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Effect of Biostimulant Application on Plant Growth, Chlorophylls and Hydrophilic Antioxidant Activity of Spinach (*Spinacia oleracea* L.) Grown under Saline Stress

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Abstract: Irrigated agricultural lands are prone to salinity problems which may imperil horticultural crops by reducing growth, yield and even qualitative traits. Eco-friendly approaches such as biostimulant application and in particular protein hydrolysates from vegetal origin are implemented to mitigate salinity stress effects on crops. For this reason, a greenhouse experiment on spinach irrigated with increasing concentrations of saline water (EC = 3 dS m⁻¹ (EC3), 6 dS m⁻¹ (EC6) and 9 dS m⁻¹ (EC9), in addition to non-saline treatment (EC0)) was organized, while plants were subjected to foliar applications of a protein hydrolysate from vegetal origin on a weekly basis. The application of this biostimulant helped mitigate the adverse effects of saline stress, by increasing the SPAD index and total chlorophylls of spinach plants. Yield was significantly boosted under biostimulant treatment in saline conditions and reached the value obtained in control treatment (no biostimulants added) × EC0 in the case of EC 3 and 6 dS m⁻¹. In addition, the number of leaves and plants m⁻¹ was increased under biostimulant treatment, and most importantly the hydrophilic antioxidant activity of spinach, thus a qualitative aspect of great importance was also increased. Such results increase the knowledge on the effects of protein hydrolysates application on an important leafy vegetable and may help growers mitigate saline conditions and maintain high crop yield and high quality of the final product when no other source of irrigation water is available.

Keywords: salt stress; abiotic stress; legume-derived protein hydrolysate; leafy vegetables; foliar application; nitrates; quality



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1. Introduction

Agricultural lands compromised by salt accumulation are on a continuous increasing rate, with more than 20% of irrigated lands being affected [1–3]. This problem is aggravated by climate change, disproportionate implication of groundwater in coastal areas, intensive farming, low quality of irrigation water, and impaired drainage [2] in addition to flooding, and rising aquifers [3]. Irrigation water of good quality is a flourishing matter when opting to reach food demand, thus leading to the suggestion of using alternative sources of water [4,5]. Under saline conditions, the electrical conductivity (EC) of the soil tends to surpass 4 dS m⁻¹ in the root zone, provoking a higher osmotic pressure and disrupting plant–water relations [6], reducing the growth, yield and quality traits of plants [2,6–8]. Nonetheless, high salinity stress induces oxidative stress and boosts the defense mechanisms of plants, which induces a higher secondary metabolite content including phenolic compounds [9].

Recently, new approaches have been introduced to ameliorate the sustainability and resilience of production systems by involving natural products such as plant biostimulants that are appraised for being eco-friendly and capable of boosting the performance and quality of crops, especially under stress conditions [6,10–12]. As stated by Tsouvaltzis et al. [13], there is a high demand for eco-friendly organic materials derived from plants to be applied in agriculture in order to boost crop yield and improve nutrient-use efficiency, while particular interest has been shown regarding mixed amino acids solutions that can improve shoots fresh and dry weight. In this context, protein hydrolysates (PHs) are a promising category of biostimulants of vegetal origin that engender positive outcomes on crop performance, via increasing nutrient availability, uptake and metabolic use, and improving the quality of vegetables with reference to phytochemical content [10–12]. As mentioned by the previous authors, PHs effect is due to their interference in phytohormone balance in plants by including peptides with hormone-like activities and precursors of phytohormones in its composition. As stated by Srivastava [14], biostimulants are oriented as gears to resist against abiotic stress, since they are endowed with bioactive molecules that regulate the physiology and metabolism of plants (signaling cascades). In particular, PHs can prevent yield losses induced by adverse soil conditions such as salinity and alkalinity [12]. When sprayed on leaves, PHs improve plant tolerance to salinity by inducing the accumulation of protective compounds with osmotic and antioxidant activities that alleviate the production cut back under stress [15–17].

Spinach is a highly appreciated leafy vegetable, known for its rich biological value, as it is abundant in antioxidant molecules that procure defensive properties in the wellbeing of humans, such as phenols and carotenoids [5,10]. These characteristics are particularly preserved when spinach is consumed raw, steamed or partially boiled [18]. Moreover, spinach is a great source of vitamins (A, C, B9, etc.), magnesium, calcium, potassium, manganese, and iron, as well as dietary fibers [18]. It is known to be available year-round and can be found in the market in different forms such as a fresh product ready for consumption or as frozen food [10]. Spinach as a glycophytic chenopod is considered a slightly salt-sensitive vegetable with an expected tolerance threshold of 2 dS m⁻¹ [5,7]. Such tolerance is crucial due to the cash value that leafy vegetables represent in comparison to field crops [5,7], and knowing that an increased salinity of soil or water irrigation can cut back spinach fresh yield is essential for the growers [4].

Based on the above-mentioned facts and considering the increasing concerns about the reduction in irrigation water availability and the degradation of water quality due to anthropogenic activities or rapidly changing environmental conditions, the aim of the present study was to determine the efficacy of a legume-derived protein hydrolysate in alleviating the detrimental effect of irrigation with saline water on yield and qualitative traits of baby spinach grown in greenhouse conditions.

2. Materials and Methods

2.1. Experimental Design, Crop Management, Saline Irrigation and Biostimulant Application

The test was carried at the experimental site (Gussone Park) of Department of Agricultural Sciences, University of Naples Federico II (Portici (NA), Italy latitude 40°49' N; longitude 14°20' E). Plants were cultivated in pots placed in a greenhouse. Pots of 58 L with 0.18 m² surface were filled with sandy soil (91.0% sand, 4.5% silt, 4.5% clay) characterized by pH 7.3, organic matter 2.45%, total N 0.9 g kg⁻¹, P₂O₅ 253.2 mg kg⁻¹ and K₂O 471.8 mg kg⁻¹, and 0.380 dS m⁻¹. Spinach seeds (*Spinacia oleracea* L.) were sown on 15 February 2022 with a seed density of 1000 m⁻², while plants were harvested on 30 March 2022. The cultivar tested was “Platypus RZ” F1 (Rijk Zwaan, De Lier, The Netherlands), a baby-leaf spinach cultivar with dark green oval leaves, that is suitable for cultivation from late autumn to spring both under field and protected conditions.

The experimental design was a split-plot design with saline stress level as the main factor (main plots) and the biostimulant application as the secondary factor (sub plots). The saline stress was applied through irrigation with saline water; four treatments were

implemented: non-saline water –EC0 (control treatment), and NaCl diluted in water solutions defined by electrical conductivity (EC) of 3.0, 6.0, and 9.0 dS m⁻¹, namely EC3, EC6, and EC9, respectively. In each saline treatment, the application of biostimulant was split between untreated Control, and that treated with Trainer[®] (a commercial legume-derived protein hydrolysate-LDPH, produced by Hello Nature Italia srl (Rivoli Veronese, Italy) plants. All treatments were replicated 3 times for a total of 24 pots (4 salinity levels (S) × biostimulant application (B) × 3 replicates).

The biostimulant product was sprayed on spinach leaves at a dose of 3 mL L⁻¹ of solution and applied on a weekly basis, starting from March 8. The crop water demand was determined with the Hargreaves method [19] and fully restored by irrigation; the desired EC of water irrigation was obtained by adding common salt to tap water according to the following formula:

$$\text{Salt}\% \text{ (g salt L}^{-1}\text{)} = 0.64 \times \text{EC}$$

At each irrigation, the EC of watering solutions were checked with a conductivity meter, Basic 30 CRISON, Spain. During the crop cycle, six irrigations were performed, the first two ones with tap water for promoting seedlings' germination and rooting, and the successive four with saline water according to the experimental design. The total amount of water was 6.5 L pot⁻¹, and salt was applied in a quantity of 12.5, 25.0, and 37.4 g pot⁻¹, for EC3, EC6, and EC9, respectively.

The crop practices were performed according to the best practice guides for this crop; as regards fertilization, only nitrogen was given at a dose of 20 kg per hectare in the form of calcium nitrate (15.5% N).

2.2. Soil Electrical Conductivity Measurements, and Temperature Monitoring

In each pot, three samplings of soil were conducted at 0–20 cm depth, to monitor electrical conductivity: one at the beginning of the trial and before sowing (11 February 2022), one at half growth cycle (17 March 2022; 30 days after sowing (DAS)), and another one at the end of the experiment (30 March 2022; 43 DAS). The EC was evaluated via a conductivity meter Basic 30 CRISON, using the 1:5 method adopted by Di Mola et al. [20], and expressed as dS m⁻¹.

The air temperature was continuously monitored during the growing period with a Vantage Pro2 weather station (Davis Instruments, Hayward, CA, USA) and the data were reported as daily means.

2.3. Yield, Growth Parameters Measurements, and Nitrate Determination

At the harvest, all plants pot⁻¹ were counted and cut; then a sample was weighed and leaves were counted. A representative sample of leaves was oven dried at 60 °C until constant weight in order to determine the dry matter (DM) percentage and the nitrate content by Foss FIAstar 5000 spectrophotometer (FOSS Italia S.r.l., Padua, Italy) continuous flow analyzer, as described by Di Mola et al. [21].

2.4. SPAD Index, and Leaves Color Determination

The Soil–Plant Analysis Development (SPAD) index was measured on a sample of ten undamaged and fully expanded leaves per each replicate with a chlorophyll meter SPAD-502 (Konica Minolta, Tokyo, Japan). On the same samples, the CIELAB color parameters were also determined with a Minolta CR-300 Chroma Meter (Minolta Camera Co. Ltd., Osaka, Japan), using the parameters L* (lightness, ranging from 0 = black to 100 = white), a* (chroma component ranging from –60 = green to +60 = red), and b* (chroma component ranging from –60 = blue to +60 = yellow).

2.5. Determination of Chlorophylls, Carotenoids, and Antioxidant Compounds and Activity

The chlorophylls a and b, and carotenoid content were assayed on a 1 g of fresh leaves sample, after the extraction with pure acetone, according to the method of Lichtenhaler and Wellburn [22]; the absorbance of the solutions was measured with a spectrophotometer

(Hach DR 2000, Hach Co., Loveland, CO, USA) at 662, 647 and 470 nm, respectively. Total chlorophylls were calculated as the sum of chlorophylls a and b.

The antioxidant activity and antioxidant compounds were determined on leaf samples after freezing and storage at $-80\text{ }^{\circ}\text{C}$, and successive lyophilizing. Hydrophilic and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) antioxidant activity, HAA and ABTS AA, respectively, were measured according to N,N-dimethyl-p-phenylenediamine (DMPD) [23] and ABTS methods [24] measuring the reduction in absorbance of the extracts with UV-Vis spectrophotometry at 505 and 734 nm, respectively.

The total ascorbic acid content (TAA) was determined spectrophotometrically according to the method previously described by Kampfenkel et al. [25] where the absorbance of extracts was measured at 525 nm. Total phenols were determined via spectrophotometer according to the procedure of Singleton et al. [26] where the absorbance of the extracts was recorded 765 nm.

2.6. Climate Characteristics of the Greenhouse

The climate characteristics of the experimental site during the crop growing period are reported in Figure 1. The maximum temperatures ranged between $13.5\text{ }^{\circ}\text{C}$, recorded at the end of February, and $34.4\text{ }^{\circ}\text{C}$ at the beginning of March; instead, the lowest value of minimum temperatures ($0.4\text{ }^{\circ}\text{C}$) was recorded on 9 March and the highest minimum temperature at the end of the cycle (Figure 1).

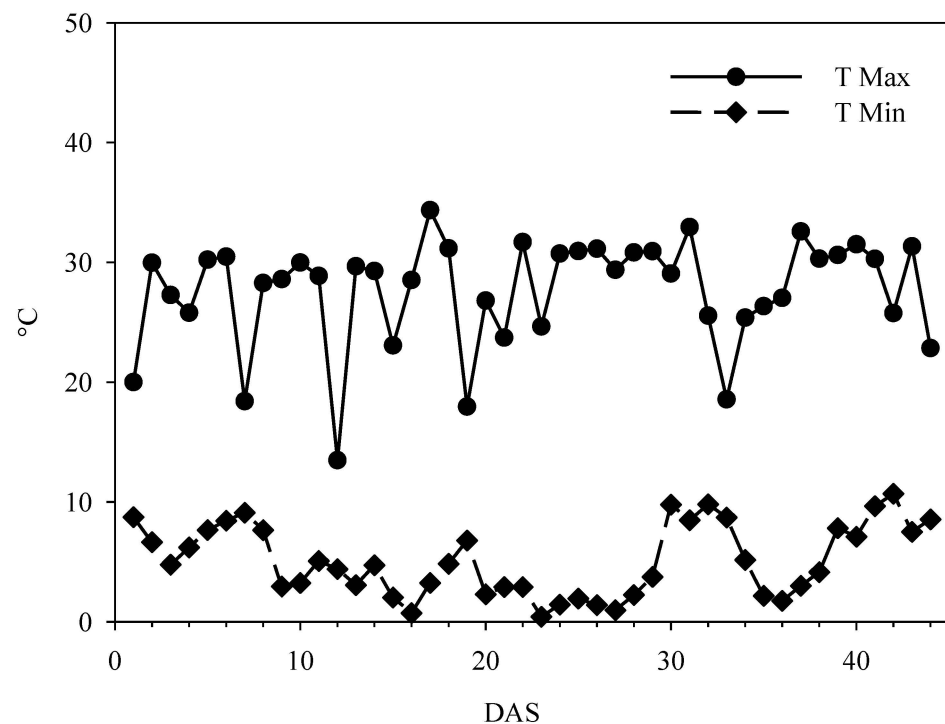


Figure 1. Daily maximum and minimum air temperature during the growing period of spinach. DAS: day after sowing.

2.7. Statistical Analysis

All data were subjected to analysis of variance (2-way ANOVA) using the SPSS software package (SPSS version 22, Chicago, IL, USA). The means were separated by the Tukey's HSD test at $p = 0.05$. All data are presented as means \pm standard error, $n = 3$.

3. Results and Discussion

3.1. Electrical Conductivity of Soil

The electrical conductivity of soil increased during the crop cycle due to saline irrigation, and the increase depended on the increase in the electrical conductivity values of irrigation water (Table 1).

Table 1. Soil electrical conductivity values (mean \pm SD; dS m^{-1}) throughout the spinach growth cycle in relation to saline water treatments.

Treatments	Electrical conductivity (dS m^{-1})		
	11 February 2022	17 March 2022	30 March 2022
Water Salinity			
EC0	0.38 ± 0.02	0.65 ± 0.06 c	0.56 ± 0.07 c
EC3	0.38 ± 0.02	1.81 ± 0.19 b	1.97 ± 0.07 b
EC6	0.38 ± 0.02	2.34 ± 0.21 ab	2.71 ± 0.18 a
EC9	0.38 ± 0.02	2.83 ± 0.18 a	3.31 ± 0.22 a
	ns	***	***

Different letters within each column indicate significant differences according to Tukey's HSD test. ns, non-significant; *** significant at $p \leq 0.001$.

On the second and third sampling dates, the treatment with tap water showed significantly lower EC values compared to the salinity treatments whereas EC6 and EC9 did not differ significantly in both samplings (Table 1). Moreover, EC values of EC3 treatment differed significantly from EC9 treatment in both dates (Table 1). This finding is in agreement with the literature reports where the use of saline water is expected to increase the electrical conductivity due to build-up of salts within the soil solution in values that depend on the soil layer and the occurrence of leaching [27] or the cultivation season [28]. Moreover, although many studies suggested a linear relationship between the EC values of soil saturated paste extract and the EC values obtained with various methods, several factors related to the physicochemical properties of soil may affect this relationship [29]. Moreover, the lack of statistical differences between EC6 and EC9 could be related to the protocol used, since, according to the literature, the 1:5 method provides information about the relative changes of EC and not the actual contents of salts in soil [29]. Another explanation could be the gradual build-up of salts in soil matrix which resulted in no significant differences at the end of the experiment.

3.2. Yield and Growth Parameters

The interaction between the salinity of irrigation water and biostimulant application was significant in the case of spinach fresh yield (Figure 2). The yield decreased when the salt stress was higher, but with a different trend between control plants and those treated with legume-derived protein hydrolysate (LDPH). The polynomial curve best fitted both trends, and sufficiently highlighted the fact that biostimulant application may mitigate the effect of salinity stress. Indeed, according to the respective equations, control plants showed a 36.1% decrease in the yield at 6.0 dS m^{-1} of water salinity, while a further increase in EC of irrigation water resulted in 60.7% loss of fresh biomass yield.

The application of biostimulant alleviated the salinity stress effects and fresh yield loss at 6 dS m^{-1} and 9 dS m^{-1} was 11.8% and 33.3%, respectively (Figure 2). Moreover, total yield loss is expected at 14.5 dS m^{-1} and 13.0 dS m^{-1} in the case of LDPH application and the control treatment, respectively. Interestingly, the treatment with LDPH significantly improved the yield of plants irrigated with tap water. In addition, for the plants irrigated with 3.0 and 6.0 dS m^{-1} water and sprayed with the biostimulant, no statistical difference in yield was recorded compared to Control-EC0 plants (Figure 2). Finally, the alleviating effect of biostimulant was again more evident at higher salinity stress levels (e.g., EC6 and 9), indeed LDPH allowed an increase in yield of 76.2% vs. 43.3% for the corresponding EC levels of untreated plants, while for the lowest EC level the fresh yield of biostimulant-treated plants was higher by 11.5% compared to the untreated ones.

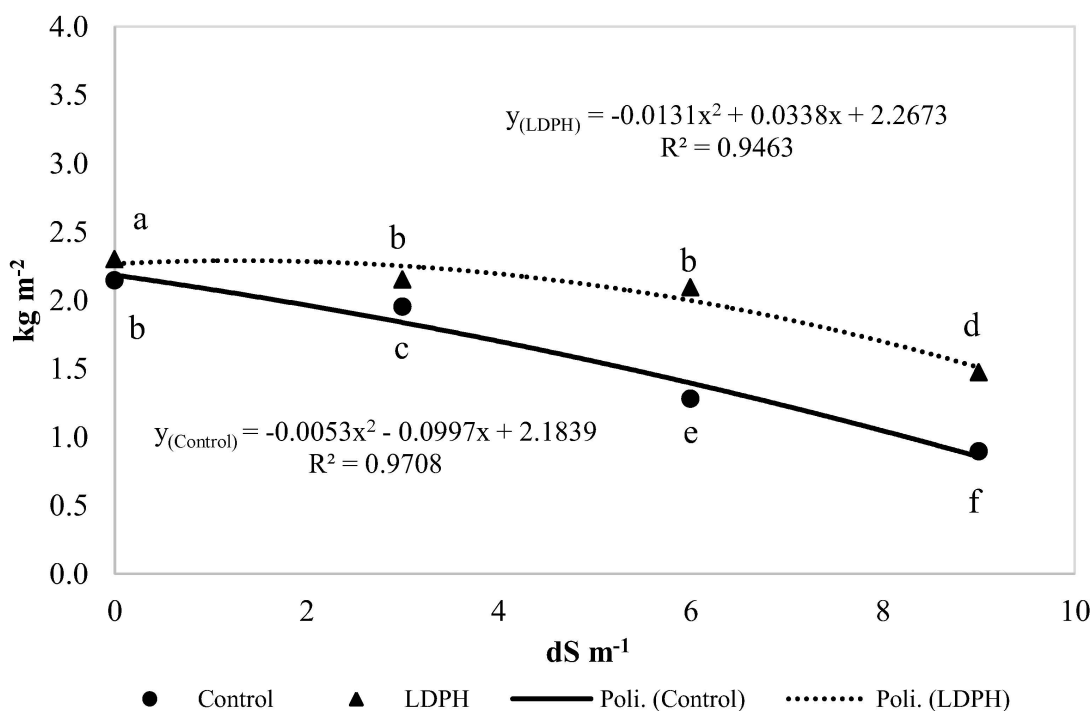


Figure 2. Spinach yield as affected by water salinity and biostimulant application. Different letters indicate statistical difference according to Tukey's HSD test ($p \leq 0.001$).

The detrimental effects of salinity stress on several crops is well documented and spinach is considered a species which is moderately sensitive to increased salinity with a tolerance threshold of 4 dS m⁻¹ [7]. However, the severity of yield losses are also dependent on the soil texture which may affect the solubility of minerals [29]. The accumulation of salts in the soil is associated with photosynthesis inhibition through the reduced stomatal and mesophyll conductance and the reduction in chlorophyll content which consequently result in reduced light absorbance and plant growth [2]. However, according to Ors and Suarez [30], the fresh yield loss in spinach was significant above EC values of 9 dS m⁻¹, whereas moderate salinity levels (4.0 dS m⁻¹) increased fresh yield over the control treatment. Similarly, Yamada et al. [31] suggested that spinach fresh yield may increase with increasing salinity up to 80 mmol L⁻¹ of NaCl. These contradictory findings could be due to differences in the growth cycle compared to our study where we harvested baby-leaf spinach, or differences in spinach genotype and soil texture. Moreover, Ors and Suarez [32] suggested differences in spinach plants' response to salinity stress depending on the growing season.

The stress-mitigating effects of biostimulant products are highly appreciated in horticultural crop production with several paradigms reported so far [33–36]. The use of plant-based protein hydrolysates as biostimulant products under stress conditions has been associated with positive effects on plant primary and secondary metabolism induction and the expression of stress-related genes [37]. However, the literature reports show contradictory results considering not only the varied responses of spinach genotypes to abiotic stressors [30], but also the genotype-dependent response of the species to biostimulant application [18,33,38]. The positive effects of LDPH application on spinach plants subjected to salinity stress conditions were profound even at low salinity levels (e.g., EC3) and they could be associated with the composition of the tested biostimulant which included amino acids and peptides. These molecules may have a hormone-like activity which induces plant growth through improved nutrient uptake and assimilation [38,39], while changes in root architecture which facilitate nutrient uptake could be also hypothesized [10,39].

The number of plants per square meter and the growth parameters were significantly affected only by the main effects of treatments (water salinity and biostimulant applica-

tion; Table 2). The number of plants per square meter decreased when the water salinity increased, but without significant difference between EC0 and EC3, and EC6 and EC9, respectively (Table 2) with the mean value of the two most stressed treatments (EC6 and EC9) being 40% lower than the mean value of the other two treatments (EC0 and EC3; 709.2 vs. 1167.5). The number of leaves per square meter also showed a linear and significant decrease, passing from EC0 and EC3 to EC9, with EC0 being different from all the other treatments. Instead, the number of leaves per plant was higher in EC6, probably due to the lower number of plants and the lower competition effects, while the average leaf weight was the lowest at EC9, without being significantly different from the control treatment EC0 (Table 2). No significant differences were recorded among the EC0, EC3 and EC6 treatments for the same parameter (average leaf weight), while no significant differences were recorded among all the salinity treatments in the case of DM content (Table 2). On the other hand, the biostimulant application improved both the number of plants and leaves per square meter by about 21.1 and 29.5%, respectively; while the dry matter percentage was higher in Control plants (Table 2). The number of leaves per plant and the average leaf weight were not affected by the biostimulant application.

Table 2. Effects of water salinity and biostimulant applications on the number of plants and leaves per square meter, number of leaves per plant, average leaf weight (ALW; g), and leaves dry matter percentage (%) of spinach at harvest. Values are presented as means \pm SD.

Treatments	Plants		Leaves		
	n° m ⁻²	n° m ⁻²	n° Plant ⁻¹	ALW (g)	D.M. (%)
Water Salinity					
EC0	1286.7 \pm 99.0 a	6952.9 \pm 455.0 a	5.4 \pm 0.4 b	0.32 \pm 0.018 ab	10.5 \pm 0.3
EC3	1048.3 \pm 91.9 a	5995.8 \pm 393.4 b	5.5 \pm 0.5 b	0.38 \pm 0.021 a	11.1 \pm 0.6
EC6	696.7 \pm 69.5 b	4802.3 \pm 360.9 bc	7.0 \pm 0.5 a	0.35 \pm 0.023 a	10.8 \pm 0.1
EC9	721.7 \pm 46.8 b	3850.0 \pm 217.9 c	5.3 \pm 0.2 b	0.30 \pm 0.016 b	11.1 \pm 0.4
Biostimulant					
Control	848.8 \pm 82.5 b	4640.1 \pm 325.6 b	5.98 \pm 0.34	0.34 \pm 0.016	11.1 \pm 0.49 a
LDPH	1027.9 \pm 71.1 a	6010.4 \pm 388.0 a	5.62 \pm 0.45	0.34 \pm 0.023	10.6 \pm 0.19 b
Significance					
Salinity (S)	***	***	**	**	ns
Biostimulant (B)	**	***	ns	ns	*
S \times B	ns	ns	ns	ns	ns

Different letters within each column indicate significant differences according to Tukey's HSD test. ns, non-significant; * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$ and *** significant at $p \leq 0.001$.

The results of our study are in agreement with literature reports which indicate spinach as a moderately salt-sensitive species [7]; therefore, increased salinity levels could affect seedling survival rates and consequently plant density [10], as indicated by the lower number of plants per m² when seedlings were subjected to EC6 and EC9 treatments. Moreover, the negative effects of salinity stress on plant growth shown in Figure 2 are extrapolated to the plant performance where a lower number of leaves per m² was recorded. However, as already reported by Ors and Suarez [30] and Yamada et al. [31], moderate salinity may induce plant growth, as indicated by the increased number of leaves per plant and the average leaf weight at EC3–EC6 and EC6, respectively. On the other hand, the beneficial effect of LDPH biostimulant on both the number of plants and the number of leaves per m² could be associated with the increased survival rate of seedlings and the improved photosynthesis and primary metabolism induction [12]. However, not all the studies reported beneficial effects of protein hydrolysates on spinach growth [18,39], which indicates the important role of genotype, growing conditions and agronomic practices (e.g., nitrogen fertilization) on the response of the species to this biostimulant [10]. Regarding the DM content, the negative effect observed for the biostimulant application in our study was also reported by Kunicki et al. [18], whereas Roupheal et al. [39] and Bonasia et al. [10] suggested positive and no-significant effects, respectively. These contradictory findings highlight the

importance of further studies in order to reveal the actual mechanisms of action and those parameters that regulate the genotypic response of spinach to biostimulant products.

3.3. SPAD Index and Color Parameters

The interaction between salinity level of irrigation water and biostimulant application significantly affected SPAD index (Figure 3) and CIELAB color parameters (Table 3). The SPAD index linearly decreased when the saline stress increased, but the LDPH application mitigated the detrimental effect of salinity. Indeed, for all the salinity treatments the biostimulant-treated plants had significant higher SPAD values than control (untreated) plants, except for the case of EC3 treatment where no significant differences were detected (Figure 3). The more profound effects were recorded for the EC9 treatment, followed by the EC6 treatment indicating the stress mitigating impact of biostimulants against salinity stress conditions. According to the literature, Di Mola et al. [40] and Roupael et al. [39] reported a beneficial effect of plant-based protein hydrolysates on the SPAD index values of baby and greenhouse spinach plants, respectively, while Carillo et al. [38] also observed increasing trends for SPAD values of plants treated with protein hydrolysates. These findings could be attributed to the improved nutrient uptake and assimilation that amino acids and peptides may incur which results in the induction of chlorophyll biosynthesis in biostimulant-treated plants compared to the untreated ones. In contrast, Ors and Suarez [30,32] suggested that SPAD values may increase with increasing salinity, while similar findings were reported for purslane plants grown under saline conditions where an increase in chlorophyll content was recorded [41].

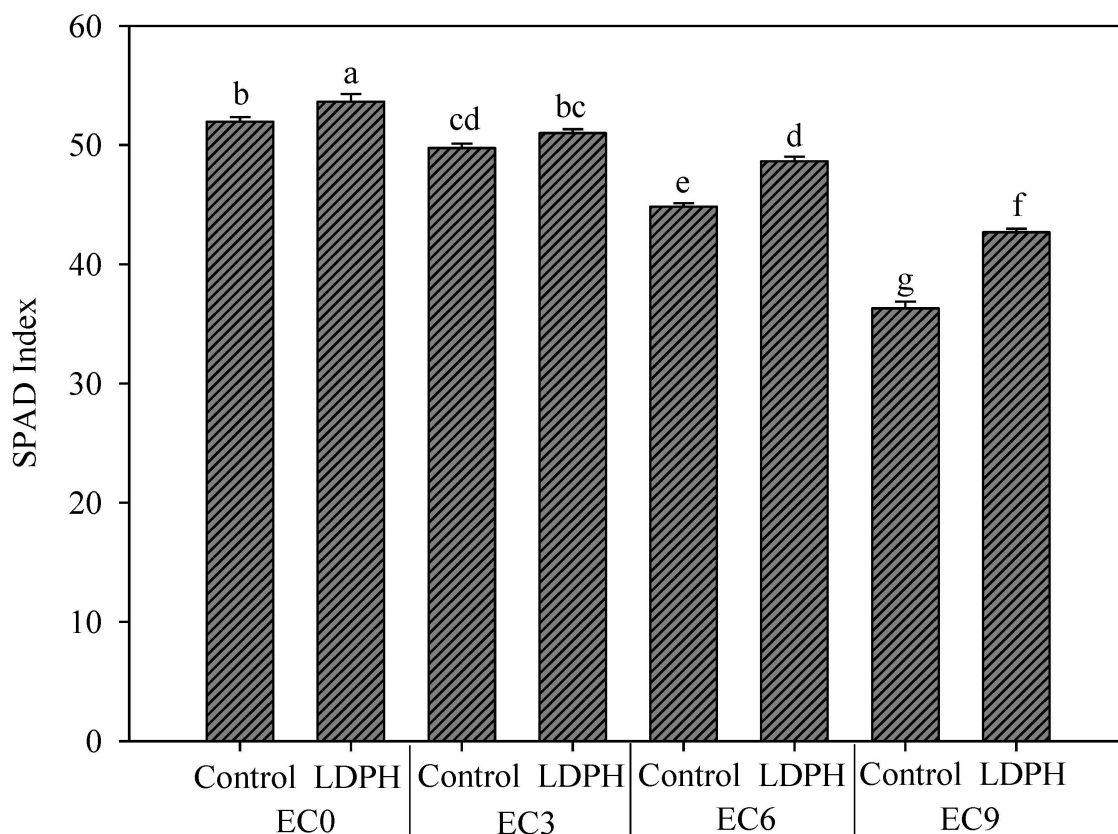


Figure 3. Soil–Plant Analysis Development (SPAD) index as affected by irrigation water salinity and biostimulant applications. Vertical bars indicate means \pm standard error; different letters indicate statistical difference according to Tukey’s HSD test ($p \leq 0.001$).

Table 3. Effects of irrigation water salinity and biostimulant applications on leaf color parameters of spinach leaves.

Water Salinity	Biostimulant	L*	a*	b*
EC0	Control	38.28 ± 0.91 bc	−12.17 ± 0.27 b	18.42 ± 0.56 b
	LDPH	36.89 ± 0.50 c	−10.83 ± 0.39 a	15.12 ± 0.81 c
EC3	Control	39.03 ± 0.39 bc	−11.52 ± 0.32 ab	16.37 ± 0.68 bc
	LDPH	38.68 ± 0.41 b	−11.81 ± 0.32 ab	17.00 ± 0.67 bc
EC6	Control	38.48 ± 0.38 bc	−11.19 ± 0.31 ab	15.97 ± 0.53 c
	LDPH	38.59 ± 0.68 bc	−11.42 ± 0.44 ab	16.92 ± 0.99 bc
EC9	Control	43.08 ± 0.73 a	−14.41 ± 0.36 c	21.93 ± 0.98 a
	LDPH	37.69 ± 0.42 bc	−11.38 ± 0.26 ab	15.10 ± 0.52 c
Significance				
Salinity (S)		***	***	**
Biostimulant (B)		***	***	***
S × B		***	***	***

Different letters within each column indicate significant differences according to Tukey's HSD test. ** significant at $p \leq 0.01$ and *** significant at $p \leq 0.001$. L* = brightness, a* = green/red; b* = blue/yellow (color parameters).

Regarding the CIELAB color parameters, a significant interaction was noted among the two factors S and B. The differences between the treatments were less marked, except for the untreated plants of the most-stressed treatment, EC9, which showed the highest overall values of brightness (L*), green intensity (a*; higher negative value), and yellow intensity (b*; higher positive value), being significantly different from the rest of the treatments (Table 3). The literature reports suggest variable effects of protein hydrolysates on leaf color of spinach plants. In particular, Rouphael et al. [39] did not observe any effects of protein hydrolysates on the leaf color parameters of spinach plants, while Bonasia et al. [10] also did not find any significant effects of animal or plant-derived protein hydrolysates on leaf brightness (L*) of baby spinach plants. On the other hand, according to Carillo et al. [38], the yellow intensity (b*) of spinach leaves was negatively affected by LDPH application. The latter finding is in agreement with our study, where the yellow intensity of leaves showed decreasing trends in the case of biostimulant-treated plants at EC0 and EC9 treatments. Moreover, the highest values of b* parameter for the untreated plants at EC9 coincide with the lowest SPAD index values recorded for the same treatment in our study (see Table 2). In the study of Corrado et al. [42], chroma parameters (L*, a* and b*) were not affected by increasing salinity in the case of lettuce leaves, while Falovo et al. [43] suggested that growing season and ion concentration have a significant impact on lettuce leaf color. Therefore, it could be suggested that the profound differences recorded in our study at EC9 (control plants) could be associated with the high ion concentration in the nutrient solution.

3.4. Chlorophylls, Carotenoids, and Nitrate Content of Spinach Leaves

The chlorophyll values (a, b, and total) showed a decreasing trend when salt stress increased, especially in the case of EC9 which was significantly lower than all the other treatments except for chlorophyll b, where it was not statistically different from EC6 (Table 4). As for the biostimulant application, it significantly affected only the total chlorophylls (Table 4). According to the literature, the effect of saline conditions on the chlorophyll content of leafy vegetables shows a variable response [44–46]. For example, Ors and Soarez [30] reported opposing trends, with increased chlorophyll content being recorded in spinach plants at high salinity levels, while similar findings were recorded by Di Mola et al. [40] in spinach plants grown under different nitrogen levels. This response could be associated with the nutrient status of plants, since, according to Ferreira et al. [4], K efficiency may alleviate the negative effects of salinity on chlorophyll content and photosynthetic capacity in spinach plants. Moreover, the variable response reported in the literature could be associated with the experimental conditions such as the severity and duration of stress or the application method which may result in a rapid or slow degradation of the mechanism of chlorophyll synthesis [44,46]. Therefore, the decreasing trends in high salinity levels observed in our

study could be associated with the growth stage of the plants when subject to salinity stress, indicating that baby spinach are more susceptible to high salinity compared to later growth stages due to inability of the plants' antioxidant systems to cope with the stress conditions [30,41]. Regarding the effect of protein hydrolysates on chlorophyll content, Bonasia et al. [10] also reported an increase in chlorophyll b and total chlorophyll content in spinach leaves, whereas no effects on chlorophyll a content were reported. According to the same authors [10], the positive effects of protein hydrolysates could be attributed to its increased content of amino acids such as glutamate which are the precursors for chlorophyll biosynthesis. Similar effects have been recorded in hydroponically grown leafy herbs where the application of protein hydrolysates increased total chlorophyll content in spearmint and peppermint leaves [47].

Table 4. Effects of irrigation water salinity and biostimulant application on chlorophylls a, b, and total, and carotenoids of spinach leaves.

Treatments	Chlorophyll a	Chlorophyll b	Total Chlorophylls	Carotenoids	Nitrates
	mg g ⁻¹ fw	mg g ⁻¹ fw	mg g ⁻¹ fw	mg g ⁻¹ fw	mg kg ⁻¹ fw
Water Salinity					
EC0	1.163 ± 0.021 a	0.743 ± 0.067 a	1.907 ± 0.087 a	0.321 ± 0.019 c	2397.2 ± 225.0 a
EC3	1.157 ± 0.029 a	0.680 ± 0.062 a	1.838 ± 0.090 a	0.340 ± 0.015 bc	2389.4 ± 244.1 a
EC6	1.162 ± 0.019 a	0.633 ± 0.042 ab	1.795 ± 0.061 a	0.360 ± 0.010 ab	2095.2 ± 187.5 a
EC9	1.038 ± 0.054 b	0.469 ± 0.050 b	1.507 ± 0.103 b	0.391 ± 0.006 a	256.9 ± 95.7 b
Biostimulant					
Control	1.111 ± 0.321	0.614 ± 0.177	1.725 ± 0.498 b	0.354 ± 0.102	1442.8 ± 244.7 b
LDPH	1.115 ± 0.332	0.649 ± 0.187	1.798 ± 0.519 a	0.352 ± 0.102	2126.6 ± 312.3 a
Significance					
Salinity (S)	***	***	**	**	***
Biostimulant (B)	ns	ns	*	ns	***
S × B	ns	ns	ns	ns	ns

Different letters within each column indicate significant differences according to Tukey's HSD test. ns, non-significant; * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$ and *** significant at $p \leq 0.001$.

The carotenoid content showed an opposite trend, but with more marked differences between the treatments, especially in the case of E6 and E9 treatments which were significantly higher than the control treatment. Moreover, the highest value of carotenoids was registered at EC9 without being significantly different from EC6 treatment, while no significant effect of the biostimulant application was recorded (Table 4). Similarly to our study, Kim et al. [1] reported that both short- and long-term irrigation of lettuce plants with saline water resulted in a significant increase in total carotenoids in lettuce plants, while deficit irrigation at 50% of normal irrigation increased carotenoid content in tomato fruit [48]. In contrast, Naz et al. [46] observed a significant decrease in the carotenoid content of spinach plants when grown in saline conditions (150 mM of NaCl), whereas Xu and Mou [44] suggested opposing trends and Bantis et al. [49] reported that the effect of salinity on total carotenoid content is associated with light conditions. These contradictory results indicate that apart from salinity, other factors related to the growing condition and stress application may also affect carotenoid content. Considering the antioxidant effects of carotenoids, it could also be suggested that its increased content at EC9 treatment is associated with the induction of antioxidant mechanisms of plants, since carotenoids are the precursors of abscisic acid which regulates plant development under stress conditions [1,9], while they are crucial for maintaining plant reactive oxygen species homeostasis in plants [50]. Regarding the biostimulant application, no significant effects were recorded on total carotenoid content, a finding which is in agreement with the results reported by Bonasia et al. [10] and Carillo et al. [38]. On the other hand, Aktsoglou et al. [47] suggested that protein hydrolysates may increase the content of total carotenoids in spearmint plants, while Di Mola et al. [51] also recorded increased total carotenoid content after the application of seaweed extracts and protein hydrolysates on baby-leaf lettuce plants. Similar results were

observed in the study of Sabatino et al. [11] and Silvana et al. [48] who evaluated the effect of protein hydrolysates on lettuce and tomato plants, respectively.

The nitrate content of spinach leaves showed decreasing trends when salinity stress was higher; however, only the most stressed treatment (EC9) was statistically lower than all the other treatments, and registered about one-tenth of the average value of the other three treatments (Table 4). In contrast, nitrate content of spinach plants that were treated with protein hydrolysates showed an increase by 47.4% compared to the untreated plants (Table 4). Regarding the effect of salinity on nitrate content, Chung et al. [52] suggested an increase in nitrates in lettuce leaves with increasing salinity up to 5 dS m⁻¹, whereas Turhan et al. [8] observed a linear decrease in nitrates in spinach leaves of up to 200 mM NaCl. It seems that at high salinity levels, the increased concentrations of Cl⁻ in soil solution have a deleterious effect resulting in reduced nitrate uptake and nitrogen deficiency [5,52]. Considering that spinach is a nitrate accumulator, mild salinity stress (EC6) may reduce its content in edible leaves and increase the quality of the final product. Regarding the effect of biostimulants, Bonasia et al. [10] also suggested an increase in nitrate content following the application of protein hydrolysates, while Di Mola et al. [40] recorded an almost nine-fold increase in nitrates for plants treated with LDPH. The results obtained from Carillo et al. [38] were along the same lines, although no such high increase was detected (approximately 30% over the control treatment). In the study of Roupheal et al. [39], no significant differences were recorded between the plants treated with LDPH and the untreated ones, which could be due to comparisons with other biostimulant products that elicited more profound increases in nitrate content over the control treatment. On the other hand, Tsouvaltzis et al. [13] and Sabatino et al. [11] reported the opposite trends for amino acids and protein hydrolysates application in lettuce plants, respectively. Moreover, similar results were reported by Aktsoglou et al. [47] regarding the positive effects of amino acids on nitrate accumulation inhibition in spearmint and peppermint plants. The main explanation for this finding could be that the amino acids included in protein hydrolysates serve as nitrogen pools for the biosynthetic activities of plants, thus reducing the intake of exogenous nitrogen [53]. Therefore, although the detected amounts in our study were below the upper threshold for EU countries (2500 mg kg⁻¹ fw), the practice of protein hydrolysate application needs further consideration, since it induces nitrate accumulation which may incur negative health effects [52].

3.5. Antioxidant Compounds and Activity of Spinach Leaves

The hydrophilic (H) and ABTS antioxidant activity (AA), and phenols were significantly affected by both experimental factors, without a significant interaction between them being observed (Table 5). Saline stress depressed the HAA when spinach plants were irrigated with EC9 treatment. Contrarily, ABTS AA linearly increased with the increase in saline stress, although no significant differences were observed between EC6 and EC9. A similar trend was also recorded for total phenols, with EC9 recording a higher value compared to EC3 and EC0 (Table 5). The total ascorbic acid content was not affected significantly by the salinity level of irrigation water, although increasing trends with increasing salinity were observed. Biostimulant application had a beneficial effect only for HAA where it elicited a 9.3% increase. In contrast, all the other parameters were negatively affected and the control plants always showed higher values by 20.8%, 15.7%, and 23.6% for ABTS AA, total phenols, and total ascorbic acid (TAA), respectively (Table 5). Sogoni et al. [54], who studied the effect of using brackish water for the irrigation of dune spinach (*Tetragonia decumbens* Mill.), suggested a contrasting response in terms of antioxidant capacity which was positively correlated with salinity in the case of the FRAP assay and total phenol content, whereas a negative correlation was observed for the ABTS assay. These findings are in agreement with our study where a variable response was recorded for the antioxidant activity assays tested (e.g., DMDP and ABTS), whereas a negative effect of salinity on total phenol content was also observed. Bantis et al. [49] also recorded a decrease in the antioxidant activity (FRAP) of spinach plants when subjected to saline conditions,

which indicates that the implemented protocol may also play a crucial role in the obtained results. Despite the numerous studies on the effect of salinity on total phenol content and the antioxidant activity, the available results show a variable response which necessitates further consideration [55,56]. Regarding the application of biostimulants, Carillo et al. [38] reported that LDPH application may result in a decreased content of polyphenols, whereas Di Mola et al. [40] did not detect any significant effect for total phenol and TAA content or ABTS AA. The same authors [40] also suggested an increase in HAA for the LPDH treatment compared to the control, a finding which is in agreement with our results. In contrast to our study, Rouphael et al. [39] reported a significant positive effect of protein hydrolysates on total phenol content and TAA in greenhouse spinach, suggesting the induction of biosynthesis of these antioxidant compounds as part of the homeostasis mechanism of plants. This difference with the results of the present work could be associated with the different growing conditions compared to the study of Rouphael et al. [39] where plants were grown later in spring, as well on the genotypes tested.

Table 5. Effects of irrigation water salinity and biostimulant application on hydrophilic and ABTS antioxidant activity (HAA and ABTS AA, respectively), total phenols, and total ascorbic acid (TAA) of spinach leaves.

Treatments	HAA	ABTS AA	Total Phenols	TAA
	mmol Ascorbic Acid equ. 100 g ⁻¹ dw	Mmol Trolox equ. 100 g ⁻¹ dw	mg Gallic Acid g ⁻¹ dw	mg g ⁻¹ fw
Water Salinity				
EC0	8.08 ± 0.30 a	9.20 ± 0.99 c	1.39 ± 0.04 c	44.87 ± 7.52
EC3	8.19 ± 0.38 a	11.70 ± 0.55 b	1.55 ± 0.08 bc	49.57 ± 6.31
EC6	8.16 ± 0.24 a	14.33 ± 0.51 a	1.77 ± 0.06 ab	52.50 ± 4.11
EC9	5.21 ± 0.62 b	15.04 ± 0.67 a	1.89 ± 0.14 a	58.29 ± 7.33
Biostimulant				
Control	7.08 ± 0.58 b	13.75 ± 0.68 a	1.77 ± 0.09 a	58.17 ± 3.32 a
LDPH	7.74 ± 0.30 a	11.38 ± 0.84 b	1.53 ± 0.06 b	44.44 ± 4.77 b
Significance				
Salinity (S)	***	***	**	ns
Biostimulant (B)	*	***	*	*
S × B	ns	ns	ns	ns

Different letters within each column indicate significant differences according to Tukey's HSD test. ns, non-significant; * significant at $p \leq 0.05$; ** significant at $p \leq 0.01$ and *** significant at $p \leq 0.001$.

4. Conclusions

The results obtained from this experiment proved that legume-derived protein hydrolysates (LDPH) mitigated the detrimental effect of saline irrigation water on baby spinach plants. Indeed, under all the salinity treatment levels, the biostimulant-treated plants showed higher yields than the untreated ones, even at the highest saline treatment when the irrigation water reached 9 dS m⁻¹. The application of LDPH helped mitigate the adverse effects of saline stress, by increasing the SPAD index, total chlorophylls and HAA of spinach plants, while yield was significantly increased under biostimulant treatment in low to moderate salinity levels (EC3 and EC6) and reached the value obtained in control treatment. On the other hand, antioxidant activity (ABTS), total phenols and total ascorbic acid content showed a decrease in biostimulant-treated plants, while nitrate content was significantly increased even though it did not exceed the upper acceptable threshold. Such results increase the knowledge on protein hydrolysates application in horticultural species grown under saline conditions and thus may help growers to mitigate the adverse effects of saline water and maintain a profitable yield when no other source of irrigation water is available. Moreover, the results of our study promote the application of biostimulants as a sustainable and eco-friendly agronomic practice in vegetable production, especially under growth limiting conditions such in the case of high salinity.

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