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Design of a hydroponic test to evaluate the biostimulant potential of new organic and organomineral products

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ABSTRACT

Currently, the use of biostimulants is increasing due to the need for greater productivity in agriculture. The European Union presented a new fertilizer regulation, UE 2019/1009, appearing for the first time the concept of biostimulants. Its main objective is to improve the efficiency of plants in the absorption and assimilation of nutrients or their tolerance to biotic or abiotic stresses, regardless of the nutrient content of the product. The objective of this work consisted in the development of a methodology to test in a short-term experiment the efficiency of commercial products as potential biostimulants in a crop of *Capsicum annuum L.* in strictly hydroponic conditions. Plants were irrigated with the respective product at the recommended dose in water, without the addition of other nutritional sources for 15 days. At the end of the test, the weights of the root and aerial part, the humidity, the chlorophyll indexes, and nutritional leaf content, as well as the volume and morphology of the roots and plants were obtained to evaluate the biostimulant effects on the plant growth and development. The water consumption was also evaluated to analyze whether any of the products generates greater water savings. The study concluded that the nutritive solutions with biostimulants produced a greater increase in the weight of the plant and a lower percentage of leaf moisture, as well as higher values of leaf chlorophyll. On the other hand, it was humic, fulvic, and algae biostimulants that presented the best values in terms of water savings. The methodology developed could be set to test in the short term the biostimulant potential of new products.

1. Introduction

Agricultural productivity has been steadily increasing through the use of chemical fertilizers that improve crop yield and allows profitable agriculture on soils either of low natural fertility or impoverished by long cultivation or erosion. However, the application of fertilizers is a highly inefficient process despite the efforts to optimize its efficiency. Typically, an important fraction of the fertilizers is lost by different means (leaching, volatilization, degradation, immobilization) causing soil and water contamination (Tissot et al., 2002). In the last decades, different alternatives have been proposed to reduce the use of chemical fertilizers. Among them, biostimulants have merited increasing interest to reduce the application of fertilizers without damaging the nutrition of the crops since they are capable of improving the absorption of nutrients (Canellas et al., 2015). Due to their biological origin, low toxicity, rapid degradation, low mobility in soil, and the absence of residues in food-related to low application rates, biostimulants have little or no negative effect on the environment or human health (Thomas et al., 2013). However, as a consequence of the complexity and variety of the biostimulant's components, their mode of action is still not fully known (García-García et al., 2020). An extended hypothesis is that they are capable of affecting the metabolism of the plant (Nardi et al., 2016); rising to growth benefits, development, or response to stressful situations (Bulgari et al., 2019). FAO and European regulatory bodies define them as "products that stimulate nutritional processes regardless of their nutrient content, with the sole objective of improving one or more of the following characteristics of the plant, its rhizosphere or its phyllosphere: efficiency in the use of nutrients, tolerance to different forms of abiotic stress, quality traits of the crop, availability of nutrients in the soil and rhizosphere, humification and degradation of soil organic compounds (Caradonia et al., 2019). Biostimulants could be grouped according to multi-component formulations and classified by the origin or mode of

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action of the active ingredient into different categories such as humic substances, complex organic materials, beneficial chemical elements, seaweed extracts, free amino acids, among others (Du Jardin, 2012; Yakhin et al., 2017). Briefly, humic substances can improve the growth and physiology of the plant, since several hormones have been identified in the structure of humus (Pizzeghello et al., 2013). Although this hormone-like activity is still not well understood, it appears that the addition of humic biostimulants produces the bioactivation of soil bacteria and fungi (Rouphael and Colla, 2020). They also increase their biostimulant potential when they are added with other biostimulants such as algae extracts (do Rosário Rosa et al., 2021). A recent study (Lima et al., 2020) showed that not only did the algae biostimulants improve the entry of nutrients into the roots, but also increased the tolerance of plants to salinity. Biostimulants based on beneficial chemical elements are nutritive solutions containing macro and micronutrients that contribute to increase specific leaf area and percentage of nitrogen in leaves (Perez-Amaro et al., 2004), plant production (Cárdenas-Navarro et al., 2004), and formation of ATP (Lazo et al., 2014) or root expansion (Sustr et al., 2019). Lastly, biostimulants based on amino acids, present in a large number of physiological processes like transport and storage to growth control (Rai, 2002), can increase tolerance to different stress factors in plants such as cold, water scarcity, or high salt concentrations (García-García et al., 2020).

Biostimulants global market is expected to grow over the next years, with an annual growth rate of 12% (Kapoore et al., 2021); reaching USD 4.14 billion by 2025 (Madende and Hayes, 2020). In 2019, the European Union presented a new fertilizer regulation (EU 2019/1009) that opens the single market for fertilizing products which are not currently covered by harmonization rules, such as plant biostimulants. This is the first European regulatory framework for biostimulants that lays down common rules on safety, quality, and labeling requirements to harmonize the biostimulant market and avoid unfair competition between operators. For that purpose, a reliable and reproducible method for testing the conformity of EU biostimulant products is essential. However, the heterogeneity of the biostimulant products makes it difficult the establishment a methodology to test their real efficiency on crops (La Torre et al., 2016). New methodologies are now being developed to characterize better the biostimulant composition (Fuentes et al., 2018) and its effects on plants roots by computer tomography scanning, which is used for the evaluation of different root parameters (Kalhoro et al., 2018); developing complete assays to evaluate the potential as biostimulant of new products by the sequential system based on two different biological model organisms (baker's yeast *Saccharomyces cerevisiae* and plant *Arabidopsis thaliana*) in six months (Saporta et al., 2019). However, the methodologies are not yet fully developed and still need long terms to be carried out.

The main objective of this study was the evaluation of a new proposed methodology to study the biostimulant potential of new products in plants and if it can be used as a standardized method of analysis. This was carried out by the evaluation of the effects on growth, chlorophyll activities, nutritional content, water consumption, and root effects of 10 products from different feedstock, chemical composition, and potential biostimulant effects on pepper plants (*Capsicum annuum* L. var. Brocanto).

2. Materials and methods

2.1. Biostimulants description and characterization

Ten liquid products supplied by different companies were used in this work, some already available in the market and others in the certification phase. They were chosen as examples of the main groups of compounds with biostimulant activity (Drobek et al., 2019). Each of these products was formed with at least one component with potential biostimulant properties of non-microbial origin. For the simplification of the ten products in terms of results, they were divided into 5 classes of biostimulants (Table 1). The criterion followed for the subdivision of the products was the kind of base substance with a biostimulant effect they contain in their formulation.

Solutions of each biostimulant in type I water were prepared at the agricultural recommended dose, i.e. 2 mL L^{-1} for all the biostimulants except for S1 and S2 which was 1 mL L^{-1}. The pH of the solutions was measured with a Thermo Orion 720A pH meter (Hach Lange, Barcelona, Spain), and the electrical conductivity with a micro CM 2200 Crison conductivity meter (Hach Lange, Barcelona, Spain). Given the disparity in the pH of the different solutions and their extreme value, e.g. HN group (Table 1), it was adjusted to 6.5 using $NaHCO_3$ and H_2SO_4 to be able to use them in hydroponics.

Total carbon (C), nitrogen (N), hydrogen (H), and sulfur (S) content of the biostimulant was determined with a LECO Element Analyzer CHNS-932 (St. Joseph, Michigan, USA) in the solid product obtained after the evaporation of the liquid at room temperature. The solid residue was also subjected to Fourier-transform infrared spectroscopy (FTIR) using a Bruker IFS66v spectrometer (Billerica, MA, USA). For that purpose, samples (1 mg) were diluted in 99 mg of KBr before the analysis. Readings were obtained in arbitrary units of diffuse reflectance. Spectra were obtained by accumulating 250 scans at a resolution of 4 cm^{-1} in a spectral range of 450–4000 cm^{-1}. Trace elements in the biostimulant solutions were determined by mass spectrometry with inductive coupling plasma (ICP-MS) (NexION 300XX, Perkin-Elmer, Waltham, MA, USA). Free amino acids were determined by high performance liquid chromatography (HPLC) Waters 2695 (Milford, MA, USA) equipped with a photodiode array detector (PDA) Waters 996 (Milford, MA, USA). A Waters AccQ·Tag™ amino acid analysis column packed with Nova-Pak™ C18 (4 μm) (Waters, Milford, MA, USA) was also used. The analysis of amino acids was performed after the derivatization of amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Operating conditions were as follows: the mobile phase was sodium acetate 140 mM with triethylamine 17 mM at pH 5.05 (A), acetonitrile (B), and Milli-Q water (C). Linear gradient elution was used at a flow rate of 1 mL min^{-1}. The column temperature was 37 ºC and the injection volume of each not-diluted biostimulant solution was 10 μL. The detector was set at 254 nm (Cooper et al., 2001).

2.2. Plant assay

Thirty days old pepper - (*Capsicum annuum* L. cv. Brocanto) seedlings supplied by Surinver (Coop. V., Alicante, Spain) were grown in pure hydroponics. Each plant was grown in a culture system consisting of a rectangular plastic bag of 15 cm x 25 cm and a volume of 500 mL held by

Table 1
Name, composition and main characteristics of the biostimulant products used in this work.

Product	Group	Composition	pH[1]	EC (μS cm^{-1})	Density (kg/L)
BC	C	Organic substances	4.9	350	1.36
H1	H	Humic substances	7.9	675	1.21
H2		Fulvic acids	6.3	264	1.07
H3		Leonardite	8.6	334	1.12
EH1	EH	Ecological humic solution	4.1	565	1.18
EH2		Ecological humic solution	5.6	367	1.12
HNS1	HNS	Nutritive solution + H2 0.2%	3.4	749	1.10
HNS2		Nutritive solution + EH2 0.2%	3.3	823	1.10
S1	S	Seaweed (phytohormones)	7.8	34	1.83
S2		Seaweed (phytohormones)	6.6	193	1.10

[1] The pH was measured in an aqueous solution of each biostimulant product at v:v of 0.2% and 0.1% (for S1 and S2) according to the recommended dose.

a methacrylate cylinder wrapped in aluminum foil. A total of 60 plants (10 products and 6 replicates for each one) were placed in a culture chamber (Dycometal-type CCK) with a temperature between 19 and 23 ºC and a humidity between 40 and 60%, with a day/night cycle of 14 and 10 h respectively with a photosynthetic photon flux density at the leaf of 1000 μmol·m^{-2}•s^{-1}, using tree different lamps of UV, white and red for warming lights. For each plant, an aeration system was used with tubes inside the bags for the oxygenation of the roots.

The addition of the biostimulants was done daily at its recommended agricultural doses. On the first day, the plants were watered with 10 mL of distilled water. From day two, 10 mL of each biostimulant solution were added to each plant for a total of 14 days. The total volume of biostimulant solution used was 840 mL (10 mL of irrigation x 6 plants x 14 days). The commercial biostimulant BC (Table 1) was used as the control to compare the other biostimulants' effects on the plant.

2.3. Plant analysis

The growth and development, weights of plant organs, and chlorophyll activities were determined. The nitrogen balance index (NBI) (ratio: chlorophyll/flavonols activities), total chlorophylls (Chl), flavonols (Flav), and anthocyanins (Anth) were obtained by the DUALEX Scientific+TM meter (Force A, Orsay, France) (Kalaji et al., 2017) on three fully developed adult leaves per plant at the end of the assay.

The nutritional status of the plant was assessed by foliar analysis. Leaves were dried in a forced-air oven at 65 ºC for 3 days, weighted to determine the leave humidity percentage, and analyzed for mineral concentration after dry digestion at 480 ºC for 2 h and acid digestion for ashes solubilization with HCl 6 M at 90 ºC. The analysis of the elements in the pepper leaves was carried out by ICP-MS (NexION 300XX, Perkin-Elmer, Waltham, MA, USA). Foliar nitrogen and carbon were measured by elemental analysis in a LECO Element Analyzer CHNS-932 (St. Joseph, Michigan, USA). Leaf organic matter percentage was done by weight measure after calcination in a muffle furnace at 450 ºC for 4 h. The total nutrient solution consumed percentage was calculated at the end of the experiment by volumetrically quantifying the remanent solution, knowing the total solution added. The Nutritional Efficiency Index (NEI) was calculated with the total content of nutrients supplied to plants and the concentration found in leaves. NEI = [Leaves nutrient concentration] / [Nutrient concentration added].

The root systems were spread out on a glass tray to be scanned using a flatbed scanner (EPSON PerfectionV700Photo, Seiko Epson Corp., Japan) at a resolution of 800 dpi. Root measurements of length, projected area, surface area, average diameter, root volume, tips, forks, and crossings number were obtained using WinRHIZO Pro 2019a image analysis software (Regent Instruments Inc., Quebec, Canada). A collection of images was carried out using a transmission electron microscope (TEM) specifically a JEOL JEM1010 transmission electron (100 kV), the Leica Ultracut S Ultramicrotome, and the 1000 series Vibratome (Leica Mycrosystems, Wetzlar, Germany). For this measurement, sample aliquots of 1 cm^2, were fixed with 1% glutaraldehyde and 4% formaldehyde in 0.1 M sodium cacodylate at pH 7.4. Afterward, degassing and treatment with 1% osmium tetroxide was done, followed by dehydration, with increasing concentrations of ethanol and finally acetone. The sample inclusion was carried out by infiltration in Durcupan epoxy resin, followed by polymerization in an oven for 48 h at 60 ºC. Samples, prepared as described before, were cut into sections, approximately 60 nm thick, that were finally subjected to a process of contrasting with heavy metals.

2.4. Statistical analysis

The data were statistically evaluated to find significant differences among samples by one-way and two-way ANOVAs followed by the Duncan post-hoc test, with a level of significance of 95% ($p \leq 0.05$), using the software IBM SPSS v20 (Armonk, NY, USA). The principal component analysis (PCA) was performed to determine the relationship between the biostimulant groups and the plant parameters. This test was done using the PAST V. 4.02 software (Natural History Museum, University of Oslo).

3. Results

3.1. Characterization of the biostimulant products

The composition of biostimulants showed a high proportion of carbon compounds if those were related to the humic substances (Fig. 1). H2 and EH2 biostimulants had more than 30% carbon content, followed by EH1 with 24%, BC and H3 with 20%, H1 with 15% a, and finally HNS1, HNS2, S1, and S2 with less than a 3% of C. The products composed of fulvic or humic acids had the highest carbon content (Table 1).

IR spectra of the biostimulants used showed clear differences among products due to their different origin (Fig. 2).

The organic nature of the compounds was elucidated by the different peaks of the spectra. Differences in the IR spectra of the products were found, based on their different origin (Fig. 2). The absorption bands in 3300–3500 cm^{-1} are due to stretching vibrations of -OH or -NH groups with varying degrees of hydrogen bonding. The aliphatic groups were confirmed by the presence of two peaks at around 2900 and around 2840 cm^{-1}, due to the asymmetrical and symmetrical stretching of methylene groups, respectively (Conselvan et al., 2018). The spectrums also showed bands at ~1610 cm^{-1} (C=C stretching vibrations in olefinic and aromatic compounds), 1380–1400 cm^{-1} (C-H deformation of -CH_2 and -CH_3 salts of carboxylic acid or aliphatic -CH), and 1200–1260 cm^{-1} (C=O stretching vibrations of esters, ethers, and phenols). The band at 1080 cm^{-1} is attributed to alcohols and carbohydrates along with Si-O vibrations due to inorganic ash forming components. The products showed a well-defined peak at 1035 cm^{-1} due to C-O stretching vibrations of ethers and phenols (Nasir et al., 2011).

The humic biostimulants (H1 and H3) had the spectra with the most acute peaks (3400 cm^{-1} and 1400 cm^{-1} regions) due to their content of complex organic matter. This was also seen for the HNS products which are also based on humic substances plus inorganic salts. In this case, the spectra of HNS1 and HNS2 overlap. Therefore, the organic substances of HNIS1 and HNS2 are the same. In the ecologic humic biostimulants (EH1 and EH2) less acute but wider peaks were found in comparison with H biostimulants in the spectra regions 3400–2400 cm^{-1} and 1800–1000 cm^{-1}. The commercial biostimulant based on the undefined organic matter used as the control (BC) showed less strong spectra with lower intensity than the Humic products (H). The S products, on the other hand, showed the spectra with the lowest intensity peaks having no interactions except in the 1400–600 cm^{-1} region. This meant less content of organic substances, which also corroborated with the C content (Fig. 1).

The H3 and H1 products presented the highest nitrogen values. However, the N content of H2 was lower than the rest of the H group, denoting clear differences among products although all of them were based on humic substances. In contrast, the products EH showed similar N contents to the HNS. The products based on seaweed extracts (S1 and S2) contained the lowest N contents.

The biostimulants based on nutritive solution, HNS1 and HNS2 showed the highest macro- and micronutrients concentration (Table 2). Although the products were grouped by their origin, differences in their nutritional compositions were clear (H2 vs H1 and H3; EH1 vs EH2 and S1 vs S2), except for HNS1 and HNS2, denoting the high variability of the nutritive content of biostimulant products.

The free amino acid profile of the samples was different for each product (Table 3). The commercial biostimulant (BC) showed low content of free amino acids. Alanine (Ala) was the sole amino acid detected in this biostimulant. The H biostimulants were different between them, H1 with no amino acids detected, H2 with low content, and H3 with the

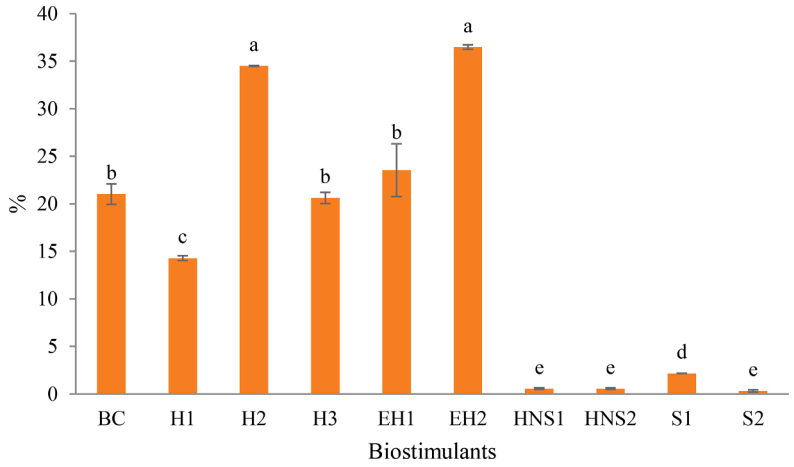

Fig. 1. Percentage of carbon (C) of the tested biostimulant products. Data were represented as the mean ± S.D, $n = 3$. Different letters indicate significant differences among biostimulant products (Duncan post-hoc test; $p \leq 0.05$).

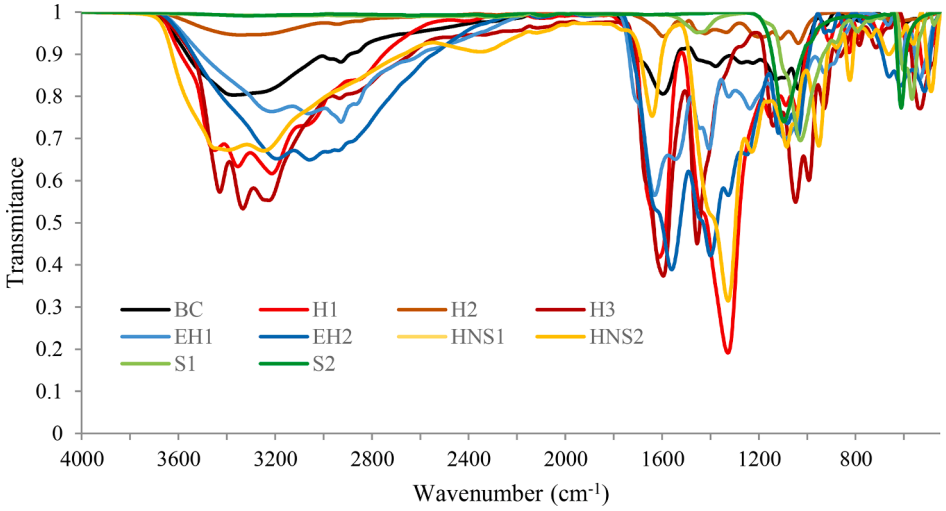

Fig. 2. FTIR spectra of the 10 biostimulant products tested. The products were grouped according to their composition in five groups according to the colors: control commercial product in black, humic products in red, ecological in blue, nutritive solution + biostimulant in yellow, and algae in green.

Table 2
Concentration (mg/L) of macro and micronutrients in the tested biostimulants.

	BC	H1	H2	H3	EH1	EH2	HNS1	HNS2	S1	S2
N	3989	275,336	12,626	284,181	93,397	133,000	115,478	115,713	1281	770
P	6	41	10	2315	4159	23	3048	2584	19,952	0.2
K	60	256	1945	1643	34,392	3975	30,332	27,266	318	22,251
Ca	4081	147	9982	31	1081	371	13,336	11,229	141,604	178
Mg	1163	35	2301	4	377	127	2678	2387	9796	153
B	25	0.03	2	33	7	55	37	35	7	2
Co	0.2	0.03	0.03	0.2	0.06	1	0.4	0.3	0.7	N.D.
Cu	N.D.	0.02	N.D.	1	0.3	3	19	17	2	N.D.
Fe	527	18	20	308	93	757	350	302	468	1
Mn	28	0.6	72	296	6	292	219	191	276	0.1
Mo	N.D.	0.44	N.D.	0.04	0.2	0.8	7	7	0.3	0.08
Zn	N.D.	N.D.	N.D.	377	0.6	280	32	27	80	N.D.

N.D.: not detected.

highest concentration of all the products. Arginine was the only amino acid detected in H3. In contrast, six different amino acids were found in H2, denoting once again the variability in the composition of biostimulant products despite their common feedstock. The EH products have the highest amino acid diversity of all biostimulants based on the humic substances and the organic matter. The HNS products contained a low concentration of free amino acids. For the seaweed products, no free amino acid content was detected.

3.2. Plant assay

The application of HNS biostimulant products resulted in the highest

Table 3
Concentration (mg/L) of free amino acids in biostimulant products.

	BC	H1	H2	H3	EH1	EH2	HNS1	HNS2	S1	S2
His	N.D.	N.D.	N.D.	N.D.	1528	2966	N.D.	6	N.D.	N.D.
Arg	N.D.	N.D.	N.D.	13,498	205	N.D.	N.D.	N.D.	N.D.	N.D.
Ala	324	N.D.	199	N.D.	N.D.	95	0.4	0.2	N.D.	N.D.
Pro	N.D.	N.D.	108	N.D.	3	25	0.2	0.05	N.D.	N.D.
Tyr	N.D.	N.D.	N.D.	N.D.	10	N.D.	N.D.	N.D.	N.D.	N.D.
Val	N.D.	N.D.	8	N.D.	43	N.D.	0.01	N.D.	N.D.	N.D.
Met	N.D.	N.D.	20	N.D.	11	N.D.	0.04	N.D.	N.D.	N.D.
Leu	N.D.	N.D.	97	N.D.	33	N.D.	0.1	N.D.	N.D.	N.D.
Ile	N.D.	N.D.	169	N.D.	N.D.	N.D.	0.4	N.D.	N.D.	N.D.
Phe	N.D.	N.D.	N.D.	N.D.	23	N.D.	N.D.	N.D.	N.D.	N.D.
Total	324	–	601	13,498	1856	3086	1	6	–	–

Histidine (His), Arginine (Arg), Alanine (Ala), Proline (Pro), Tyrosine (Tyr), Valine (Val), Methionine (Met), Leucine (Leu), Isoleucine (Ile), and Phenylalanine (Phe). N.D. not detected.

plant biomass increment (Table 4) due to their higher content of nutrients (Table 2). The seaweed-based biostimulant products (S) meant in general the lowest plant growth. The highest root growth was found for the HNS2 product followed by two products with humic substances EH2 and H2. In contrast, the lowest growth was found for the H1 product. The different treatments showed significant differences in leaf moisture (Table 4). Treatments BC, S1, and S2 presented a significantly higher percentage of leaf moisture than the other treatments. The lowest percentages of leaf moisture were found for the H1 and EH2 treatments. Concerning the total nutrient solution consumed, the highest consumption was found for plants treated with the products H3, EH2, and HNS2, being significantly higher than the BC, H1, and S2 treatments. Humic, fulvic, and algae biostimulants produced less growth, but do lead to greater water savings. The humic (H2, H3, EH2) and the humic + nutrient solution (HNS1 and HNS2) treatments showed the highest values of solution consumed, indicating low water savings.

The different Dualex parameters showed differences among treatments (Table 5). For NBI, the highest values were measured for the H3, and the lowest for the S1, S2, BC, and H2. The highest Chl values were found for H3 and EH2. Biostimulants S1, S2, B, C, and H2 presented the lowest values of chlorophyll in leaves. In contrast, the treatments S1 and S2 showed the highest values for Flav and Anth. The EH products showed in general the lowest values of Flav and Anth.

The highest value of leaves C was found for the H3, H1, and EH1 biostimulant products (Fig. 3). However, the lowest values were found for the H2 and BC, indicating clear differences among products of a similar origin. The rest of the treatments showed similar leaf carbon percentages.

The concentration of macro- and micronutrients in leaves differed among biostimulant products applied. For macronutrients, the highest nitrogen values were found for the H3 followed by the H1 treatments. However, the other humic product H2 meant the lowest N content also with the S and BC products. The concentration of P in leaves followed the opposite pattern, BC, H2, HNS, and S biostimulant treatments reached the highest content. EH2 and H3 showed the lowest content of P and K among the biostimulants. The highest leaf concentration of Ca was achieved by the biostimulant products BC, H2, and S1. For Mg, most of the biostimulant products reached a high concentration of this macronutrient. The biostimulant products with humic substances H1, H3, EH1, and EH2 produced the lowest concentration of Mg in pepper leaves. Moreover, the biostimulant group EH had in general the lowest macronutrient content in pepper leaves despite its medium-high concentration of macronutrients (Table 2).

The micronutrient concentration of B and Co was not affected by the product used (Table 6). For Cu, the only significant difference was found between EH1 and H3, being higher than the first one. The S1 product showed significantly higher Fe content in leaves than the H1, EH, and HNS products. For Mn, H2 was significantly higher than H1 and EH1. In Mo concentration, significant differences were found for H2, BC, H1, H3, and S2, following a decreasing concentration order. For the Zn micronutrient, the treatment H3 produced significantly higher values than the rest of the biostimulant products except for EH2.

In root parameters, several trends were found depending on the treatment applied. In general, the treatment HNS2 showed the highest values of P.A., S.A., A.D., and V. The other treatment with nutritional solution plus biostimulant (HNS1) also meant in general high values of P.A. and S.A. The treatments H1 and EH1 were the worst in terms of root growth with the lowest values of P.A., S.A. and V. The product H2 generated higher values in those parameters than H3 and the EH2 higher than EH1 in general. The S1 and S2 treatments generated the lowest A.D. of roots.

For tips number, the treatment EH1 meant the highest number, and the commercial product BC was the lowest. A significantly higher number of forks were found for H3 with respect to EH1. The other biostimulant products did not show significant differences. Finally, no differences among treatments were found for the crossings number.

N NEI values showed that the S products increased their N content in the highest proportion, meaning the highest biostimulation rate for this nutrient assimilation rate. For P, the S2 product generated the highest index, followed by the BC, H2, and EH2 products. K was higher for the BC product, followed by the H and S1 products. Ca and Mg followed a similar trend with higher values for H3, followed by H1, S2 and EH2.

For the NEIs of micronutrients, the product S2 generated the highest values, implying that lower nutrient addition, generated the highest concentration in leaves. The H products had high values for certain elements such as B, Cu, Mn, and Mo. EH products, except for Mo,

Table 4
Leaves, stem, and root fresh weights (mg) leaves humidity percentage (%LH), and percentage of biostimulant solution consumption (% CS) of pepper plants treated with the biostimulant products. Data were represented as the mean ± S.D., Different letters indicate significant differences among treatments (Duncan post-hoc test; $p \leq 0.05$, $n = 6$).

	BC	H1	H2	H3	EH1	EH2	HNS1	HNS2	S1	S2
Leaves	3.1 ± 0.2^{bc}	2.9 ± 0.7^{bc}	3.1 ± 0.7^{bc}	3.8 ± 0.8^{ab}	3.3 ± 0.4^{bc}	3 ± 1^{bc}	3.8 ± 0.9^{ab}	4.4 ± 0.6^{a}	2.8 ± 0.3^{c}	3.2 ± 0.4^{bc}
Stem	2.7 ± 0.8^{cd}	2.9 ± 0.3^{cd}	2.8 ± 0.2^{cd}	3.3 ± 0.2^{ab}	3.2 ± 0.3^{bc}	3.2 ± 0.4^{bc}	3.7 ± 0.5^{a}	3.6 ± 0.3^{ab}	2.7 ± 0.2^{d}	2.8 ± 0.3^{cd}
Root	3.5 ± 0.8^{bcd}	1.4 ± 0.5^{e}	4.3 ± 0.5^{abc}	3 ± 1^{bcd}	3.2 ± 0.4^{cd}	4 ± 1^{ab}	3 ± 1^{d}	5.4 ± 0.4^{a}	3.6 ± 0.6^{bcd}	2.8 ± 0.5^{d}
%LH	88.2 ± 0.5^{a}	84.0 ± 0.7^{d}	87.6 ± 0.8^{ab}	85.6 ± 0.8^{cd}	87.2 ± 0.6^{abc}	84 ± 2^{d}	86 ± 1^{bcd}	85.8 ± 0.5^{bcd}	87.8 ± 0.4^{a}	87.8 ± 0.5^{a}
%SC	94 ± 3^{bc}	93 ± 2^{c}	96.1 ± 0.9^{ab}	97 ± 1^{a}	95 ± 2^{abc}	97 ± 1^{a}	96.4 ± 0.4^{ab}	97 ± 1^{a}	95 ± 2^{abc}	94 ± 3^{bc}

Table 5
Dualex indexes of nitrogen balance index (NBI), total chlorophylls (Chl), flavonols (Flav), and anthocyanins (Anth) were measured in the fresh leaves of pepper plants treated with the biostimulant products. Data are represented as the mean ± S.D. Different letters indicate significant differences among treatments (Duncan post-hoc test; $p \leq 0.05$, $n = 6$).

	BC	H1	H2	H3	EH1	EH2	HNS1	HNS2	S1	S2
NBI	13± 2^{e}	19.8 ± 0.9^{cd}	12.7 ± 0.8^{e}	30 ± 1^{a}	22.3 ± 0.1^{bc}	25 ± 3^{b}	17.2 ± 0.4^{d}	19 ± 2^{cd}	12 ± 1^{e}	10.8 ± 0.9^{e}
Chl	21 ± 2^{ef}	29± 3^{cd}	21± 1^{ef}	36 ± 3^{a}	29.5 ± 0.4^{bc}	34 ± 4^{ab}	24.2 ± 0.5^{de}	28 ± 2^{cd}	19 ± 1^{ef}	18.9 ± 0.8^{f}
Flav	1.65 ± 0.09^{b}	1.45 ± 0.07^{cd}	1.66 ± 0.01^{b}	1.27 ± 0.03^{e}	1.35 ± 0de	1.42 ± 0.01^{cd}	1.44 ± 0.01^{cd}	1.49 ± 0.03^{c}	1.68 ± 0.05^{ab}	1.78 ± 0.07^{a}
Anth	0.13 ± 0.00^{ab}	0.11 ± 0.01^{bc}	0.15 ± 0.01^{a}	0.11 ± 0.01^{bc}	0.11 ± 0.01^{bc}	0.10 ± 0.02^{c}	0.12 ± 0.01^{bc}	0.12 ± 0.01^{bc}	0.16 ± 0.01^{a}	0.16 ± 0.01^{a}

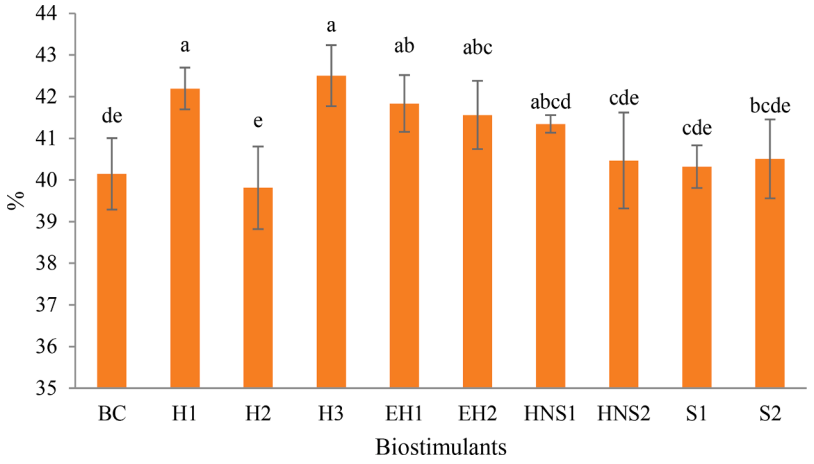

Fig. 3. Carbon percentages of plant leaves. Data were represented as the mean ± S.D. Different letters indicate significant differences among treatments (Duncan post-hoc test; $p \leq 0.05$, $n = 6$).

Table 6
Concentration of macro- and micronutrients in the plant leaves. Data were represented as the mean ± S.D. Different letters indicate significant differences among treatments (Duncan post-hoc test; $p \leq 0.05$, $n = 6$).

	BC	H1	H2	H3	EH1	EH2	HNS1	HNS2	S1	S2
g/kg										
N	16.1 ± 2^{f}	26± 3^{b}	16 ± 1^{f}	33 ± 4^{a}	23 ± 2^{c}	21 ± 1^{d}	20 ± 2^{d}	19 ± 1de	17.2 ± 0.8^{ef}	17 ± 1^{ef}
P	1.6 ± 0.1^{abc}	0.96±0.03^{d}	1.61±0.06^{ab}	1.3 ± 0.2^{c}	1.4 ± 0.2^{bc}	0.9 ± 0.1^{d}	1.6 ± 0.1^{abc}	1.8 ± 0.2^{a}	1.8 ± 0.2^{a}	1.6 ± 0.1^{ab}
K	41.4 ± 0.8^{a}	35 ± 1 ^{bc}	39 ± 1^{ab}	30 ± 4 ^{c}	40 ± 3^{a}	31 ± 2^{c}	38 ± 3^{ab}	38 ± 2^{ab}	40 ± 3^{a}	40 ± 3^{a}
Ca	13.5 ± 0.9^{ab}	8.1 ± 0.4^{d}	14 ± 1^{a}	8.2 ± 0.5^{d}	8.8 ± 0.8^{d}	9 ± 1^{d}	10.5 ± 0.7^{c}	12 ± 2^{c}	13.8 ± 0.5^{a}	12.1 ± 0.7^{bc}
Mg	3.2 ± 0.2^{a}	2.2 ± 0.2^{b}	3.2 ± 0.2^{a}	2.0 ± 0.3^{b}	2.3 ± 0.2^{b}	2.2 ± 0.2^{b}	3.0 ± 0.4^{a}	2.9 ± 0.1^{a}	3.3 ± 0.3^{a}	3.0 ± 0.1^{a}
mg/kg										
B	63 ± 25^{a}	29 ± 10^{a}	92 ± 87^{a}	44 ± 1^{a}	35 ± 16^{a}	51 ± 4^{a}	45 ± 10^{a}	47 ± 7^{a}	62 ± 31^{a}	55 ± 33^{a}
Co	0.07±0.02^{a}	0.06±0.04^{a}	0.0 ± 0.03^{a}	0.05±0.02^{a}	0.04±0.03^{a}	0.2 ± 0.2^{a}	0.06±0.03^{a}	0.04±0.01^{a}	0.05±0.02^{a}	0.5 ± 0.7^{a}
Cu	5 ± 3^{ab}	3 ± 2^{ab}	2 ± 1^{ab}	2 ± 2^{b}	6 ± 3^{a}	2 ± 2^{ab}	2 ± 1^{ab}	2 ± 1^{ab}	5 ± 1^{ab}	3.2 ± 0.6^{ab}
Fe	72 ± 19^{ab}	46 ± 4^{b}	67 ± 13^{ab}	59 ± 18^{ab}	52.1 ± 0.3^{b}	49 ± 13^{b}	52 ± 3^{b}	54 ± 7^{b}	82 ± 25^{a}	58 ± 8^{ab}
Mn	45 ± 1^{ab}	26 ± 2^{c}	50 ± 3^{a}	41 ± 19^{ab}	32 ± 5^{bc}	37 ± 4^{abc}	42 ± 8^{ab}	41 ± 5^{ab}	40 ± 1^{ab}	39 ± 1^{ab}
Mo	22 ± 5^{b}	11 ± 4^{d}	37 ± 5^{a}	13 ± 5^{cd}	14 ± 7^{bcd}	17 ± 3^{bcd}	14± 2^{bcd}	14 ± 3^{bcd}	21 ± 5^{bc}	13 ± 2^{cd}
Zn	37 ± 2^{b}	30 ± 6^{b}	31 ± 5^{b}	61 ± 31^{a}	29 ± 4^{b}	43 ± 8^{ab}	34 ± 7^{b}	33 ± 6^{b}	34 ± 2^{b}	39 ± 7^{b}

Table 7
Root parameters were obtained with the Win-RHIZO software. Data were represented as the mean ± S.D. Different letters indicate significant differences among treatments (Duncan post-hoc test; $p \leq 0.05$, $n = 6$).

	BC	H1	H2	H3	EH1	EH2	HNS1	HNS2	S1	S2
L.	234 ± 28^{ab}	184 ± 30^{c}	226 ± 46^{abc}	232 ± 29^{ab}	190 ± 31^{bc}	209 ± 33^{abc}	249 ± 24^{a}	228 ± 43^{abc}	256 ± 26^{a}	235 ± 26^{ab}
P.A.	30 ± 3^{bcd}	23 ± 3^{f}	31 ± 4^{abc}	28 ± 5^{cde}	24 ± 3^{ef}	30 ± 3 ^{abcd}	33 ± 2^{ab}	35 ± 5^{a}	29 ± 2^{bcd}	26 ± 3^{def}
S.A.	93 ± 11^{bcd}	72 ± 9^{f}	98 ± 13^{abc}	88 ± 14 ^{cde}	76 ± 9^{ef}	95 ± 10^{abcd}	104 ± 5^{ab}	109 ± 15^{a}	92 ± 6^{bcd}	82 ± 9 ^{def}
A.D.	1.3 ± 0.1^{abcd}	1.3 ± 0.2^{abcd}	1.4 ± 0.2^{abc}	1.2 ± 0.2^{bcd}	1.3 ± 0.1^{abcd}	1.5 ± 0.4^{ab}	1.3 ± 0.1^{abcd}	1.5 ± 0.1^{a}	1.2 ± 0.2^{cd}	1.1 ± 0.1^{d}
V.	3.0 ± 0.6^{bcd}	2.3 ± 0.5^{d}	3.4 ± 0.4^{abc}	3 ± 1^{bcd}	2.4 ± 0.3^{d}	4 ± 1 ^{ab}	3.5 ± 0.4^{abc}	4.2 ± 0.4^{a}	2.7 ± 0.5^{cd}	2.3 ± 0.4 ^{d}
Tips	186 ± 30^{c}	278 ± 84^{ab}	239 ± 38^{bc}	234 ± 39 ^{bc}	312 ± 78^{a}	260 ± 64^{abc}	229 ± 9^{bc}	224 ± 21^{bc}	255 ± 37^{abc}	240 ± 23^{bc}
Forks	1787 ± 115^{ab}	2056 ± 581^{ab}	1982 ± 325^{ab}	2290 ± 526^{a}	1704 ± 325^{b}	1861 ± 577 ^{ab}	1983 ± 262^{ab}	1740 ± 428^{ab}	1999 ±193^{ab}	1981 ± 291^{ab}
Crossings	31 ± 11^{a}	51 ± 20^{a}	36 ± 7^{a}	44 ± 37^{a}	26 ± 10^{a}	27 ± 21^{a}	35 ± 12^{a}	34 ± 13^{a}	41 ± 11^{a}	28 ± 8^{a}

L. Length, cm; P.A. Projected Area, cm^2; S.A. Surface Area, cm^2; A.D. Average Diameter, mm; V. Volume, cm^3.

Table 8
Nutritional Efficiency Index of macro and micronutrients. Data were represented as the mean ± S.D. Different letters indicate significant differences among treatments (Duncan post-hoc test; $p \leq 0.05$, $n = 6$).

	BC	H1	H2	H3	EH1	EH2	HNS1	HNS2	S1	S2
N	1.24 ± 0.06^{c}	0.03 ± 0.01 g	0.33 ± 0.02^{d}	0.04 ± 0.01^{fg}	0.08 ± 0.01^{e}	0.06 ± 0.01^{ef}	0.06 ± 0.01^{ef}	0.07 ± 0.01^{e}	10.5 ± 0.7^{b}	12 ± 2^{a}
P	65 ± 6^{b}	7 ± 1^{e}	38 ± 2^{c}	0.18 ± 0.03 g	0.09 ± 0.01^{h}	14 ± 4^{d}	0.16 ± 0.05 g	0.26 ± 0.05^{f}	0.04 ± 0.01^{h}	4176 ± 271^{a}
K	156 ± 7^{a}	40 ± 7^{c}	4.7 ± 0.3^{e}	5.7 ± 0.9^{d}	0.31 ± 0.04^{i}	2.6 ± 0.5^{f}	0.4 ± 0.1^{hi}	0.53 ± 0.09^{h}	53 ± 7^{b}	0.9 ± 0.1 g
Ca	0.75 ± 0.07^{f}	16 ± 4^{c}	0.33 ± 0.04 g^{h}	83 ± 19^{a}	2.1 ± 0.3^{e}	8 ± 2^{d}	0.25 ± 0.08^{h}	0.4 ± 0.1 g	0.04 ± 0.01^{i}	33 ± 3^{b}
Mg	0.63 ± 0.07 g	18 ± 2^{b}	0.33 ± 0.03^{h}	162 ± 25^{a}	1.6 ± 0.2^{f}	6 ± 1^{e}	0.34 ± 0.06^{h}	0.46 ± 0.04^{h}	0.15 ± 0.02^{i}	9.5 ± 0.7^{c}
B	0.6 ± 0.3^{d}	315 ± 75^{a}	10 ± 9^{bc}	0.4 ± 0.1^{d}	1.4 ± 0.7^{cd}	0.30 ± 0.07^{d}	0.4 ± 0.1^{d}	0.51 ± 0.09^{d}	4 ± 2^{cd}	17 ± 10^{b}
Co	0.09 ± 0.03^{c}	0.6 ± 0.5^{b}	0.4 ± 0.3^{bc}	0.09 ± 0.04^{c}	0.2 ± 0.1^{c}	0.06 ± 0.06^{c}	0.05 ± 0.03^{c}	0.05 ± 0.01^{c}	0.03 ± 0.01^{c}	60 ± 83^{a}
Cu	0^{e}	63 ± 20^{a}	0^{e}	0.3 ± 0.3^{d}	5 ± 2^{b}	0.2 ± 0.2^{de}	0.03 ± 0.02^{e}	0.05 ± 0.03^{e}	1.2 ± 0.3^{c}	0^{e}
Fe	0.03 ± 0.01^{fg}	0.77 ± 0.09^{b}	0.8 ± 0.2^{b}	0.058 ± 0.003^{de}	0.148 ± 0.007^{c}	0.021 ± 0.006 g	0.05 ± 0.01^{ef}	0.07 ± 0.01^{d}	0.07 ± 0.02^{d}	22.2 ± 0.6^{a}
Mn	0.36 ± 0.02^{d}	13 ± 3^{b}	0.16 ± 0.01^{e}	0.04 ± 0.01 g	1.4 ± 0.3^{c}	0.04 ± 0.01 g	0.06 ± 0.01^{fg}	0.08 ± 0.02^{f}	0.06 ± 0.01^{fg}	135 ± 20^{a}
Mo	0^{f}	7 ± 3^{d}	0^{f}	96 ± 49^{a}	21 ± 10^{c}	7 ± 2^{d}	0.59 ± 0.08^{e}	0.8 ± 0.2^{e}	31 ± 9^{b}	76 ± 8^{a}
Zn	0^{f}	0^{f}	0^{f}	0.05 ± 0.01^{e}	12 ± 2^{a}	0.05 ± 0.01^{e}	0.3 ± 0.1^{c}	0.45 ± 0.05^{b}	0.18 ± 0.02^{d}	0^{f}

generated lower micronutrient NEIs. Among all the biostimulants, in general, HNS products showed the lowest NEIs values for micronutrients.

The comparison of the products grouped by their nature showed several trends and differences (Table 9). Biostimulant products HNS get higher weights of leaves and stems than the other treatments, but no significant differences were found for root weight among treatments. S and the commercial product BC get the highest water contents in plant leaves and the lowest total solution consumed by plants together with H products. The EH products were better for the chlorophyll content (NBI and Chl) but Flav and Anth were the S products. H, EH, and S generated the highest content of Carbon in leaves.

For the macro and micronutrients, the differences were less clear, generating the commercial product BC the higher values in general. The humic products H and EH generated the highest content of N and the lowest concentrations for the rest of the macronutrients. The only micronutrient concentration differences among the groups were the Cu and Fe, having H and HNS the lowest values for the first and C being significantly higher than EH for the second. Root parameters were higher when the nutritional solution was used HNS; except for the tips number which was significantly lower than for EH.

Because the carbon was obtained by the plant through the air, the NEI value for this element was not calculated, instead, a Pearson correlation ($p \leq 0.05$) was done between the carbon added with the biostimulants and the carbon found in leaves. No correlation was found (sig. 0.803) indicating no relation between C concentration and biostimulant use.

The study of the Nutritional Balance Index (NEI) of the biostimulant groups showed that no differences were found for Ca, Mg, B, Co, and Cu assimilation and concentration in plant leaves. For N, the S group had the highest NEI, followed by C. The P NEI was also found to be the highest if S products were used. K NEI highest value was for C followed by H and S. For micronutrients, S products showed the highest values for Fe, Mn, and Mo (for Mo only significantly higher than C and HNS). However, Zn was found higher for the EH products.

The TEM images showed differences in the root cell morphology (Fig. 4). Three different groups were done depending on the size of the root vacuole area generated by the biostimulants. The biggest vacuole size was generated by the products H1, H3, EH1, HNS1, and S2, with mainly the whole-cell occupied by them. Medium vacuole size was obtained by the products BC, HNS2, and S1. And finally, the lowest vacuole volume was produced by the products H2 and EH2. These results denote once again the variability of effects produced in plants by biostimulant products of the same group.

4. Discussion

All the products tested had a different composition in terms of nutritional content (Table 2), carbon content and functional groups (Figs. 1 and 2). Several parameters like free amino acids could explain the activity shown for the pepper plants. In the ecological humic products (EH), the organic matter or the metal cations that it contained could protect the amino acids from degradation, supplying nutrients to the plants (Ghasemi et al., 2012). The amino acid alanine is used by plants as nitrogen storage (Carillo et al., 2019); meanwhile, glycine is believed to enhance flowering and maintain the water balance between the plant cell and its environment (Khan et al., 2020). This better nutrition also meant a higher value of total solution consumed (EH product treatments). This measure is especially important because it highlights the water savings of each of the biostimulants. If we overlap growth and solution consumption, we observe that, in general, biostimulants with nutritional solutions produce greater growth in plants as was also seen by Da Cunha Leme Filho et al. (2021). This is shown in Fig. 5A where the plant parameters were represented depending on the biostimulant group. Commercial (C) and Seaweed (S) were related with the Flav, Anth, and %LH, as was previously studied (Salvi et al., 2019). The biostimulant with nutritional solution (HNS) showed a positive relationship with the leaves and stem growth since more nutrients were supplied for the plants and nutrient solution consumption. The ecologic humic group (EH) was related to higher contents of Chl and NBI, showing the ability of humic substances to positively affect the protein and chlorophyll contents (Pizzeghello et al., 2013). This ability was also seen for the humic biostimulant group (H) which was in general positively correlated with all the plant parameters except Flav, Anth, and LH. However, to fully understand the multiple responses of plants treated with different biostimulants, they must be correlated with the phenotype changes to connect molecular changes with activated physiological pathways via the omics studies (Franzoni et al., 2022).

The humic products (H group) were correlated with all the nutrients due to the high dispersion they had (Fig. 5B). The H products had clear relations with the component 1 and negative with the component 2 (H2); positive (H3) and negative (H1) for the component 2 with both

Table 9
ANOVA analysis of the plant parameters grouped the different products according to their composition: C (commercial product), H (humic), EH (ecological and humic), HNS (humic + nutritional solution), and S (seaweed). (Duncan post-hoc test; $p \leq 0.05$).

Plant Parameters	Nutritional Content									Root Analysis					NEI					
	C	H	EH	HNS	S		C	H	EH	HNS	S		C	H	EH	HNS	S		C	H	EH	HNS	S
Leaves (g)	b	b	b	a	b	N	d	a	ab	bc	cd	L.	a	ab	b	a	a	N	b	c	c	c	a
Stem (g)	c	bc	b	a	c	P	ab	bc	c	a	a	P.A.	b	b	b	a	b	P	b	b	b	b	a
Root (g)	a	a	a	a	a	K	a	c	bc	abc	ab	S.A.	b	b	b	a	b	K	a	b	c	c	b
LH (%)	a	b	b	b	a	Ca	a	c	c	ab	a	A.D.	ab	ab	a	a	b	Ca	a	a	a	a	a
SC (%)	c	abc	ab	a	bc	Mg	a	bc	c	ab	a	V.	b	b	b	a	b	Mg	a	a	a	a	a
NBI	bc	ab	a	abc	c	B	a	a	a	a	a	Tips	c	ab	a	bc	ab	B	a	a	a	a	a
Chl	bc	ab	a	abc	c	Co	a	a	a	a	a	Forks	a	a	a	a	a	Co	a	a	a	a	a
Flav	ab	bc	c	bc	a	Cu	a	c	ab	c	ab	Crossings	a	a	a	a	a	Cu	a	a	a	a	a
Anth	ab	b	b	b	a	Fe	a	ab	b	ab	ab							Fe	b	b	b	b	a
C (%)	c	ab	a	abc	bc	Mn	a	a	a	a	a							Mn	b	b	b	b	a
						Mo	a	a	a	a	a							Mo	b	ab	ab	b	a
						Zn	a	a	a	a	a							Zn	b	b	a	b	b

L. Length, cm; P.A. Projected Area, cm^2; S.A. Surface Area, cm^2; A.D. Average Diameter, mm; V. Volume, cm^3.

negative correlations with the component 1. Although the differences between H products led to different correlations, a direct relationship between fertilization with nutrients and the increase in chlorophylls showed in Table 2 (Latsague et al., 2014). Nitrogen is closely linked to the concentration of chlorophylls, since this is necessary for the formation of chlorophylls there are chlorophyll meters that are used to add nitrogen to crops for optimal fertilizations that meet the optimal requirement (Dordas and Sioulas, 2008; Gianquinto et al., 2004). Humic substances are able to improve plant mineral nutrition through the activation of the main actors involved in nutrient root uptake and further transport and metabolism within the plant (Olaetxea et al., 2018). In general, the commercial biostimulant (C) and the seaweed (S) products had the same behavior being positively correlated with component 1 (Cu, Mo, K, Mg, Ca, and P) and the EH products being negatively correlated with this component. The HNS biostimulants were between those two groups. Although biostimulants can increase the leaf nutrient content of plants, the concentrations vary depending on the biostimulant and the nutrient itself (Dehkordi et al., 2021; Rady and Rehman, 2016; Wang et al., 2022).

The H group was the most correlated with all the root parameters and the commercial biostimulant group (C) was the least (Fig. 5C). Humic biostimulants are able to increase the root system volume (Rady and Rehman, 2016). The S group was correlated with the number of crossings, tips, and forks, and EH with tips number and average root diameter (AD) as seen by Dehkordi et al. (2021). Lastly, HNS was correlated with project and surface areas (PA and SA), root volume (V), and AD. The study of the root is key since root length, surface area, and the number of tips are important indicators of water and nutrient uptake potential, and root diameter is an important parameter for rhizosphere modeling (Pang et al., 2011). Several of the parameters obtained with the Win Rhizo are closely related to nutrient absorption and transportation. Thus, the total root length was correlated with the phosphorus absorption; the diameter of roots with nitrogen absorption; the root surface area with soil moisture; the average root link length with droughts; and the root surface area and volume with soil nitrogen concentration (Xiaoting et al., 2019).

The Nutritional Efficiency Index (NEI) differed more than other parameters among the biostimulant groups. Clear relation was found for Mg, Cu, and B and the H group (Fig. 5D). The HNS biostimulants group was related to K and Zn accumulation in leaves and the S with the nutrients Co, Mn, Fe, P, and N (component 1). However, groups C and EH were negatively correlated with components 1 and 2, indicating the same behavior. The absorption and accumulation of Zn, Cu, Co, Mo, and Mn were also improved when these minerals were applied together with biostimulants (Messias et al., 2015). The addition of biostimulant was previously seen to maintain plant status in reduced macronutrient fertilization (Koleška et al., 2017), also seen with its mixture with inorganic nutrients (Raposo et al., 2013). The study of foliar mineral status is important because is directly related to different organs of the plants (Zouari et al., 2020), affecting the total plant growth (Vătcă et al., 2020).

The effects on the root morphology were more related to the particular product used than to the group of biostimulants (Fig. 5). Several biostimulants like the humic-based have the ability to induce the formation of a large vacuole in root cells, with the dimension reaching almost the complete cell volume (Antón-Herrero et al., 2020). In our study, several products produced higher cell vacuole volume than the commercial product BC, meaning a potential higher water savings and hydric stress resistance by increasing the water retention in roots. Root morphology and architecture reflect the efficiency of root structural carbon investment in the root area to maximize nutrient and water uptake. Changes in surface area per unit of root biomass mean more rapid nutrient uptake per unit of root mass (Xiang et al., 2013). The vacuoles of plants have several physiological functions shifting their function and form following physiological situations and developmental. Plant roots optimize their root architecture to acquire water and

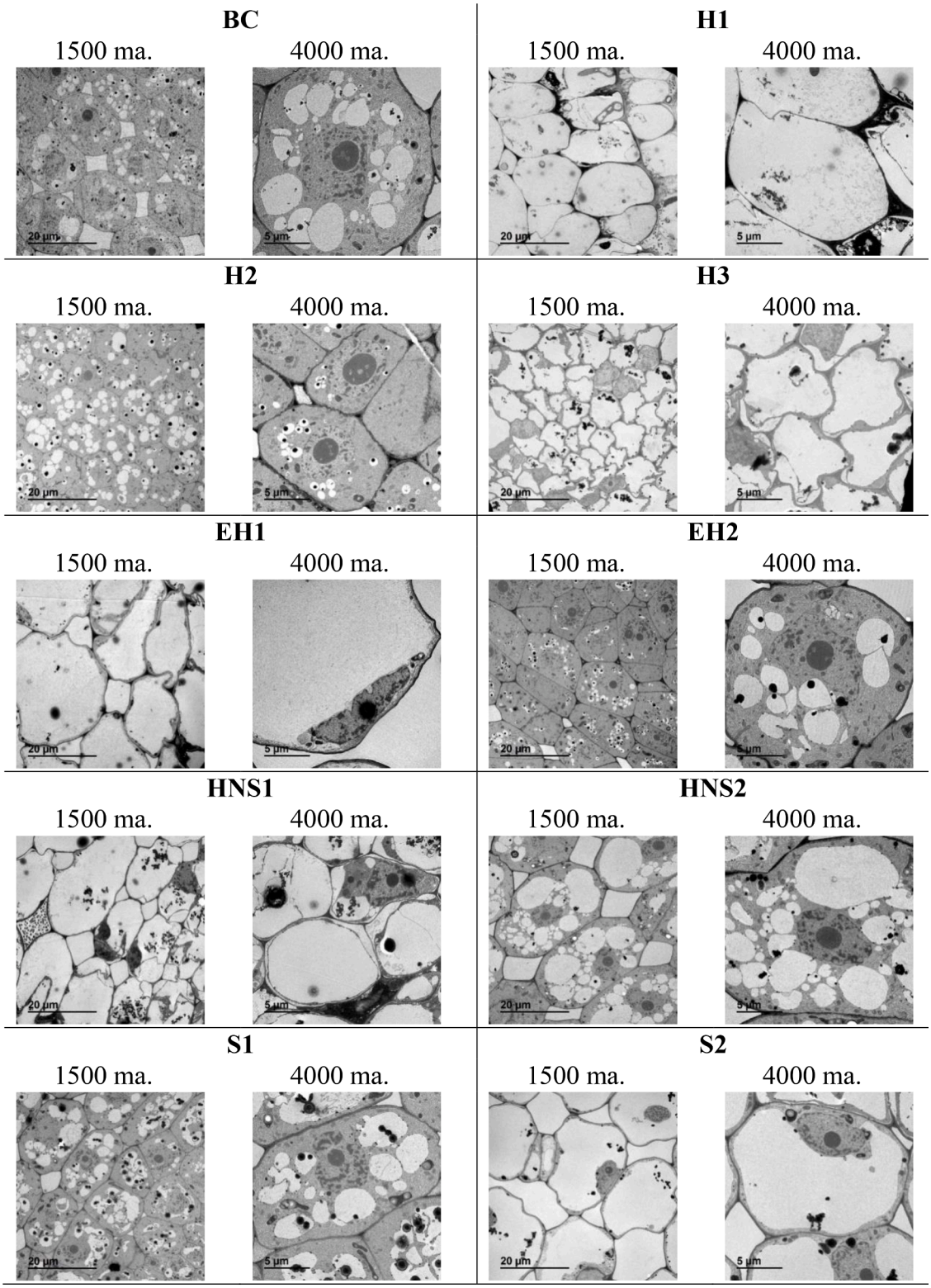

Fig. 4. Transversal images of the roots of pepper plants fertigated with the biostimulant solutions at 1500 and 4000 magnifications obtained by transmission electron microscopy (TEM).

essential nutrients from the soil (Aljuaifari et al., 2018). In fact, a direct relation was found between the vacuole volume in cells and the percentage of solution consumed, with for example the product EH2 generating the highest %SC (Table 4) and the lowest vacuole volume (Fig. 4) and the product H1 with the lowest solution consumption but a higher vacuole volume. This trend meant that in general, the higher the volume of the vacuoles was, the lowest solution those plants consumed, implying a potential water saving.

Recently, studies on how to applicate biostimulants are increasing, developing new forms of protecting the active compounds (Amirkhani et al., 2019). However, methodologies of how to test the biostimulant effects are still scarce. Several are based on the measurement of

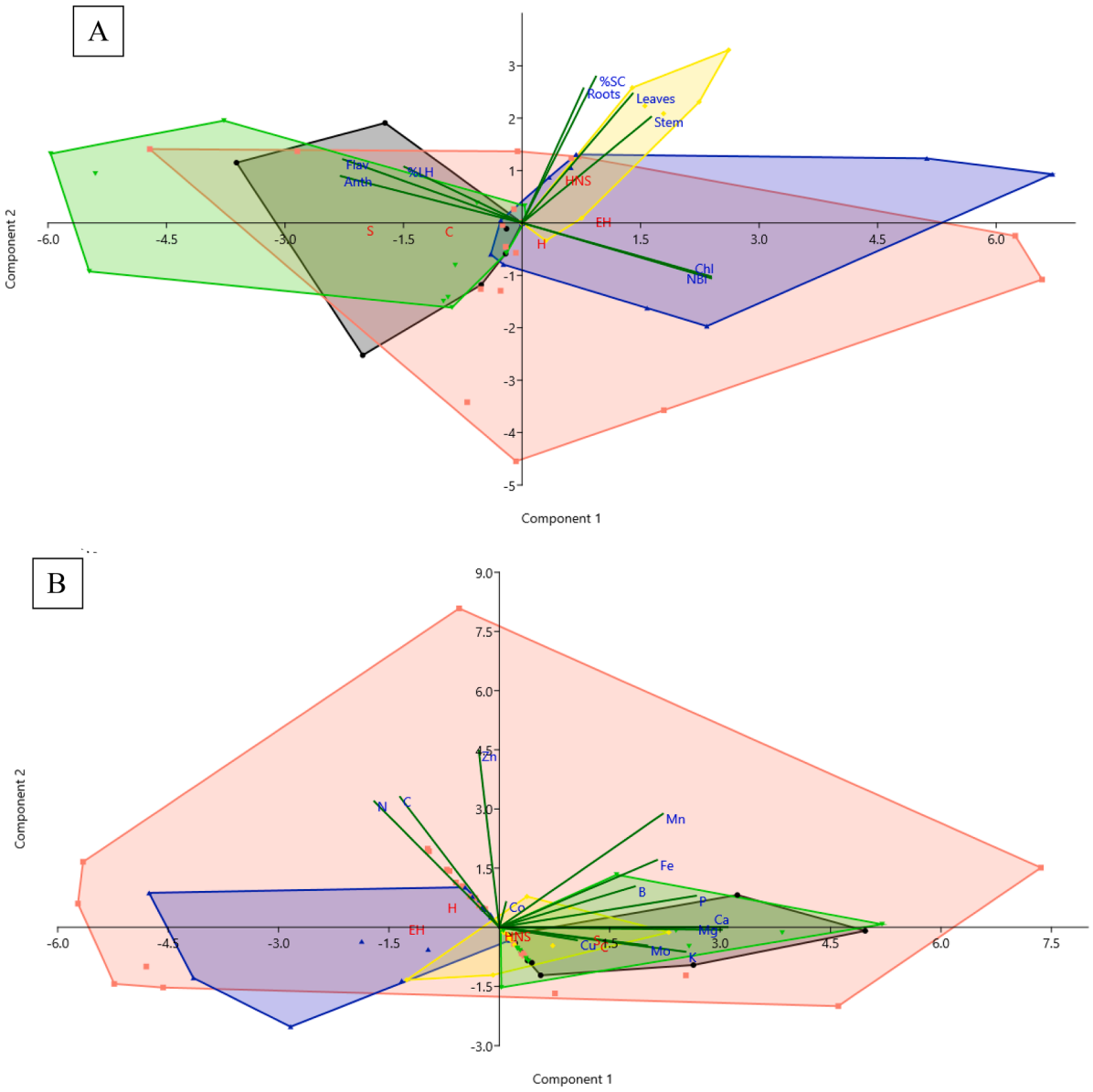

Fig. 5. Principal component analysis of the studied parameters depending on their group: C (commercial product) in black, H (humic) in red, EH (ecological and humic) in blue, HNS (biostimulant + nutritional solution) in yellow, and S (seaweed) in green. A: plant growth and chlorophyll activities (leaves humidity percentage,%LH; percentage of the total solution consumed by the plants,%SC; nitrogen balance index, NBI; chlorophylls activity, Chl; flavonols, Flav and anthocyanins, Anth). B: nutritional leaf content. C: root parameters (length, L.; Projected Area, P.A.; Surface Area, S.A; Average Diameter, A.D.; Volume, V., number of crossings, tips, and forks). D: Nutritional Efficiency Index off each nutrient.

inhibition of watercress root or chicory hypocotyledon; since bioassays are useful as a preliminary screen of biostimulants effects on plants (Migliore et al., 2012; Summerer et al., 2012). Other proposed methodologies were based also on yeast plus plant assays over a period of six months. With the proposed methodology of controlled hydroponic plant growth with the study of growth, nutritional and morphological parameters, we have found interesting patterns of the way different biostimulants affected plants. Completed with the NEI data, this methodology could be used to preliminary test the efficiency and the effects of different potentially commercial products.

5. Conclusions

The biostimulant products tested had different effects on plant parameters depending on the origin they have. Among them, the humic-based products seem to have the best overall properties. The proposed methodology seems to be a promising way to analyze the biostimulant activity of a commercial product in a short-term assay, being able to be used as a screening tool to develop new functional products for the market.

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CRediT authorship contribution statement

Rafael Antón-Herrero: Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Carlos**

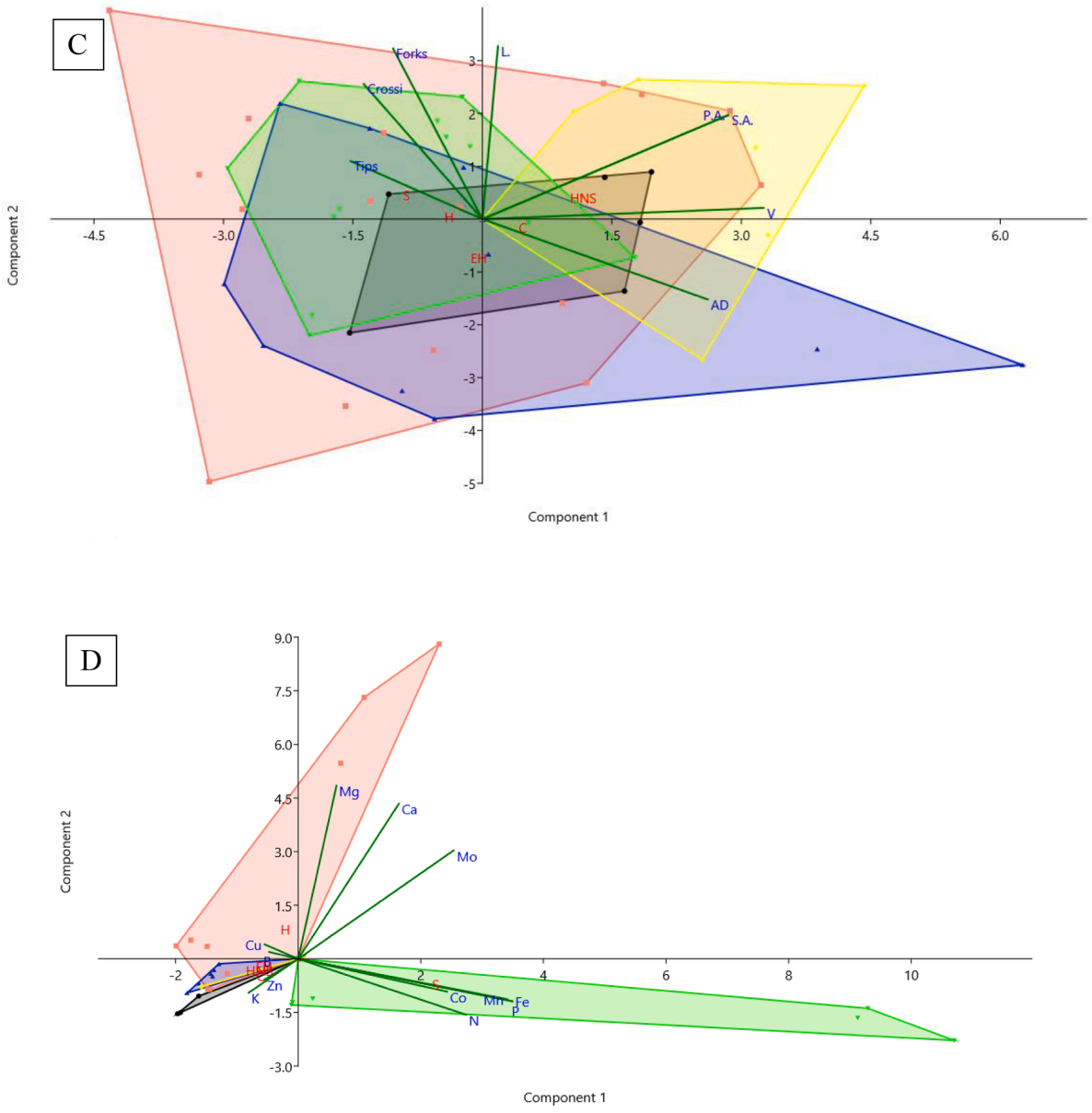

Fig. 5. (*continued*).

García-Delgado: Methodology, Writing – original draft, Supervision, Project administration, Writing – review & editing. **Gabriel Antón-Herrero:** Writing – review & editing. **Begoña Mayans:** Validation, Formal analysis, Investigation, Writing – review & editing. **Laura Delgado-Moreno:** Supervision, Writing – review & editing. **Enrique Eymar:** Methodology, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data, and in the writing of the manuscript.

Data Availability

Data will be made available on request.

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