaweeds on a tomato crop, observing positive effects on growth, development, and crop productivity. Carillo et al. [25] observed improvements in morphological and colorimetric parameters, as well as mineral composition in a greenhouse-grown spinach crop after applying a plant-derived protein hydrolysate biostimulant. Agliassa et al. [26] tested the efficacy of a protein-hydrolysate-based biostimulant on pepper crops, observing improvements in growth and an increase in yield. Francesca et al. [27] observed an increase in tomato crop yield under limited water availability conditions when applying a biostimulant derived from protein hydrolysates. Furthermore, Wang et al. [28] applied a pig blood-derived protein hydrolysate biostimulant to a tomato crop, observing improvements in the plant's photosynthetic capacity and mineral nutrition.

There are also studies that indicate the positive effect of biostimulants obtained via enzymatic hydrolysis processes on other non-horticultural crops. Tejada et al. [9] observed significant improvements in the growth, mineral nutrition, production, and quality of corn after the application of biostimulants derived from sewage sludge and chicken feathers. Additionally, Tejada et al. [7] reported enhanced mineral nutrition, chlorophyll contents, and olive production following the application of a sewage sludge biostimulant produced through enzymatic processes.

Several authors have suggested that the beneficial effects of biostimulants containing amino acids and low-molecular-weight peptides on crop growth, development, and yield can be attributed to their ability to enhance plant physiology. These enhancements include the reinforcement of natural defenses and direct influence on plant metabolism [27,29].

Similarly, there are many studies in the literature that indicate the positive effect of organic matter on the mineral nutrition of plants, thereby influencing the growth, development, and production of crops [5,10,30].

Furthermore, several authors also suggest that the foliar application of humic substances can increase the permeability of the cuticle, facilitating the improved entry of chemical compounds from fertilizers into plant cells [5,7].

In our experiment, the application of the biostimulant notably improved the mineral nutrition of bell pepper crops. Adequate crop mineral nutrition is essential for optimal plant growth and development [11]. Both macro- and micronutrients play essential roles in regulating various physiological processes and facilitating metabolic reactions, including photosynthesis and nitrogen assimilation, while also serving as precursors or activators of various enzyme systems [11,31,32].

According to Colla et al. [33], the reason for observing superior mineral nutrition in plants when the biostimulant was applied through the root route as opposed to the foliar route could be attributed to root application leading to more vigorous root development. This, in turn, results in increased root biomass, length, volume, and branching, all of which enhance nutrient absorption.

Cristofano et al. [34] suggest that the uptake of amino acids and peptides by plants is influenced by various factors, including the method of treatment application, environmental conditions, and specific modalities.

On the other hand, Colla et al. [33] and Pecha et al. [35] suggest that substrate application can cause plants to take up 6–25% of amino acids due to microbial competition, while foliar uptake depends on factors such as moisture levels, stomata opening and number, and cuticle thickness.

The application of the biostimulant also led to an increase in chlorophyll and carotenoid contents, with a more substantial increase observed in plants that received the biostimulant through root application. These findings agree with those obtained by Ávila-Pozo et al. [5], who examined the effects of applying the same biostimulant (also through root and foliar application) to tomato crops. Parađiković et al. [6,18] and Ahmad et al. [11] also observed elevated concentrations of photosynthetic pigments in pepper plants treated with various biostimulants composed of amino acids and organic matter. These authors suggest that the increased content of photosynthetic pigments is a result of enhanced nitrogen (N) absorption by the plant.

Mandal et al. [8], Parađiković et al. [16], and Agliassa et al. [26] have suggested that biostimulants enhance the photosynthetic activity of plants. We believe that a higher uptake of amino acids and low-molecular-weight peptides occurred in the plants when the biostimulant was applied via the roots of the plants, which likely contributed to the observed increases in the chlorophyll contents and photosynthetic activity.

There is ample evidence supporting the close relationship between carotenoid and chlorophyll contents [15]. According to these authors, an increase in chlorophyll content leads to a corresponding increase in carotenoid content, as carotenoids serve to protect chlorophyll from photo-oxidation.

It is likely that the enhanced mineral nutrition and improved photosynthetic activity observed in plants treated with the biostimulant via root application played a significant role in increasing production and improving harvest quality. These findings are consistent with those reported by Ávila-Pozo et al. [5] after they applied the same biostimulant to tomato crops. Similarly, Parađiković et al. [16] observed improved plant mineral nutrition, increased photosynthetic pigment concentrations, higher fruit production, and enhanced fruit quality when various biostimulants based on amino acids were applied to pepper plants. These studies also established a direct relationship between photosynthetic pigment concentration and production, as higher pigment concentrations support the synthesis of carbohydrates crucial for plant productivity.

The results obtained from this study underscore the potential benefits of using this biostimulant in green pepper cultivation. Its application appears to enhance nutrient utilization efficiency, positively impacting both the morphological and nutritional characteristics of the plants.

5. Conclusions

The results of this study show that the use of the biostimulant obtained from slaughterhouse sludge significantly improved the mineral nutrition of the green pepper crops, as well as the content of photosynthetic pigments, nutritional quality, and fruit yield. These positive results were obtained when the biostimulant was applied successively to the root of the plant at a dose of 1.4 g L^{-1}.

Hence, the application of this biostimulant presents a promising sustainable strategy for achieving high crop yields while enhancing their nutritional value.

The use of this biostimulant should, however, be further studied. The dosage of biostimulant, type of crop (horticultural and non-horticultural), and number of applications and their timing must be taken into consideration in order to gain a deeper understanding of the action of this biostimulant on crops and provide practical recommendations for its use.

Regarding non-horticultural crops, it is also necessary to check the response of the biostimulant when applied to these crops when they are grown in different soil types.

Furthermore, future research should focus on studying the impact of this biostimulant on both horticultural and non-horticultural crops subjected to environmental stress conditions, such as variations in temperature and drought.

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