horticulturae

MDPI

Article

Use of a Biostimulant Obtained from Slaughterhouse Sludge in a Greenhouse Tomato Crop

Paloma Ávila-Pozo [1], Juan Parrado [2], Pablo Caballero [2] and Manuel Tejada [2,*]

[1] Grupo de Investigación Edafología Ambiental, Departamento de Cristalografía, Mineralogía y Química Agrícola, Escuela Técnica Superior de Ingeniería Agronómica, Universidad de Sevilla, Crta de Utrera km. 1, 41013 Sevilla, Spain; pavila@us.es

[2] Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad de Sevilla, C/Prof. García González 2, 41012 Sevilla, Spain; parrado@us.es (J.P.); pcaballero2@us.es (P.C.)

* Correspondence: mtmoral@us.es

Abstract: Currently, the use of biostimulants is widespread in sustainable agriculture because they represent an alternative to chemical fertilizers. In this manuscript, we investigate the response of a greenhouse tomato crop grown in pots to a biostimulant obtained from slaughterhouse sludge applied continuously to the substrate under which the tomato crop was grown or applied directly via the foliar route. Two doses of biostimulant (0.7 and 1.4 g L^{-1}), applied four times throughout the crop growth period (120 days), were used. During this period, the height of the plants, number of flowers and number of fruits obtained were measured. After 90 days of the growth period, the nutrient contents in leaves as well as the chlorophyll a and b contents were analyzed. For fruits, nutrient and lycopene contents were determined, and the equatorial diameter and average fresh and dry weight were measured. The results indicate a higher content of nutrients and chlorophyll a and b in the leaves of plants treated with the highest dose of biostimulant and applied continuously to the substrate. This higher content of photosynthesis pigments in the plant is possibly responsible for a greater absorption of N by the plant and, consequently, for better growth.

Keywords: protein hydrolysates; plant nutrition; lycopene; tomato yield

Citation: Ávila-Pozo, P.; Parrado, J.; Caballero, P.; Tejada, M. Use of a Biostimulant Obtained from Slaughterhouse Sludge in a Greenhouse Tomato Crop. *Horticulturae* **2022**, *8*, 622. https://doi.org/10.3390/horticulturae8070622

Academic Editor: Julė Jankauskienė

Received: 11 May 2022
Accepted: 7 July 2022
Published: 9 July 2022

1. Introduction

For many years, the application of chemical fertilizers has been a widely used agricultural practice in the development of intensive agriculture [1,2]. However, the continuous use and abuse of these synthetic fertilizers has caused many negative effects related to the depletion of natural resources as well as the generation of greenhouse gases, eutrophication of water, salinization of the soil and security problems of food and quality deterioration [2–4]. These chemicals also increase the susceptibility of plants to pathogens, altering the soil microbiome and influencing plant health, posing a significant threat to consumers [5].

For this reason, the sustainability of agricultural production is necessary both to meet consumer demand for healthy products and to try to eliminate or reduce the aforementioned problems [2,4–6].

To achieve this agricultural sustainability in recent years, a number of biostimulants consisting of protein hydrolysates have been used [6]. These substances, which are generally obtained by enzymatic hydrolysis processes, typically consist of low-molecular-weight peptides, amino acids, polysaccharides, etc., used in a great variety of crops such as cereals, fruits and vegetables and have been shown to have a large number of positive effects on these crops, such as increased productivity and better quality [7–10].

According to Searchinger [11] and Kapoore et al. [12], the use of these biostimulants in agriculture has two objectives, namely increasing the supply and quality of food for the world population and reducing the negative effect of agriculture on the environment and human health.

The production of biostimulants from industrial waste is an alternative of great environmental interest since the recovery of these wastes results in their elimination, and consequently a reduction in their environmental impact, and also increases their value, thus coinciding with the circular economy concept proposed by the EU to convert this waste into new energy sources [13].

There has been a marked increase in the production of slaughterhouse sludge in recent decades as a consequence of the increase in the number of such facilities [14]. These authors obtained a biostimulant from slaughterhouse sludge by way of enzymatic hydrolysis processes and used it for the bioremediation of soil contaminated with the herbicide oxyfluorfen. However, we are not aware of the use of this biostimulant in crop production. As such, the application of this biostimulant, which has a high content of low-molecular-weight peptides and macro- and micronutrients, to the soil or leaves could be an alternative use for these new products.

Tomato (*Lycopersicon esculentum*) is one of the most widely consumed horticultural crops due to its nutritional value given that it is a source of a large number of bioactive compounds such as minerals, vitamins and antioxidants [2,15,16]. Consequently, a study of the reaction of this crop to this new biostimulant could be of enormous scientific and agricultural interest, since its use could constitute a solution to the challenge of improving the sustainability of agricultural systems by reducing the use of synthetic inorganic fertilizers.

There are no studies using biostimulants obtained from slaughterhouse sludge obtained by enzymatic hydrolysis in crops. We presume that the application of this biostimulant to crops will improve the mineral nutrition of the plant and, consequently, will have a positive impact on its growth, production and quality of the fruit obtained. In the same way, we do not know if this positive effect on the plant will be better when the biostimulant is applied directly to the substrate on which the crop grows or when it is applied by the foliar route. As such, the main objective of this study was to investigate the response of a tomato crop to a biostimulant obtained from slaughterhouse sludge using enzymatic hydrolysis processes. This biostimulant was applied either directly to the substrate under which the tomato crop was grown or was applied directly to the leaves.

2. Materials and Methods

2.1. Biostimulant Properties

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

Before the enzymatic hydrolysis process, the slaughterhouse sludge was autoclaved. According to Rodríguez-Morgado et al. [17], the autoclaving process favors the elimination of pathogens. In addition, this sterilization process can enhance the ability of enzymes to degrade proteins of higher molecular weight into others of lower molecular weight.

To obtain the biostimulant, and according to Ávila-Pozo et al. [14], slaughterhouse sludge was subjected to two treatments. In the first treatment, the slaughterhouse sludge was concentrated at 75 °C with a rotary evaporator until reaching a dry matter value of around 15%. This was done because under these conditions the slaughterhouse sludge was more manageable.

Next, the concentrated sludge was obtained through an enzymatic hydrolysis process using the pH-stat methodology [18] using an endoprotease obtained by liquid fermentation of Bacillus licheniformis ATCC 21.415 as a hydrolytic agent.

The enzyme used in this work is subtilisin (EC3.4.21.62), a protease characteristic of Bacillus, and its enzymatic activity has been determined by the azocasein method (340 ± 5 U/mL). The enzyme is produced in our laboratory and has been sequenced by proteomic analysis [19]. It was used in the enzymatic process of sludge without purification.

This process and enzymatic hydrolysis were performed in a bioreactor. The conditions were the following: pH = 9, temperature = 55°, take = 180 min, enzyme concentration = 1 mL L^{-1} of substrate and substrate concentration = 10%.

After this hydrolysis process, the product was centrifuged, and a soluble solution and an insoluble paste were obtained.

The biostimulant was obtained by concentrating this soluble solution.

Table 1 shows the chemical composition of the resulting biostimulant. The methodology used to determine each chemical parameter for the biostimulant is detailed in the work of Rodríguez-Morgado et al. [17].

Table 1. Chemical characteristics and protein molecular weight distribution of biostimulant obtained from slaughterhouse sludge by enzymatic hydrolysis process.

Chemical Composition	
Dry matter (%)	15.3 ± 2.1
Organic matter (g kg^{-1})	658 ± 351
N (g kg^{-1})	4.3 ± 1.3
P (g kg^{-1})	6.2 ± 1.6
K (g kg^{-1})	9.3 ± 2.1
S (g kg^{-1})	12.4 ± 2.7
Ca (g kg^{-1})	17.1 ± 3.1
Mg (g kg^{-1})	2.1 ± 0.9
Fe (g kg^{-1})	3.7 ± 1.2
Cu (mg kg^{-1})	81.3 ± 11.6
Mn (mg kg^{-1})	39.8 ± 12.4
Zn (mg kg^{-1})	298 ± 37
Pb (mg kg^{-1})	6.4 ± 1.8
Ni (mg kg^{-1})	5.2 ± 1.3
Protein Molecular Weight Distribution (Da)	
>10,000	48.7 ± 1.5
10,000–5000	4.1 ± 1.9
5000–3000	2.0 ± 0.7
3000–1000	5.7 ± 1.1
1000–300	7.2 ± 1.8
<300	32.3 ± 2.5

2.2. Experimental Design

The experiment was carried out in a greenhouse under controlled conditions of temperature and humidity (temperature: 25 °C and humidity: 80%). During the experiment, the light that prevailed was only natural.

Tomato seedlings (*Lycopersicon esculentum* Mill. cv. Mina) were purchased from a commercial nursery. These seedlings had a height of approximately 20 cm, as measured from the cotyledons. They were transplanted into 25 L pots containing a universal substrate (Blumenerde, Gramoflor) as a culture medium. This substrate was a mixture of Sphagnum peat, wood fiber and perlite, with pH ($CaCl_2$) 5.4–6.2; electrical conductivity 80 mS cm^{-1}; and amounts of added N, P and K of 210, 150 and 270 mg L^{-1}, respectively.

The plants were allowed to grow for 40 days before application of the experimental biostimulant to allow them to adapt well to the pot.

Two doses of the experimental biostimulant were used (0.7 and 1.4 g L^{-1}, respectively) on the basis of the results obtained by Tejada et al. [20] after the foliar application of two leachates from the vermicomposting of cow manure and green forage in tomato crops. Although these doses were selected randomly, they were sufficient to ensure that the tomato plant did not suffer any type of nutritional deficiency during growth [21].

The biostimulant was applied foliarly with the help of a hydraulic sprayer at a pressure of 0.017 MPa or to the substrate every 20 days. The reason for application every 20 days is that, according to Tejada et al. [9], the biostimulants obtained using enzymatic hydrolysis processes have a very short residence time in the soil because they are quickly absorbed by microorganisms. As a result, the biostimulant was applied at 20, 40, 60 and 80 days after the previously described adaptation period.

The different fertilizer treatments are detailed as follows:

1. C treatment: control, plants were not fertilized with the biostimulant;
2. S1 treatment: plants amended with the biostimulant applied to the substrate at a dose of 0.7 g L^{-1};
3. S2 treatment: plants amended with the biostimulant applied to the substrate at a dose of 1.4 g L^{-1};
4. F1 treatment: plants fertilized foliarly with the biostimulant at a dose of 0.7 g L^{-1};
5. F2 treatment: plants fertilized foliarly with the biostimulant at a dose of 1.4 g L^{-1}.

Only foliar spray control is shown. We created two controls, one root and one foliar. However, in both cases, the water supply was provided via the root every 2–3 days, depending on the water status of the substrate. Both controls only differ in the foliar application of water (20 mL $\times$ 4). This amount applied during the vegetative period of the crop did not produce any effect on any of the parameters studied.

As such, the total doses of biostimulant used in the experiment were 2.8 and 5.6 g L^{-1}, respectively.

A total of 60 tomato plants were used for each fertilizer treatment, and the crop growth time was 120 days. During this period, the height of the plants was measured for each fertilizer treatment, as were the number of flowers and number of fruits obtained. These measurements were performed 15 days after the application of the biostimulants as well as at the end of the experimental period.

Ninety days after having applied the biostimulant to the substrate or foliarly, tomato leaves were taken in order to carry out a nutritional monitoring of the crop. Thus, on each of these days and for each fertilizer treatment, 30 leaves corresponding to the fourth and fifth most developed leaves from the apex were collected.

This plant material was washed, dried and crushed according to the procedure reported by Madejón et al. [22]. The determination of macro- and micronutrients (P, K, S, Ca, Mg, Fe, Cu, Mn and Zn) in the extracts was carried out by ICP-OES. Kjeldahl-N was determined using the MAPA method [23] for fresh matter.

The photosynthetic pigments chlorophyll a and chlorophyll b were extracted with 95% ethanol. Absorption maxima were recorded at pigment-specific wavelengths (λmax), in this case, 663 nm for chlorophyll a, 645 nm for chlorophyll b and 663 nm and 646 nm for total chlorophyll. Chlorophyll contents were calculated according to Lichtenthaler [24].

The selected fruit was lyophilized and crushed prior to analysis. Determination of the macro- and micronutrients in this fruit was carried out according to the methodology described for the leaves.

In addition, 90 days after application of the biostimulant, the lycopene content was determined according to the methodology described by Fish et al. [25].

Finally, the equatorial diameter and the average fresh and dry weight of these fruits were measured for each fertilizer treatment.

2.3. Statistical Analysis

The results obtained were analyzed by performing an analysis of variance (ANOVA) using the Statgraphics Plus 2.1 software package, with the fertilizer treatment being considered as an independent variable, followed by Tukey's significant difference as a post hoc test, considering a significance level of $p < 0.05$ throughout the study.

3. Results

The height of the tomato plant, the number of flowers per plant and the number of fruits per plant were influenced by the application of the biostimulant, with all these parameters being higher in the plants fertilized with the biostimulant at a dose of 1.4 g L^{-1} and applied to the substrate in a regular manner (Table 2).

Table 2. Effect of biostimulant obtained from slaughterhouse sludge on plant height, number of flowers per plant and number of fruits per plant.

Parameter	Crop Time (Days)	C Treatment	S1 Treatment	S2 Treatment	F1 Treatment	F2 Treatment
Plant height (cm)	35	33.4 ± 3.5 a	35.0 ± 2.7 a	40.2 ± 2.9 a	36.2 ± 1.9 a	39.4 ± 2.2 a
	55	55.2 ± 4.4 a	64.8 ± 3.8 b	66.7 ± 3.0 b	61.8 ± 2.7 b	64.2 ± 2.0 b
	75	65.8 ± 3.1 a	79.6 ± 4.0 b	84.1 ± 2.4 c	73.2 ± 3.6 b	79.2 ± 3.1 b
	95	70.3 ± 3.7 a	87.8 ± 4.1 b	98.2 ± 4.7 c	80.9 ± 3.2 b	91.4 ± 2.9 bc
	120	74.3 ± 4.1 a	96.2 ± 3.8 b	109.3 ± 5.2 c	86.8 ± 4.1 b	95.9 ± 3.7 b
Number of flowers per plant	35	-	-	-	-	-
	55	3.2 ± 1.3 a	5.7 ± 1.0 b	6.2 ± 1.3 b	8.2 ± 1.1 c	11.3 ± 1.8 d
	75	5.7 ± 1.1 a	6.8 ± 1.4 b	7.9 ± 1.6 b	8.4 ± 1.3 bc	9.5 ± 1.2 c
	95	4.1 ± 1.0 a	9.5 ± 1.4 bc	10.3 ± 2.1 c	6.1 ± 1.7 ab	7.8 ± 1.2 b
	120	1.2 ± 0.5 a	2.6 ± 0.3 b	4.1 ± 0.6 c	2.5 ± 0.3 b	2.3 ± 0.5 b
Number of fruits per plant	35	-	-	-	-	-
	55	1.4 ± 0.2 a	3.3 ± 0.3 b	3.9 ± 0.4 c	2.5 ± 0.5 b	3.7 ± 0.4 b
	75	1.7 ± 0.3 a	6.0 ± 0.8 b	7.2 ± 1.0 c	5.1 ± 0.6 b	5.9 ± 0.9 b
	95	2.1 ± 0.3 a	9.6 ± 1.2 b	12.9 ± 1.5 c	8.2 ± 1.0 b	9.7 ± 1.2 b
	120	1.2 ± 0.2 a	2.7 ± 0.4 b	3.2 ± 0.3 b	2.2 ± 0.6 b	2.8 ± 0.3 b

Rows followed by the same letter(s) are not significantly different ($p < 0.05$).

Table 3 shows the foliar content of macro- and micronutrients for all fertilizer treatments expressed on a dry matter basis. Compared to the control treatment, the results indicate that continuous application of the biostimulant, both foliarly and in the substrate, significantly increased the content of these nutrients in the tomato leaves ($p < 0.05$). Application of the experimental biostimulant both foliarly and continuously to the substrate also resulted in differences in the content of these macro- and micronutrients in the leaf. With regard to the macronutrients analyzed, the highest values were found when the biostimulant was applied continuously to the substrate and at a dose of 1.4 g L^{-1}, compared to the application of this biostimulant foliarly. Thus, a significant increase ($p < 0.05$) in N (26.7%), P (12.1%), K (21.9%) and Ca (18.1%) was found for treatment S2 compared to treatment F2. Similarly, and with regard to the micronutrients analyzed, the results also indicate a significant increase ($p < 0.05$) in Fe (17.8%), Mn (15.3%) and Cu (20.2%) for treatment S2 compared to treatment F2.

Table 3. Effect of biostimulant obtained from slaughterhouse sludge on tomato leaf mineral nutrient content.

Parameter (Unit)	C Treatment	S1 Treatment	S2 Treatment	F1 Treatment	F2 Treatment
N ‡ (%)	1.3 ± 0.2 a	1.9 ± 0.2 b	3.0 ± 0.3 c	1.7 ± 0.1 b	2.2 ± 0.2 bc
P (%)	0.56 ± 0.18 a	0.79 ±0.11 b	0.83 ± 0.13 c	0.72 ± 0.17 b	0.73 ± 0.15 b
K (%)	2.0 ± 0.4 a	2.7 ± 0.6 b	3.2 ± 0.5 c	2.3 ± 0.7 a	2.5 ± 0.5 ab
S (%)	0.25 ± 0.1 a	0.45 ± 0.14 b	0.57 ± 0.17 b	0.32 ± 0.11 ab	0.43 ± 0.14 b
Ca (%)	6.1 ± 0.8 a	8.7 ± 0.9 b	10.5 ± 1.3 c	7.3 ± 0.7 b	8.6 ± 1.0 b
Mg (%)	0.40 ± 0.11 a	0.50 ± 0.08 b	0.53 ± 0.10 b	0.48 ± 0.07 ab	0.51 ± 0.11 b
Fe (mg kg^{-1})	79.2 ± 8.9 a	99.2 ± 9.6 b	128.3 ± 12.4 c	93.5 ± 10.1 b	105.4 ± 11.3 b
Mn (mg kg^{-1})	119.2 ± 12.3 a	159.3 ± 15.4 b	177.4 ± 14.9 c	143.4 ± 13.5 b	150.3 ± 15.8 b
Cu (mg kg^{-1})	4.8 ± 0.8 a	6.3 ± 0.5 b	8.4 ± 1.0 c	5.5 ± 0.6 ab	6.7 ± 0.7 b
Zn (mg kg^{-1})	25.4 ± 0.7 a	30.4 ± 1.8 b	32.6 ± 1.0 b	30.8 ± 1.5 b	31.7 ± 1.7 b

‡ Fresh matter. Rows followed by the same letter(s) are not significantly different ($p < 0.05$).

Table 4 shows the chlorophyll a and chlorophyll b contents obtained. The results indicate a higher content of chlorophyll a and b and total chlorophyll for S2 treatment, followed by F2, S1, F1 and C treatments.

Table 4. Effect of biostimulant obtained from slaughterhouse sludge on chlorophyll a and b content in tomato leaves.

Parameter	C Treatment	S1 Treatment	S2 Treatment	F1 Treatment	F2 Treatment
Chlorophyll a (mg g^{-1})	2.4 ± 0.6 a	4.5 ± 1.3 b	6.7 ± 1.8 c	3.9 ± 0.8 b	5.5 ± 1.2 b
Chlorophyll b (mg g^{-1})	1.3 ± 0.2 a	2.9 ± 0.5 b	3.8 ± 0.9 c	2.4 ± 0.7 b	3.1 ± 0.6 b
Total chlorophyll (mg g^{-1})	4.7 ± 0.9 a	10.3 ± 1.2 b	15.5 ± 1.5 c	8.3 ± 1.1 b	12.3 ± 1.7 b

Rows followed by the same letter(s) are not significantly different ($p < 0.05$).

With respect to the control treatment, the content of macro- and micronutrients in tomato was also significantly ($p < 0.05$) higher for all treatments in which the biostimulant was applied (Table 5). As for the foliar macro- and micronutrient contents, these nutrients also showed differences when the biostimulant was applied foliarly or continuously to the substrate. Again, the highest macronutrient contents were found when the biostimulant was applied to the substrate continuously and at a dose of 1.4 g L−1, compared to the application of this biostimulant foliarly. Thus, a significant increase ($p < 0.05$) in N (26.1%), P (15.1%), K (18.4%) and Ca (20.8%) was observed for S2 treatment in comparison with F2 treatment. Similarly, and with regards to the micronutrients analyzed, a significant increase ($p < 0.05$) in Fe (27.5%), Mn (19.1%) and Cu (20%) was observed for S2 treatment in comparison with F2 treatment.

Table 5. Effect of biostimulant obtained from slaughterhouse sludge on chemical analysis (fresh wt.) in tomatoes harvested.

Parameter (Unit)	C Treatment	S1 Treatment	S2 Treatment	F1 Treatment	F2 Treatment
N (%)	1.1 ± 0.1 a	1.8 ± 0.3 b	2.3 ± 0.4 c	2.3 ± 0.4 c	1.7 ± 0.2 b
P (%)	0.31 ± 0.12 a	0.44 ±0.10 b	0.53 ± 0.14 c	0.39 ± 0.12 b	0.45 ± 0.11 b
K (%)	2.5 ± 0.6 a	4.2 ± 1.2 b	4.9 ± 1.3 c	3.5 ± 1.0 b	4.0 ± 1.1 b
S (%)	0.11 ± 0.1 a	0.17 ± 0.03 b	0.19 ± 0.05 b	0.13 ± 0.04 ab	0.14 ± 0.04 b
Ca (%)	0.11 ± 0.1 a	0.19 ± 0.2 bb	0.24 ± 0.3 c	0.16 ± 0.2 b	0.19 ± 0.3 b
Mg (%)	0.09 ± 0.02 a	0.14 ± 0.02 b	0.20 ± 0.10 b	0.14 ± 0.037 b	0.17 ± 0.10 b
Fe (mg kg^{-1})	19.3 ± 1.4 a	34.9 ± 4.1 b	49.5 ± 4.6 c	29.6 ± 3.7 b	35.9 ± 2.9 b
Mn (mg kg^{-1})	8.8 ± 1.1 a	9.6 ± 1.5 b	13.1 ± 1.5 c	9.1 ± 1.2 b	10.6 ± 1.1 b
Cu (mg kg^{-1})	1.7 ± 0.2 a	2.6 ± 0.7 b	3.5 ± 1.2 c	2.3 ± 0.6 b	2.8 ± 0.9 b
Zn (mg kg^{-1})	9.8 ± 1.2 a	12.1 ± 0.8 ab	16.5 ± 1.3 b	13.4 ± 1.0 b	14.2 ± 1.0 b

Rows followed by the same letter(s) are not significantly different ($p < 0.05$).

The lycopene content of the fruits was also higher when the biostimulant was applied continuously to the substrate at the highest dose than when the biostimulant by the foliar route (Table 6). Thus, a 17.8% higher lycopene content was found for S2 treatment than for F2 treatment.

Table 6. Effect of biostimulant obtained from slaughterhouse sludge on lycopene content (mg kg^{-1} fresh wt.) in tomatoes harvested.

C treatment	S1 Treatment	S2 Treatment	F1 Treatment	F2 Treatment
62.1 ± 1.5 a	80.7 ± 1.8 bc	93.8 ± 2.6 c	71.8 ± 2.0 b	77.1 ± 1.4 b

Rows followed by the same letter(s) are not significantly different ($p < 0.05$).

The equatorial diameter of the tomato was also greater when the biostimulant was applied at a dose of 1.4 g L^{-1} and continuously to the substrate than when it was applied foliarly to the plant (Table 7). Thus, the statistical analysis showed a significant increase ($p < 0.05$) of 17.3% in the tomatoes obtained in the S2 treatment compared to the F2 treatment. Similarly, both the fresh weight and dry weight of the fruits were significantly ($p < 0.05$) higher when the dose of experimental biostimulant was applied in the S2 treatment than in the F2 treatment.

Table 7. Equatorial diameter, fresh weight and dry weight of the tomatoes obtained during the experimental period for each fertilizer treatment.

Parameter (Unit)	C Treatment	S1 Treatment	S2 Treatment	F1 Treatment	F2 Treatment
Equatorial diameter (cm)	17.2 ± 1.4 a	25.2 ± 1.0 b	28.4 ± 1.3 c	20.3 ± 1.2 b	23.5 ± 1.1 b
Fresh weight (g)	97.6 ± 3.5 a	159.3 ± 4.8 c	220.6 ± 5.6 d	125.3 ± 4.0 b	139.6 ± 3.9 b
Dry weight (g)	58.9 ± 2.3 a	87.6 ± 3.5 c	119.7 ± 3.8 d	68.6 ± 2.6 b	72.6 ± 2.9 b

Rows followed by the same letter(s) are not significantly different ($p < 0.05$).

4. Discussion

Our results indicate that this new biostimulant obtained from slaughterhouse sludge using enzymatic hydrolysis processes stimulates tomato plant growth and also has a positive effect on plant mineral nutrition, fruit quality and production.

These results are in agreement with those obtained by other authors when using different types of biostimulants in a tomato crop. Thus, Rouphael et al. [26] observed a significant increase in the total amount of soluble solids, lipophilic and hydrophilic antioxidant activities, lycopene, total phenolic content and total ascorbic acid in tomatoes after foliar application of a biostimulant derived from legumes. Similarly, Francesca et al. [27] found a significant increase in tomato plant growth and number of tomato fruits after soil application of a biostimulant comprising plant and yeast extracts; amino acids; and micronutrients such as boron, zinc and manganese. Moreover, de Paula et al. [28] observed an increase in the production of tomatoes as well as in their quality after the application of two biostimulants comprising algae extracts to the soil.

This stimulating effect on growth, mineral nutrition, quality and production in tomato cultivation is mainly a consequence of the chemical composition of the experimental biostimulant used, especially its content of protein hydrolysates and organic matter. There is a currently large amount of information available regarding the biostimulant properties of these protein hydrolysates (consisting of a mixture of amino acids and soluble peptides) and organic matter in various crops [9,10,29,30]. Indeed, it has been found that the positive effects of protein hydrolysates are associated with the upregulation of metabolites involved in plant growth processes and the provocation of various activities similar to those caused by the hormones [31,32]. This enhancement of plant metabolism promotes plant respiration, photosynthesis and protein synthesis processes, thus improving crop yield and quality [30,33].

It has been observed that when organic matter is applied to the soil, it improves the growth and morphology of the roots, increases the absorption of nutrients and the efficiency of their use, improves crop yields and, finally, increases the quality of the fruit [32,33]. According to Garcia et al. [34] and Conselvan et al. [35], humic substances could promote plant growth by producing effects very similar to those of hormones.

Similarly, Tejada et al. [9,10] have suggested that the foliar application of humic substances improves the permeability of the cuticle, thus favoring penetration of the different chemical compounds found in biostimulants into plant cells.

Our results indicate that the different applications of the biostimulant studied herein on the tomato crop stimulated the plant in different ways, with a greater positive effect on the growth of the plant, as well as on the mineral nutrition of the tomato, mineral nutrition and quality of the fruit and yield, being obtained when the biostimulant was applied continuously at the dose of 1.4 g L^{-1} to the substrate than when it was applied foliarly. These results are in agreement with those obtained by Sestili et al. [36], who observed that continuous application of a plant-derived biostimulant to the substrate was more effective in improving plant growth and total N uptake in tomato than foliar application. According to these authors, continuous application of the biostimulant to the substrate resulted in greater improvement of the physiological parameters of tomato plants, leading to greater stomatal conductance and a better rate of plant transpiration and efficiency of the use of transpiration.

Zhao et al. [37] indicated that a high content of photosynthetic pigments in plants could be responsible for an increase in their photosynthetic activity. Consequently, it is very likely that the higher chlorophyll a and b contents found in plants after S2 treatment result in a higher photosynthetic activity.

This increase in the photosynthetic capacity of the plant, as well as the supply of energy for cellular metabolism generated by the application of protein hydrolysates, could be responsible for a greater absorption of N by the plant and, consequently, for better growth [27]. According to Huang et al. [38], the availability of N for plants plays a fundamental role in the biosynthesis of photosynthetic pigments and in plant growth. Similarly, these authors suggest that a good absorption of N by a plant would lead to a nutritional improvement of other chemical elements in that plant.

Rouphael et al. [26] reported that the application of a protein hydrolysate with a high amino acid content increased the height of the plant, number of leaves before the beginning of the fruit harvest and biomass of the shoots and also increased the average weight of the fruit and the number of fruits. We propose that all these values were higher in S2 treatment as the photosynthetic activity of these plants, as well as their mineral nutrition, was better. Similarly, the better mineral nutrition of the plant in S2 treatment is likely to explain why the mineral composition of the fruit was also higher. This fact is of great interest since we now know that minerals play a fundamental role in human metabolism, thus meaning that a deficiency of minerals in human nutrition could cause various nutritional disorders [39].

Within the mineral content of the fruit, one of the most important macroelements to highlight apart from N, which is directly involved in protein formation, is the K content. Indeed, it has been shown that this macronutrient is directly related to the lycopene content in the fruit [26]. These authors also emphasized that lycopene is essential for the pyruvic, kinase and acetic thiokinase enzymes, which are directly involved in lycopene synthesis. The higher absorption of K in the plant in treatment S2 may therefore explain the higher lycopene content in the fruit.

5. Conclusions

It can be concluded that the best results were obtained when the biostimulant was applied continuously to the substrate at a dose of 1.4 g/L^{-1} compared to when it was applied foliarly. Under these conditions, the highest chlorophyll contents were found in tomato plants. These higher chlorophyll contents are possibly responsible for a higher absorption of N by the plant, which leads to a nutritional improvement of other chemical elements in that plant and consequently to greater production and quality of fruits.

We believe that this study could be a starting point for future studies on the use of this type of biostimulant in which the greater efficacy of this compound when applied continuously to the substrate than when applied foliarly is corroborated, as is the optimal dose to use. In the same way, it is also necessary to study the behavior of this new biostimulant on crops other than tomato.

Author Contributions: P.Á.-P.: formal analysis, investigation; J.P.: formal analysis, investigation; P.C.: formal analysis, investigation; M.T.: validation, writing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by "Proyectos I + D+i FEDER Andalucía 2014–2020", grant number US-1263885; "Comisión Europea, Horizonte 2020", grant number 3052/0048; "Ministerio de Ciencia, Innovación y Universidades (Spain), Plan Nacional I + D+I", grant number RTI2018-097425-B-100; and PAIDI Project P18-HO-2049 Junta de Andalucía (Ministry of Economy and Knowledge).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Da Costa, P.B.; Beneduzi, A.; de Souza, R.; Schoenfeld, R.; Vargas, L.K.; Passaglia, L.M.P. The effects of different fertilization conditions on bacterial plant growth promoting traits: Guidelines for directed bacterial prospection and testing. *Plant Soil* **2013**, *368*, 267–280. [CrossRef]
2. Ye, L.; Zhao, X.; Bao, E.; Li, J.; Zou, Z.; Cao, K. Bio-organic fertilizer with reduced rates of chemical fertilization improves soil fertility and enhances tomato yield and quality. *Sci. Rep.* **2020**, *10*, 177. [CrossRef] [PubMed]
3. González, M.; Cea, M.; Medina, J.; González, A.; Díez, M.; Cartes, P.; Monreal, C.; Naria, R. Evaluation of biodegradable polymers as encapsulating agents for the development of a urea controlled-release fertilizer using biochar as support material. *Sci. Total Environ.* **2015**, *505*, 446–453. [CrossRef] [PubMed]
4. Ashour, M.; Hassan, S.M.; Elshobary, M.E.; Ammar, G.A.G.; Gaber, A.; Alsanie, W.F.; Mansour, A.T.; El-Shenody, R. Impact of commercial seaweed liquid estract (TAM) biostimulant and its bioactive molecules on growth and antioxidant activities of hot pepper (*Capsicum annuum*). *Plants* **2021**, *10*, 1045. [CrossRef] [PubMed]
5. Hassan, S.M.; Ashour, M.; Soliman, A.A.E.; Hassanien, H.A.; Alsanie, W.F.; Gaber, A.; ElShobary, M.E. The potential of a new commercial seaweed extract in stimulating morpho-agronomic and bioactive properties of *Eruca vesicaria* (L.) Cav. *Sustainability* **2021**, *13*, 4485. [CrossRef]
6. Colla, G.; Rouphael, Y. Biostimulants in agriculture. *Sci. Hortic.* **2015**, *196*, 1–2. [CrossRef]
7. Colla, G.; Rouphael, Y.; Canaguier, R.; Svecova, E.; Cardarelli, M. Biostimulant action of a plant-derived protein hydrolyzates produced through enzymatic hydrolysis. *Front. Plant Sci.* **2014**, *5*, 448. [CrossRef]
8. Ertani, A.; Pizzeghello, D.; Francioso, O.; Sambo, P.; Sanchez-Cortes, S.; Nardi, S. *Capsium chinensis* L. growth and nutraceutical properties are enhanced by biostimulants in a long-term period: Chemical and metabolomic approaches. *Front. Plant Sci.* **2014**, *5*, 1–2. [CrossRef]
9. Tejada, M.; Rodríguez-Morgado, B.; Gómez, I.; Franco-Andreu, L.; Benítez, C.; Parrado, J. Use of bioferilizers obtained from sewage sludges on maize yield. *Eur. J. Agron.* **2016**, *78*, 13–19. [CrossRef]
10. Tejada, M.; Rodríguez-Morgado, B.; Paneque, P.; Parrado, J. Effects of foliar fertilization of a biostimulant obtained from chicken feathers on maize yield. *Eur. J. Agron.* **2018**, *96*, 54–59. [CrossRef]
11. Searchinger, T. *The Great Balancing CCT: Installment 1 of Creating a Sustainable Food Future*; World Resources Institute: Washington, DC, USA, 2013; pp. 1–15.
12. Kapoore, R.V.; Wodd, E.E.; Llewellyn, C.A. Algae biostimulants: A critical look at microalgal biostimulants for sustainable agricultural practices. *Biotechnol. Adv.* **2021**, *49*, 107754. [CrossRef] [PubMed]
13. European Comission (Ed.) *Circular Economy Package-Proposal for a Regulation of the European Parlament and of the Council*; 2016/0084; European Comission: Brussels, Belgium, 2016.
14. Ávila-Pozo, P.; Parrado, J.; Caballero, P.; Díaz-López, M.; Bastida, F.; Tejada, M. Use of slaughterhouse sludge in the bioremediation of an oxyfluorfen-polluted soil. *Int. J. Environ. Res.* **2021**, *15*, 723–731. [CrossRef]
15. Bilalis, D.; Krokida, M.; Roussis, I.; Papastylianou, P.; Travlos, I.; Cheimona, N.; Dede, A. Effects of organic and inorganic fertilization on yield and quality of processing tomato (*Lycopersicon esculentum* Mill.). *Folia Hort.* **2018**, *30*, 321–332. [CrossRef]
16. Maach, M.; Boudouasar, K.; Akodad, M.; Skalli, A.; Moumen, A.; Baghour, M. Application of biostimulants improves yield and fruit quality in tomato. *Int. J. Veg. Sci.* **2021**, *27*, 288–293. [CrossRef]
17. Rodríguez-Morgado, B.; Gómez, I.; Parrado, J.; García-Martínez, A.M.; Aragón, C.; Tejada, M. Obtaining edaphic biostimulants/biofertiliaers from different sewage sludges. Effects on soil biological properties. *Environ. Technol.* **2015**, *36*, 2217–2226. [CrossRef]
18. Adler-Nissen, J. Enzymatic hydrolysis of food protein. *Proc. Biochem.* **1977**, *12*, 15–32.
19. Parrado, J.; Rodríguez-Morgado, B.; Tejada, M.; Hernandez, T.; García, C. Proteomic analysis of enzyme production by *Bacillus licheniformis* using different feather wastes as the sole fermentation media. *Enzyme Microb. Technol.* **2014**, *57*, 1–7. [CrossRef]
20. Tejada, M.; Gonzalez, J.L.; Hernandez, M.T.; Garcia, C. Agricultural use of leachates obtained from two different vermicomposting processes. *Biores. Technol.* **2008**, *99*, 6228–6232. [CrossRef]
21. Nuez, F. *El Cultivo del Tomate*; Mundi-Prensa: Madrid, Spain, 1995.
22. Madejón, P.; Xiong, J.; Cabrera, F.; Madejón, E. Quality of trace element contaminated soils amended with compost under fast growing tree *Paulownia fortune* plantation. *J. Environ. Manag.* **2014**, *144*, 176–185. [CrossRef]
23. MAPA. *Métodos Oficiales de Análisis*; Secretaría General Técnica del Ministerio de Agricultura, Pesca y Alimentación: Madrid, Spain, 1986; Volume 1, pp. 221–285.
24. Lichtenthaler, H.K. Chlorophyll and carotenoids: Pigments of photosynthetic biomembranes. In *Methods Enzymology*; Packer, L., Douce, R., Eds.; Academic Press: Sandiego, Spain, 1987; pp. 350–382.
25. Fish, W.W.; Perkins-Veazie, P.; Collins, J.K. A quantitative assay for lycopene that utilizes reduced volumes of organic solvents. *J. Food Compos. Anal.* **2002**, *15*, 309–317. [CrossRef]

26. Rouphael, Y.; Colla, G.; Giordano, M.; El-Nakhel, C.; Kyriacou, M.C.; De Pascale, S. Foliar applications of a legume-derived protein hydrolysate elicit dose-dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* **2017**, *226*, 353–360. [CrossRef]
27. Francesca, S.; Arena, C.; Mele, B.H.; Schettini, C.; Ambrosino, P.; Barone, A.; Rigano, M.M. The use of a plant-based biostimulant improves plant performances and fruit quality in tomato plants grown at elevated temperatures. *Agronomy* **2020**, *10*, 363. [CrossRef]
28. De Paula, B.S.; Feltrim, D.; Engel, D.C.H.; Baptistella, J.L.C.; Rodrigues, M.; Engel, E.; Mazzafera, P. Algae-based biostimulants increase yield and quality of mini tomatoes under protected cultivation. *JSFA Rep.* **2022**, *2*, 155–160. [CrossRef]
29. Colla, G.; Nardi, S.; Cardarelli, M.; Ertani, A.; Lucini, L.; Canaguier, R.; Rouphael, Y. Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* **2015**, *196*, 28–38. [CrossRef]
30. Kocira, A.; Lamorska, J.; Kornas, R.; Nowosad, N.; Tomaszewska, M.; Leszczyńska, D.; Kozłowicz, K.; Sylwester Tabor, S. Changes in biochemistry and yield in response to biostimulants applied in bean (*Phaseolus vulgaris* L.). *Agronomy* **2020**, *10*, 189. [CrossRef]
31. Ertani, A.; Cavani, L.; Pizzeghello, D.; Brandellero, E.; Altissimo, A.; Ciavatta, C.; Nardi, S. Biostimulant activity of two protein hydrolyzates in the growth and nitrogen metabolism of maize seedlings. *J. Plant Nutr. Soil Sci.* **2009**, *172*, 237–244. [CrossRef]
32. Colla, G.; Hoagland, L.; Ruzzi, M.; Cardarelli, M.; Bonini, P.; Canaguier, R.; Rouphael, Y. Biostimulant action of protein hydrolysates: Unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* **2017**, *8*, 2202. [CrossRef]
33. Shahrajabian, M.H.; Chaski, C.; Polyzos, N.; Petropoulos, S.A. Biostimulants application: A low input cropping management tool for sustainable farming of vegetables. *Biomolecules* **2021**, *11*, 698. [CrossRef]
34. Garcia, A.C.; Olaetxea, M.; Santos, L.A.; Mora, V.; Baigorri, R.; Fuentes, M.; Zamarreño, A.M.; Berbara, R.L.; Garcia-Mina, J.M. Involvement of hormone- and ROS-signaling pathways in the beneficial action of humic substances on plants growing under normal and stressing conditions. *BioMed. Res. Int.* **2016**, *2016*, 3747501. [CrossRef]
35. Conselvan, G.B.; Fuentes, D.; Merchant, A.; Peggion, C.; Francioso, O.; Carletti, P. Effects of humic substances and indole-3-acetic acid on *Arabidopsis* sugar and amino acid metabolic profile. *Plant Soil* **2018**, *426*, 17–32. [CrossRef]
36. Sestili, F.; Rouphael, Y.; Cardarelli, M.; Pucci, A.; Bonini, P.; Canaguier, R.; Colla, G. Protein hydrolysate stimulates growth in tomato coupled with N-dependent gene expression involved in N assimilation. *Front. Plant Sci.* **2018**, *9*, 1233. [CrossRef] [PubMed]
37. Zhao, L.-S.; Su, H.N.; Li, K.; Xie, B.B.; Liu, L.N.; Zhang, X.Y.; Chen, X.L.; Huang, F.; Zhou, B.C.; Zhang, Y.Z. Supramolecular architecture of photosynthetic membrane in red algae in response to nitrogen starvation. *Biochim. Biophys. Acta* **2016**, *1857*, 1751–1758. [CrossRef] [PubMed]
38. Huang, W.-T.; Xie, Y.-Z.; Chen, X.-F.; Zhang, J.; Chen, H.-H.; Ye, X.; Guo, J.; Yang, L.-T.; Chen, L.-S. Growth, mineral nutrients, photosynthesis and related physiological parameters of citrus in response to nitrogen deficiency. *Agronomy* **2021**, *11*, 1859. [CrossRef]
39. Gharibzahedi, S.M.T.; Jafari, S.M. The importance of minerals in human nutrition: Bioavailability, food fortification, processing effects and nanoencapsulation. *Trends Food Sci. Technol.* **2017**, *62*, 119–132. [CrossRef]