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The Alleviation Effects of Biostimulants Application on Lettuce Plants Grown under Deficit Irrigation

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Abstract: The aim of this study was to examine the potential of using biostimulants for the amelioration of deficit irrigation effects on field-grown lettuce plants growth parameters (cv. Doris (Romaine type) and cv. Manchester (Batavia type)). Therefore, five biostimulatory products that differed in their composition were evaluated, including seaweed extracts, amino acids, humic and fulvic acids, macronutrients, Si, and vegetable proteins, while a control treatment with no biostimulants applied on plants was also considered. Plants were subjected to three irrigation regimes, e.g., rain-fed plants (RF), deficit irrigation (I1; 50% of field capacity) and normal irrigation (I2; 100 of field capacity). The results indicate that the application of seaweed extracts, macronutrients, and amino acids (SW treatment) alleviated the negative effects of deficit irrigation on plant growth and chlorophyll content of Romaine-type plants. On the other hand, Batavia-type plants were more susceptible to water stress, since the highest crop yield plant was observed under the full irrigation treatment and the application of vegetal proteins and amino acids (VP treatment). In general, the application of biostimulants on the Romaine type improved plant growth under water shortage conditions compared with fully irrigated plants in almost all measurements, whereas the Batavia-type plants appeared to be more sensitive to deficit irrigation. Therefore, the ecofriendly practices of deficit irrigation and biostimulant application could be useful in leafy vegetable production on a genotype-depended manner.

Keywords: *Lactuca sativa*; seaweed extracts; humic and fulvic acids; silicon; amino acids; water stress; chlorophyll content; proline

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1. Introduction

The increasing water shortage in many regions of the world is one of the key abiotic variables that endanger agricultural output within a climate change environment [1]. The lack of irrigation water, combined with other biotic and abiotic pressures, is detrimental to plant productivity and product quality in both open-field and protected cropping systems [2] since these stressors may impair crop growth and output by changing plant morphological, biochemical, and molecular characteristics [3]. The intensification of cropping systems due to increased needs for food amounts and food availability throughout the year has put high pressure on water availability for irrigation purposes [4]. In this context, the current farming systems have to be re-evaluated and re-designed focusing on the sustainable use of natural resources, while considering farmers' income and further environmental perspectives [5]. The integration of novel and traditional agronomic practices in modern agriculture is pivotal for securing food security, especially in developing and undeveloped parts of the world where food shortage is expected to increase over the years [6,7]. Therefore, the sustainable management of irrigation water needs to be amplified considering the contribution of irrigation land to overall crop production at the global scale (40% of world crop production is obtained from 20% of irrigated cultivated land) [5].

So far, irrigation water management was not among the first priorities from the farmers' point of view due to low associated costs and the low adoption of precision agriculture practices that may ensure sustainable water use [8]. However, the ongoing issue of climate change and high production costs have introduced the concept of water footprint which determines the sustainable use of irrigation water [9]. Although irrigation is necessary to obtain high crop yields, the use of water is not linearly associated with crop yield and high yields are not proportional to water consumption [10,11]. Among the various suggested strategies for water management, deficit irrigation becomes a necessity in the regions of the world where there is a shortage of irrigation water, since it ensures high water use efficiency without severe effects on the quality of the final product [12]. In regards to vegetables, water availability is pivotal to obtaining high crop yield and quality, thus ensuring economic viability of farming businesses and conserving water reserves [13]. However, considering the shallow root system and the high water content of most vegetables, especially the leafy ones, practices that regulate irrigation water availability to crops need special attention to obtain the best result in terms of both water saving and crop performance [14].

The use of plant biostimulants (PBs) in a variety of crops is a novel, cutting-edge, and ecofriendly agronomic strategy. This practice considers the application of various products (organic or inorganic compounds and/or microbes) to crops aiming to promote the growth and development of plants, their defense mechanisms against pathogens, and stress tolerance [15]. The use of biostimulants has high practical interest within the scope of mitigating negative effects of climate change due to anthropogenic activities, since they are capable of improving nutrient availability and inducing the morphological and physiological changes that allow plants to cope with external stressors [2]. Therefore, biostimulator products could be rendered as a promising weapon in the quiver of farmers against climate change effects on crop production and the resulting crop yield losses [16,17]. Du Jardin [18] suggested different categories of plant biostimulatory products according to their composition, including fulvic and humic compounds, extracts from seaweeds, derivatives of chitosan and chitin, compounds with antitranspiratory effects, free amino acids, chemicals that contain nitrogen, etc. Its use is becoming more and more popular in a wide range of crops, such as tree and field crops or vegetables, with confirmed benefits in crop performance and final product quality on several occasions [19–22]. In the case of horticultural crops, the production of products with high added value and the intensification of cropping systems (e.g., greenhouse production) justifies the use of biostimulants, especially under stress-imposing conditions, without compromising farmer's income and food safety [20,23,24]. Water-stress alleviation is among the beneficial properties of biostimulants and several reports highlighted the positive effects on horticultural species which are more prone to shortage of water than other crops [23,25,26].

Lettuce is one of the most important leafy vegetables throughout the globe, which is mostly used in a variety of salads [27]. The use of lettuce and the corresponding cultivar choice is determined by the visual appearance of the plants, as well as by its nutritional and functional properties, especially in the case of colored cultivars which are associated with increased health benefits due to their chemical composition and bioactive compounds content [28–30]. The annual global production and cultivated area of lettuce (including chicory) for 2020 were approximately 27.7 million tones and 12.2 million hectares, respectively [31]. The increasing consumer demand for lettuce and lettuce-based ready-to-eat salads throughout the year has been linked to its health-promoting qualities, particularly its high content of macro- and micro-nutrients and bioactive molecules [32]. Considering the current market trends and the high water requirements of lettuce, intensification of cropping systems has to be carefully implemented to focus on the sustainable management of irrigation water and increased efficiency of water use from crops, as well as the blue water footprint of the final product [33]. The use of biostimulants towards this aim is pivotal and several reports have suggested their use in sustainable lettuce production under abiotic stressors [13,22,34].

Considering the pressure from abiotic stressors on modern agriculture and the increasing need for vegetable crop intensification, the current study assessed the impact of five biostimulatory products with varying content on plant development and growth, as well as on the chemical composition of leaves (free proline, chlorophyll, and total carotenoids content) of lettuce plants grown in the field under water shortage conditions.

2. Materials and Methods

2.1. Description of Biostimulant Treatments and Experimental Design

The experiment was conducted in the spring–summer growing period of 2021 at the experimental farm of the University of Thessaly. The experimental parameters were previously described in detail by the authors [35]. In brief, lettuce seedlings of two varieties (*Lactuca sativa* L.: Romaine type cv. *Doris* and *Lactuca sativa* L.: Batavia type cv. Manchester) were transferred to the field on 1 April at the stage of 3–5 true leaves, and plants were harvested 57 days after transplantation (DAT) (May 7). Each experimental plot was 2.5 m^2 and plants were planted in double rows at a plant density of 160,000 plants/ha (distance of 0.5 m between the centers of double rows and 0.25 m between the plants in each row) and according to the split-plot design (n = 3). Five biostimulants were tested as previously described by the authors [35], namely: (a) SW: plants and seaweed extracts, amino acids, and trace elements; (b) HF: humic and fulvic acids; (c) SiC: CaO and SiO_2 combined with a calcium utilization, mobilization and translocation factor; (d) Si: orthosilicic acid (Si); and (e) VP: vegetable proteins and amino acids, plus the C_{NB} (control) treatment where no biostimulants were added. The biostimulants application and irrigation regimes were previously described in the study of Chaski and Petropoulos [35]. In brief, three irrigation treatments were studied, namely, rain-fed plants (RF); plants that received water according to 50% of field capacity (I1); plants that received water according to 100% of field capacity (I2). Prior to transplantation, plants were treated with the biostimulant products (except for control plants that were treated with tap water) by immersing the whole seed trays in tubs containing the biostimulant products, while three more applications of biostimulants were conducted at regular intervals of 10 days starting 5 days after transplantation (DAT), either by direct application on roots (HF and SiC) or foliage spraying (SW, VP, and Si). Soil conditions were the following: 48% sand; 29% silt; 23% clay; 1.3%; organic matter; pH 7.9; EC: 1.4 mS/cm; NO_3^-: 9.49 mg/kg; P: 74.53 mg/kg; K_{exch}: 0.98 cmolc/kg; Ca_{exch}: 13.96 $cmol_c$/kg; and Mg: 4.32 $cmol_c$/kg.

2.2. Irrigation Treatments

The irrigation was applied according to three different regimes as described above. The irrigation dates (including rain incidences) and the cumulative water supply are presented in Figures 1 and 2, respectively. The first irrigation after transplantation was applied approximately three weeks after transplantation, since two rain incidences occurred one and seven days after transplantation. The total amount of water supplied in plants (including precipitation in the case of I1 and I2 treatments) was the following: rain-fed = 515 m^3/ha, I1 = 1828 m^3/ha, and I2 = 3528 m^3/ha (Figure 2). Irrigation for I1 and I2 treatments was carried out based on the readings of sensors (Delta T PR2/4 + HH2; Delta-T devices Ltd., Burwell, UK) that recorded the soil moisture profile at 40 cm of depth. The irrigation was applied via drip irrigation, using a common dripline between each double row and one emitter every 0.5 m.

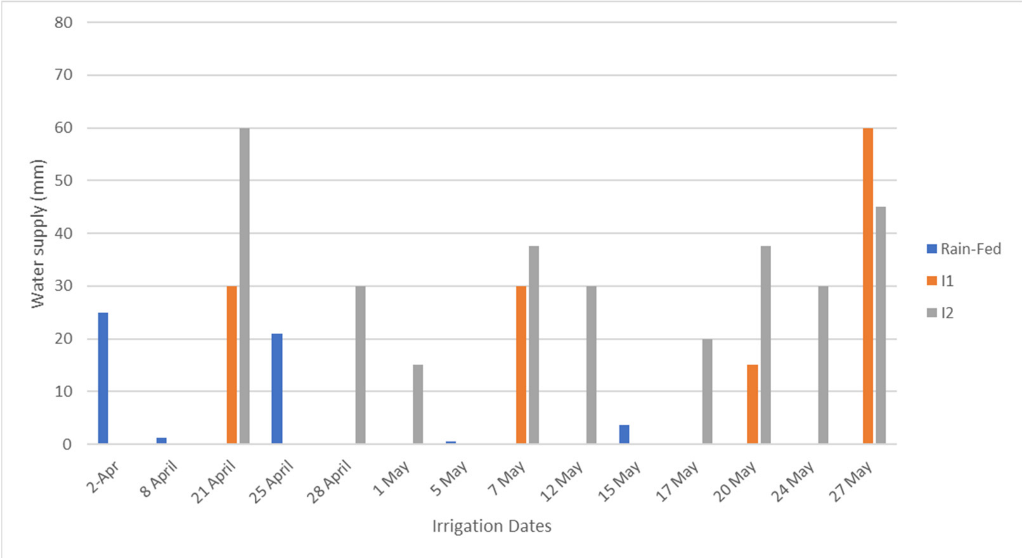

Figure 1. Water supply (mm) during the growing period.

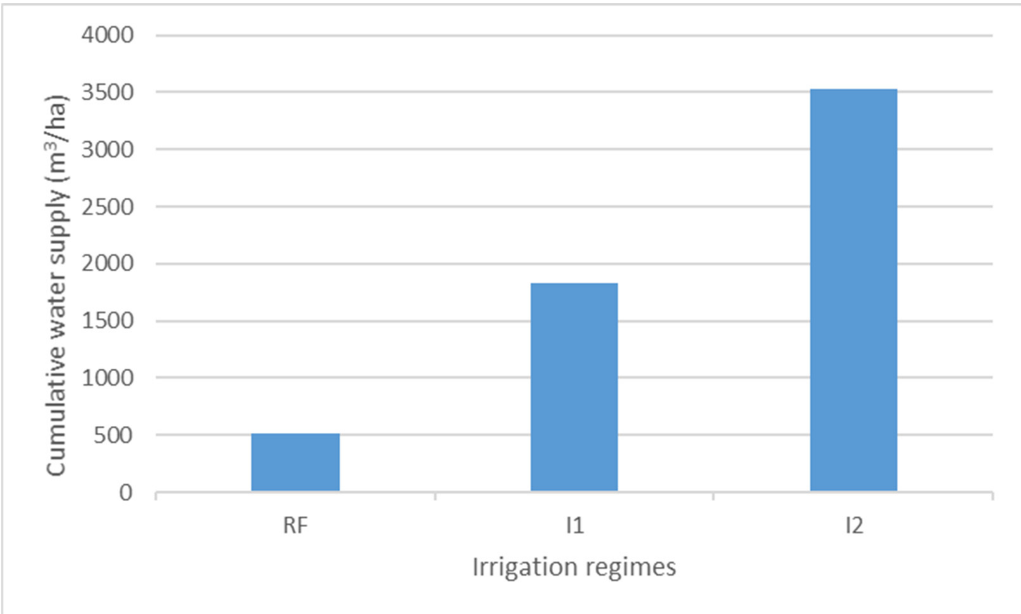

Figure 2. Cumulative water supply (m^3/ha) during the growing period. RF: rain-fed plants; I1: 50% of field capacity; I2: 100% of field capacity.

2.3. Plant Growth and Crop Perfomance Determination

Plants where harvested when they reached a marketable size at 57 DAT. At harvesting day, plant total fresh weight (aerial part), number of leaves, fresh and dry weight of leaves, leaf area index (LAI), and specific leaf area (SLA) were evaluated [35]. Dry weight was determined after forced air drying for approximately after 72 h at 72 °C and until constant weight, while for chlorophyll content (SPAD index) a portable chlorophyll meter (SPAD-502; Konica Minolta Inc., Osaka, Japan) was implemented. The sampling for SPAD determination included three measurements on mature leaves of the same plant, repeated in ten plants from each treatment. The total leaf area (cm^2) was measured in five plants from each replication with the LI-3100C Area Meter (LI-COR Biosciences; Hellamco S.A., Athens, Greece). Specific leaf area (SLA) value was determined the using the formula: SLA = total leaf area/dry weight and was expressed in m^2/kg. Fresh biomass yield was calculated after harvesting the plants of each plot, excluding the borderlines and expressed in kg/ha. Water use efficiency (WUE) was calculated according to the equation [36]:

$$\text{WUE} = \frac{\text{Fresh Yield (kg/ha)}}{\text{Cumulative water supply (mm or m}^3\text{/ha)}}$$

2.4. Chemical Analyses

Free proline content in leaf samples was determined according to the ninhydrin reaction method [37]. Leaf samples (100 mg) were extracted in 10 mL of sulfosalicylic acid (3%) and after filtration the homogenate was put in a water bath at 85 °C for 1 h. The next step included the addition of nihydrin solution including the same amounts of proline, ninhydrin acid, and glacial acetic acid (1:1:1), and then incubated at 90 °C for 1 h. The reaction was stopped after cooling in an ice bath. Finally, proline was extracted using 2 mL of toluene and its absorbance was determined using a spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK) at 520 nm. Proline content was expressed in mg/g fresh weight (f.w.) after calibration with a standard curve from 0 to 2.5 mg/mL of L-proline.

Chlorophyll content was determined after the extraction of leaf samples with acetone, as described by Alexopoulos et al. [38]. In brief, 50 mg of leaf tissue was extracted into 3 mL of acetone and stored at 23 °C in darkness for 2 h prior to analysis. The absorbance of the extracts at 663 and 647 nm was determined with a spectrophotometer (Evolution 210, Thermo Scientific, Abingdon, UK) and chlorophyll a and chlorophyll b content were calculated based on the equations suggested by Lichtenthaler and Buschmann [39]. Chlorophyll a, chlorophyll b, and total chlorophyll content were expressed in mg/g f.w.

Carotenoid content was determined colorimetrically based on the methodology previously described in the literature [39] with slight modifications [38]. Leaf samples were extracted in 80% acetone and then centrifuged at 14,000 rpm for 20 min. After centrifugation, total carotenoids were measured in the supernatants by reading its absorbance at 470 nm. The content was expressed in mg/g f.w.

2.5. Statistical Analysis

The data of growth parameters were collected from 15 plants for each treatment (n = 15). Chemical analyses were performed in triplicate in three batch samples obtained from the fresh tissues harvested from each treatment (n = 3). Statistical analysis was performed with the statistical software JMP v. 16.1 (SAS Institute Inc., Cary, NC, USA). Prior to statistical analysis, the normal distribution of raw data was tested according to the Shaphiro–Wilk test and then a two-way analysis of variance (ANOVA) for each cultivar was performed. When significant differences were detected, means were compared according to the Tukey HSD test (p = 0.05). All the results are expressed as mean values and standard deviations (mean ± SD).

3. Results and Discussion

3.1. Plant Biomass and Growth Parameters

Results regarding the plant height of the two varieties (Romaine and Batavia type, respectively) are presented in Table 1. Concerning the Romaine type, slight differences in plant height were detected at the first sampling date (data not shown), whereas the effect of irrigation treatments and biostimulant application stood out at harvest (Table 1). More specifically, a variable response was detected in the case of Romaine-type lettuce plants where the highest values were measured for the Si treatment at deficit irrigation conditions (Si × I1), although there were no significant differences from C_{NB}, SiC, and HF × RF, and C_{NB}, HF, and VP × I1 treatments. On the other hand, the lowest overall values were recorded for the SiC × I1 treatment, without significant differences from the rest of the treatments being detected (except for the abovementioned ones). Moreover, deficit irrigation conditions resulted in lower height of plants compared with the RF treatment (rain-fed plants) for all the biostimulant products tested, apart from the Si treatment where no significant differences were observed. On the other hand, in the case of Batavia-type lettuce plants, the highest overall height was recorded for plants grown under deficit irrigation and treated with the HF biostimulant, while there were no significant differences with water-stressed plants (I2) treated with SiC treatment or no biostimulants (C_{NB}). In contrast to the Romaine type, deficit irrigation resulted in higher plant height for Batavia plants compared with the control (rain-fed plants) for all the treatments of biostimulants, apart from the SW treatment. According to the literature, the varied response of lettuce genotypes to deficit irrigation in regards to plant height could be attributed to susceptibility to flowering induction, since Izzeldin et al. [40] suggested that increased soil moisture deficit promoted stalk elongation in iceberg lettuce, while Rosental et al. [41] identified specific genetic loci that affect bolting and stalk elongation in lettuce. Regarding the varied response to biostimulant treatments, several studies suggested contrasting reports for the effect of biostimulants to lettuce plant growth depending on the composition of the biostimulant product and the differential mechanisms of action [42], the application method (e.g., foliar or root application) [43] and dose [44,45], while Di Mola et al. [43] highlighted the crop-specific variability of response to specific biostimulants.

Table 1. Plant height (cm) of lettuce plants at harvest.

Biostimulants	Irrigation	Romaine	Batavia
C_{NB}	RF	28.7 ± 3.1 Aab	19.1 ± 2.6 Bcd
	I1	28.3 ± 3.7 Aab	23.1 ± 3.1 Ab
	I2	26.9 ± 3.1 Bbc	25.9 ± 5.8 Aab
SiC	RF	29.3 ± 1.3 Aab	19.2 ± 3.0 Bcd
	I1	24.0 ± 2.2 Bc	20.5 ± 2.3 Bcd
	I2	24.4 ± 2.5 Bc	26.8 ± 4.0 Aa
HF	RF	28.8 ± 2.1 Aab	22.7 ± 2.5 Bbc
	I1	28.1 ± 2.6 Aab	20.1 ± 4.8 Bcd
	I2	26.0 ± 2.8 Bbc	28.5 ± 4.3 Aa
SW	RF	27.7 ± 3.0 Abc	21.2 ± 3.4 Abc
	I1	26.8 ± 2.4 Abbc	19.3 ± 1.9 Acd
	I2	25.2 ± 2.7 Bc	20.9 ± 3.8 Acd
Si	RF	24.7 ± 1.4 Bc	17.9 ± 2.5 Bd
	I1	30.1 ± 3.1 Aa	22.7 ± 4.0 Abc
	I2	24.9 ± 2.5 Bc	24.3 ± 3.1 Ab
VP	RF	27.6 ± 2.9 Abc	18.8 ± 2.3 Bcd
	I1	28.1 ± 2.2 Aab	19.9 ± 3.5 Bcd
	I2	25.7 ± 1.9 Bbc	23.9 ± 3.6 Ab

Means in the same column of the same biostimulant treatment followed by different capital letters are significantly different according to Tukey's HSD test at $p = 0.05$. When means of the same column and for the same irrigation treatment are followed by different lowercase letters, they are significantly different according to Tukey's HSD test at $p = 0.05$. SW: algae extracts + macronutrients + amino acids; HF: humic + fulvic acids; SiC: Si + Ca; Si: Si; VP: plant proteins + amino acids; C_{NB}: without addition of biostimulants; RF: rain-fed plants; I1: 50% of field capacity; I2: 100% of field capacity.

SPAD index values (chlorophyll content) are presented in Table 2. Regarding the Romaine type, the highest and lowest SPAD index values were recorded for the SW × I1 and VP × I2 treatments, respectively, while most of the biostimulants and/or I1 treatments resulted in higher chlorophyll content compared with the I2 irrigation treatment. Similar effects were detected in the case of the Batavia type, where rain-fed conditions and/or I1 treatments resulted in the highest SPAD values for most of the biostimulants, apart from the case of SiC where the differences among the irrigation treatments were not statistically significant. According to El-Nakhel et al. [46], the application of protein hydrolysates may increase SPAD index values in spinach plants and also alleviate the negative effects of salinity stress, while similar results were suggested by Rouphael et al. [47] for the same species and the same category of biostimulants. Moreover, Abdipour et al. [48] suggested that increasing doses of humic acids were beneficial to SPAD index values of green basil plants, whereas Caruso et al. [49] suggested an increase in the SPAD index for plants treated with plant extracts or protein hydrolysates over the control (untreated plants) without significant differences between the two biostimulants. In contrast, Lucini et al. [50] did not record any differences between the type of application (root or root and foliar application) of a plant-derived biostimulant on lettuce SPAD values, although they reported an adverse effect of salinity on the same parameter, whereas Bulgari et al. [45] observed no effect of salinity on total chlorophyll content of lettuce plants. Similarly, Di Mola et al. [43] suggested a variable effect of different biostimulant extracts on SPAD values of baby lettuce depending on nitrogen availability. These contrasting reports highlight the crop-specific response to biostimulant application under limiting conditions which could be attributed to different mechanisms of action depending on the chemical composition of the biostimulant product [51].

Table 2. Values SPAD index of lettuce plants at harvest (means ± SD).

Biostimulant	Irrigation Treatment	Romaine	Batavia
C_{NB}	RF	26.7 ± 1.5 Ab	24.8 ± 1.6 Aa
	I1	28.1 ± 2.1 Aab	17.4 ± 3.2 Bc
	I2	19.5 ± 1.6 Bc	15.5 ± 3.2 Bd
SiC	RF	31.3 ± 1.2 Aab	18.4 ± 3.6 Ac
	I1	28.9 ± 1.5 Bab	17.1 ± 2.3 Ac
	I2	20.5 ± 1.5 Cc	17.2 ± 2.6 Ac
HF	RF	31.2 ± 1.8 Aab	17.9 ± 3.5 Bc
	I1	25.4 ± 1.0 Bb	21.3 ± 4.3 Ab
	I2	24.6 ± 1.0 Bb	14.5 ± 2.9 Cd
SW	RF	27.3 ± 1.2 Bab	24.3 ± 7.0 Aa
	I1	33.3 ± 1.2 Aa	18.4 ± 2.2 Bab
	I2	19.0 ± 1.0 Cc	15.5 ± 2.8 Cd
Si	RF	29.5 ± 1.5 Aab	24.0 ± 4.0 Aa
	I1	29.9 ± 1.0 Aab	15.3 ± 2.9 Bd
	I2	19.7 ± 1.3 Bc	15.6 ± 4.5 Bd
VP	RF	31.9 ± 1.8 Aab	25.7 ± 4.3 Aa
	I1	26.5 ± 1.3 Bb	19.8 ± 4.2 Bbc
	I2	14.9 ± 2.0 Cd	14.8 ± 2.7 Cd

Means in the same column of the same biostimulant treatment followed by different capital letters are significantly different according to Tukey's HSD test at $p = 0.05$. Means in the same column of the same irrigation treatment followed by different lowercase letters are significantly different according to Tukey's HSD test at $p = 0.05$. SW: algae extracts + macronutrients + amino acids; HF: humic + fulvic acids; SiC: Si + Ca; Si: Si; VP: plant proteins + amino acids; C_{NB}: without addition of biostimulants; RF: rain-fed plants; I1: 50% of field capacity; I2: 100% of field capacity.

Plant growth-related parameters for Romaine- and Batavia-type lettuce plants are shown in Tables 3 and 4, respectively. For the Romaine type, total plant weight, leaf weight and leaf area were the highest in the I1 treatment for plants treated with the SW treatment. However, in the case of total plant weight, no significant differences were detected from plants subjected to the same irrigation conditions and treated with HF and Si biostimulant treatments or the C_{NB} (no biostimulants) treatment. The number of leaves was significantly higher for the Si × I1, SiC × I1, and HF × RF (rain-fed conditions) treatments, whereas the lowest overall values were detected for the C_{NB} × RF and SiC × I2 treatments. The weight of leaves was the highest in the case of the SW treatment under deficit irrigation (I1), with no significant differences being detected from plants treated with the same biostimulant under full irrigation (I2) or the plants subjected to deficit irrigation (I1) and no biostimulant application. Similar trends were detected for the leaf area values where the SW treatment under deficit or full irrigation (I1 and I2 treatments, respectively) resulted in significantly higher values compared with the rest of the treatments, indicating that the weight of plant and leaves was higher due to larger leaves and not to the formation of more leaves. Dry weight of leaves differed significantly among the studied treatments, while significantly higher values were recorded for the rain-fed plants that received Si or no biostimulants. Moreover, I1 treatment resulted in higher plant weight and weight of leaves compared with the rain-fed plants for most of the tested biostimulants, apart from the case of VP and SiC application, whereas the I2 treatment resulted in lower dry weight for all the tested biostimulants compared with the rain-fed plants. Finally, specific leaf area (SLA) values were the highest for the I2 treatment, independently of the biostimulant treatment, apart from the VP treatment where RF treatment increased SLA values.

Table 3. Growth-related parameters of Romaine-type lettuce plants concerning the irrigation regime and the biostimulant treatment (means ± SD).

Biostimulant	Irrigation Treatment	Plant Weight (g)	Number of Leaves	Weight of Leaves (g)	Leaf Area (cm^2)	Dry Weight (%)	Specific Leaf Area (m^2/kg)
C_{NB}	RF	402.7 ± 12.0 Bb	36 ± 1 Be	298.5 ± 7.1 Bc	5905.4 ± 173.6 Bb	8.3 ± 3.9 Aa	26.8 ± 1.2 Cd
	I1	437.4 ± 10.6 Ab	42 ± 1.4 Ac	362.4 ± 6.9 Ab	6647.6 ± 108.3 Ab	5.0 ± 0.3 Bc	36.6 ± 1.5 Ba
	I2	363.1 ± 18.3 Cb	36.8 ± 1.6 Bc	284.8 ± 5.9 Bb	5209.1 ± 134.9 Cb	3.8 ± 0.8 Cb	51.1 ± 1.6 Aa
SiC	RF	429.1 ± 12.8 Aa	43.6 ± 1.3 Bb	346.6 ± 18.5 Aa	5997.0 ± 129.7 Ab	7.4 ± 0.7 Aab	23.9 ± 2.6 Ce
	I1	312.9 ± 11.0 Cd	44 ± 1.8 Ab	257.8 ± 13.9 Cd	4630.9 ± 198.6 Be	6.9 ± 0.6 Ba	27.8 ± 2.9 Bc
	I2	348.1 ± 8.1 Bc	36.2 ± 1.3 Cc	280.4 ± 14.7 Bb	4808.8 ± 109.0 Bc	5.6 ± 0.5 Ca	32.1 ± 1.9 Ad
HF	RF	392.1 ± 10.4 Bc	45.4 ± 1.6 Aa	322.5 ± 9.2 Bb	6375.5 ± 120.8 Aa	6.6 ± 0.6 Ab	31.0 ± 1.0 Bb
	I1	438.9 ± 14.2 Ab	37.6 ± 1.0 Ce	355.5 ± 12.4 Ab	6472.7 ± 193.1 Ac	6.2 ± 0.4 Bb	30.0 ± 1.6 Bb
	I2	311.5 ± 8.4 Cd	42 ± 1.8 Ba	253.0 ± 8.7 Cc	4813.7 ± 163.3 Bc	5.5 ± 0.5 Ca	35.3 ± 2.0 Ac
SW	RF	323.6 ± 18.8 Cd	41.2 ± 2.2 Ac	260.4 ± 12.9 Bd	5176.5 ± 198.0 Be	6.9 ± 1.4 Ab	29.5 ± 1.2 Bc
	I1	460.5 ± 10.4 Aa	42.6 ± 1.9 Ac	379.3 ± 8.0 Aa	6928.8 ± 147.6 Aa	6.4 ± 0.7 Bab	28.8 ± 1.9 Bbc
	I2	440.1 ± 14.4 Ba	37.2 ± 1.6 Bc	362.8 ± 7.5 Aa	6718.7 ± 146.3 Aa	4.2 ± 0.5 Cb	44.5 ± 1.9 Ab
Si	RF	325.4 ± 11.2 Cd	43.2 ± 1.8 Bb	267.5 ± 6.4 Bd	5392.1 ± 118.0 Bd	8.1 ± 1.7 Aa	25.8 ± 1.9 Cd
	I1	451.2 ± 12.8 Aa	46.8 ± 1.0 Aa	357.3 ± 7.3 Ab	6542.8 ± 109.4 Abc	6.2 ± 0.7 Bb	30.3 ± 1.8 Bb
	I2	361.3 ± 11.8 Bb	40.4 ± 1.9 Cb	283.4 ± 5.2 Bb	5167.4 ± 124.9 Bb	5.6 ± 0.7 Ca	33.1 ± 1.7 Ad
VP	RF	417.9 ± 19.1 Aab	41.2 ± 1.6 Ac	324.9 ± 6.7 Ab	5679.3 ± 109.1 Ac	4.5 ± 1.7 Cc	46.8 ± 2.0 Aa
	I1	381.3 ± 13.8 Bc	39.6 ± 1.4 Bd	297.3 ± 9.9 Bc	5125.4 ± 152.7 Bd	6.9 ± 0.4 Aa	25.4 ± 1.5 Cd
	I2	302.7 ± 14.2 Ce	37.4 ± 1.10 Cc	245.6 ± 1.0 Cc	4495.3 ± 105.8 Cd	5.2 ± 0.6 Ba	36.1 ± 1.4 Bc

Means in the same column of the same biostimulant treatment followed by different capital letters are significantly different according to Tukey's HSD test at p = 0.05. Means in the same column of the same irrigation treatment followed by different lowercase letters are significantly different according to Tukey's HSD test at p = 0.05. SW: algae extracts + macronutrients + amino acids; HF: humic + fulvic acids; SiC: Si + Ca; Si: Si; VP: plant proteins + amino acids; C_{NB}: without addition of biostimulants; RF: rain-fed plants; I1: 50% of field capacity; I2: 100% of field capacity.

Regarding the growth parameters of Batavia-type lettuce plants, total plant weight and also the weight of leaves was significantly higher for the plants treated with full irrigation and the VP biostimulant, whereas the lowest values were detected for the rain-fed plants that received VP, SiC, and C_{NB} treatments (Table 4). Moreover, full irrigation resulted in significantly higher total plant and leaf weight, regardless of the biostimulant treatment. The number of leaves was significantly increased for the SiC and SW treatments under full irrigation, while total leaf area was significantly higher for the Si (I1 and I2 treatments) and VP × I2 treatment. Dry matter content was increased under deficit or full irrigation regime for most of the biostimulants (except for the case of HF treatment), while the highest overall values were recorded for the C_{NB}, SiC, and VP of rain-fed plants. Finally, specific leaf area was significantly increased under deficit and/or full irrigation regime for all the tested biostimulants, while the values recorded for I2 × SiC, I2 × HF, and Si × I1 and I2 were significantly higher than the rest of the treatments. Moreover, both leaf and specific leaf area showed an increase under full irrigation for most of the biostimulants tested.

Table 4. Growth parameters of Batavia-type lettuce plants concerning the irrigation regime and the biostimulant treatment (means ± SD).

Biostimulant	Irrigation Treatment	Plant Weight (g)	Number of Leaves	Weight of Leaves (g)	Leaf Area (cm^2)	Dry Weight (%)	Specific Leaf Area (m^2/kg)
C_{NB}	RF	240.2 ± 16.9 Cc	25.4 ± 1.6 Bb	172.6 ± 12.2 Cc	3645 ± 168 Cd	8.4 ± 1.1 Aa	25.8 ± 2.2 Cd
	I1	388.1 ± 14.4 Ba	27.6 ± 1.1 Abc	318.8 ± 10.7 Ba	6700 ± 113 Bb	5.5 ± 0.7 Bab	39.3 ± 1.2 Bd
	I2	431 ± 24 Ad	28.0 ± 0.7 Ab	340 ± 10 Ac	7025 ± 70 Ac	4.3 ± 0.4 Ca	48.5 ± 2.2 Ac
SiC	RF	236.9 ± 19.7 Cc	26.4 ± 1.1 Bb	183.5 ± 16.8 Cc	3852 ± 139 Cc	7.6 ± 1.7 Aa	29.4 ± 8.2 Cc
	I1	358.0 ± 16.4 Bb	27.2 ± 1.6 Bcd	278.4 ± 12.3 Bb	5695 ± 129 Bc	5.0 ± 0.5 Bb	41.6 ± 2.0 Bc
	I2	482.6 ± 13.8 Ab	30.8 ± 1.6 Aa	384.6 ± 11.4 Ab	7479 ± 64 Ab	3.8 ± 0.6 Cb	53.1 ± 1.8 Aa
HF	RF	333.6 ± 13.1 Bb	29.6 ± 1.2 Aa	266.2 ± 10.4 Ba	5065 ± 166 Bb	6.0 ± 0.6 Abc	31.8 ± 2.1 Cb
	I1	296.2 ± 12.9 Cd	28 ± 1.7 Bab	231.0 ± 14.7 Cd	5173 ± 206 Bd	5.9 ± 1.2 Aa	39.9 ± 1.7 Bcd
	I2	442.2 ± 13.9 Acd	29 ± 2 Aab	336.7 ± 16.0 Ac	7045 ± 60 Ac	4.1 ± 0.5 Bab	51.3 ± 1.5 Ab
SW	RF	350.1 ± 14.2 Ba	28.8 ± 1.9 Ba	262.8 ± 10.2 Ba	5499 ± 174 Aa	5.8 ± 0.6 Ac	36.2 ± 2.3 Ba
	I1	333.1 ± 11.5 Bc	28.4 ± 1.4 Ba	252.8 ± 11.0 Bc	5548 ± 112 Ac	5.2 ± 0.9 Bbc	44.1 ± 3.0 Ab
	I2	472.9 ± 18.1 Ab	31.6 ± 1.2 Aa	385.6 ± 12.7 Ab	5678 ± 154 Ad	3.7 ± 0.6 Cb	45.5 ± 1.5 Ad
Si	RF	338.5 ± 9.1 Cab	25.6 ± 0.9 Bb	204.6 ± 12.5 Cb	3818 ± 103 Bc	6.7 ± 0.9 Ab	28.7 ± 1.4 Bc
	I1	396.2 ± 11.6 Ba	26.4 ± 1.4 Bd	315.3 ± 9.6 Ba	7515 ± 254 Aa	4.6 ± 0.7 Bc	52.2 ± 2.0 Aa
	I2	451.3 ± 6.7 Ac	28.4 ± 0.6 Ab	342.9 ± 15.6 Ac	7678 ± 117 Aa	4.3 ± 0.8 Bab	54.3 ± 2.1 Aa
VP	RF	235.8 ± 11.4 Cc	26.0 ± 1.9 Cb	187.5 ± 7.9 Cc	4012 ± 176 Cc	7.7 ± 2.0 Aa	30.1 ± 3.6 Cbc
	I1	287.4 ± 11.9 Bd	28.4 ± 2.1 Ba	239.3 ± 6.9 Bd	5062 ± 83 Bd	5.8 ± 0.4 Ba	37.5 ± 1.6 Be
	I2	507.4 ± 14.6 Aa	30.4 ± 2 Aa	413.2 ± 10.6 Aa	7662 ± 177 Aa	4.5 ± 0.4 Ca	41.4 ± 1.6 Ae

Means in the same column of the same biostimulant treatment followed by different capital letters are significantly different according to Tukey's HSD test at $p = 0.05$. Means in the same column of the same irrigation treatment followed by different lowercase letters are significantly different according to Tukey's HSD test at $p = 0.05$. SW: algae extracts + macronutrients + amino acids; HF: humic + fulvic acids; SiC: Si + Ca; Si: Si; VP: plant proteins + amino acids; C_{NB}: without addition of biostimulants; RF: rain-fed plants; I1: 50% of field capacity; I2: 100% of field capacity.

According to the literature reports, abiotic stressors had a significant effect on plant growth-related parameters of lettuce and resulted in decreased plant and leaf weight with increasing salinity [46,50], nitrogen deprivation [43], or deficit irrigation [34,52]. However, the results of our study indicate a genotype-dependent response, since Batavia-type lettuce plants were more susceptible to deficit irrigation than Romaine-type plants by showing a decrease in plant and leaf weight compared with the full irrigation regime. Malejane et al. [53] also recorded a variable response of two lettuce cultivars to deficit irrigation, although both genotypes showed a significant decrease with increasing water stress, while Adhikari et al. [54] suggested a genotype-dependent response to salinity stress for thirty-two lettuce genotypes. Moreover, several studies reported a beneficial impact of biostimulant application on the growth of leafy vegetable plants cultivated under stressful conditions. For example, Malécange et al. [55] suggested that the application of a biostimulant rich in free amino acids improved lettuce plant growth under deficit irrigation, while Di Mola et al. [43] reported a beneficial impact of biostimulants (seaweed extracts, protein hydrolysates, and plant extracts) on baby lettuce growth under nitrogen deprivation. Moreover, Liang et al. [56] suggested that Si may accelerate cell division and cell elongation, strengthen the plant immune system, and promote plant development through altering the water balance in plants. Similarly, Bulgari et al. [45] and Abdipour et al. [48] suggested the positive impact of biostimulants (organic extracts and humic acids) on lettuce and basil plants, respectively; while they reported a dose-dependent response. Hernandez et al. [57] also claimed that applying humates to lettuce plants may speed up development, allowing for earlier harvesting of the plants while also increasing yields by encouraging the growth of more leaves, while positive effects on lettuce plants have also been recorded for amino acids and bacterial–algal biostimulants [58,59]. In contrast, El-Nakhel et al. [46] did not report a significant impact of protein hydrolysates obtained from legumes on the average leaf weight of *Spinacia oleracea* plants grown under saline conditions, while according to Caruso et al. [49], no significant differences between tropical plant extracts and legume-derived protein hydrolysates were observed regarding the yield and mean weight of marketable leaves of wall rocket plants.

Regarding the leaf area and specific leaf area values, a varied response to the irrigation regime was observed depending on the type of lettuce plants and the biostimulant tested. Moreover, the observed values of leaf area were concomitant of increased leaf weight only in the case of Romaine-type lettuce plants, which could be associated with the differences in leaf morphology between the types of lettuce (Romaine vs. Batavia). Specific leaf area increased under full irrigation in both lettuce types regardless of the biostimulant tested (except for the case of the VP biostimulant in Romaine-type lettuce plants), while the opposite trend was recorded for the dry matter content which was higher in rain-fed plants for most of the biostimulant treatments (except for the VP biostimulant in Romaine-type plants). The literature reports are in agreement with the results of our study and also indicate a negative effect of water deficit and abiotic stressors on total leaf area of leafy vegetables, such as baby leaf lettuce [60]. Moreover, biostimulant application was beneficial for the leaf area values of various species, such as *Brassica rapa* L. subsp. *sylvestris* [61], zucchini [62] and lettuce plants [43,63] treated with seaweed extracts, lettuce plants treated with bacterial inoculum [26], spinach and lettuce plants treated with legume-derived protein hydrolysates [64,65], or lettuce plants treated with various biostimulants (e.g., legume-derived protein hydrolysates, seaweed extracts, vegetal oils + seaweed extracts + herbal extracts) [47]. Moreover, Asgharipour and Masapour [66] results were in line with our study, since they also reported that silicon foliar spray under water deficiency conditions demonstrated positive effects on leaf area. The increased leaf area under deficit and/or full irrigation conditions recorded in the Batavia plants for most of the biostimulant treatments could be attributed to the improved water relations that biostimulants may induce, as well as to leaf morphology and tenderness of leaf tissues which render them more susceptible to water deficit conditions [62]. On the other hand, Romaine-type lettuce plants seem to be more tolerant to deficit irrigation due to the morphology of the head and the texture of leaves; thus, on several occasions, full irrigation resulted in lower leaf area compared with rain-fed or deficit irrigation regimes. Moreover, according to the literature, water or salinity stress conditions may result in lower leaf expansion and specific leaf area values in lettuce plants as part of the adaptation mechanism of plants under stress [67], a result that agrees with the results of the present study.

The results related to water use efficiency (WUE) of Romaine-type lettuce plants are presented in Figure 3. The highest WUE values were recorded for the rain-fed plants due to the low amounts of water that plants received over the growing period (only 14.6% of full irrigation and 28.2% of deficit irrigation; see Figure 2), regardless of the biostimulant treatment, although some biostimulant products (e.g., HF, SW, and Si treatments) resulted in higher WUE values compared with the rest of the treatments. On the other hand, deficit irrigation (I1) also resulted in higher WUE values compared with full irrigation (I2) for all the biostimulant treatments, especially in the case of C_{NB}, SiC, and Si treatments, apart from the VP treatment where no significant differences between the deficit and full irrigation were recorded. Similar trends for the WUE values in response to the irrigation regime and biostimulant application were also recorded in the case of Batavia-type lettuce plants where rain-fed plants recorded the highest values compared with the other two irrigation regimes, regardless of the biostimulant treatment (Figure 4). However, in this type of lettuce, the SiC treatment was the most beneficial biostimulant, followed by the VP treatment, indicating a variable response to water use efficiency depending on the genotype. Moreover, Si and C_{NB} treatments were also the most effective in increasing water use efficiency under deficit irrigation, while the seaweed extracts were the most efficient under full irrigation conditions.

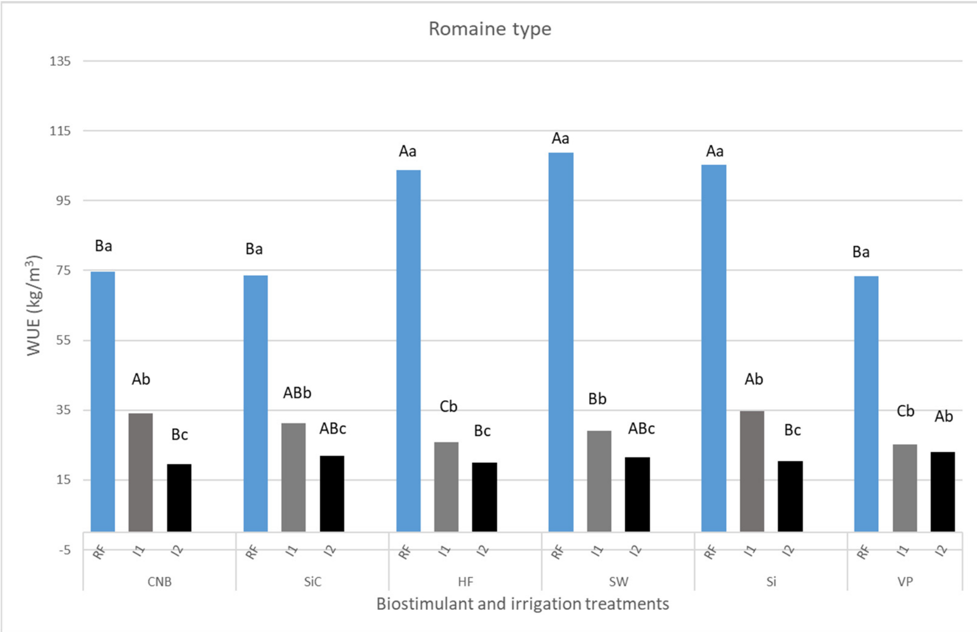

Figure 3. Water use efficiency (WUE) of Romaine-type lettuce plants in relation to biostimulants and irrigation regime. Capital letters above bars indicate significant differences between the means of the same irrigation regime, according to Tukey's HSD test at $p = 0.05$. Lowercase letters above bars indicate significant differences between the means of the same biostimulant treatment, according to Tukey's HSD test at $p = 0.05$. SW: algae extracts + macronutrients + amino acids; HF: humic + fulvic acids; SiC: Si + Ca; Si: Si; VP: plant proteins + amino acids; C_{NB}: without addition of biostimulants; RF: rain-fed plants; IR.1: 50% of field capacity; IR.2: 100% of field capacity.

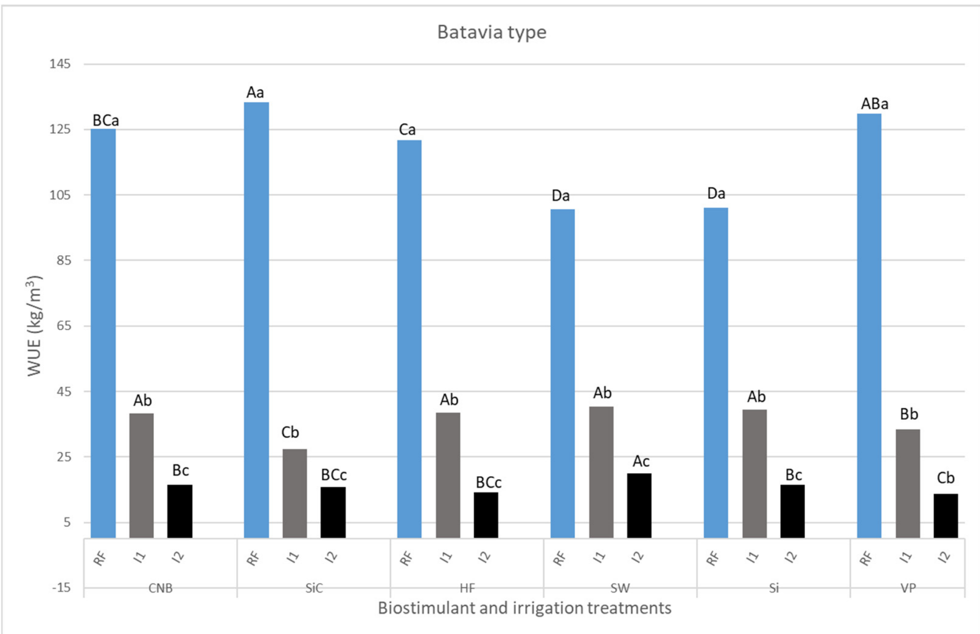

Figure 4. Water use efficiency (WUE) of Batavia-type lettuce plants in relation to biostimulants and irrigation regime. Capital letters above bars indicate significant differences between the means of the same irrigation regime, according to Tukey's HSD test at $p = 0.05$. Lowercase letters above bars indicate significant differences between the means of the same biostimulant treatment, according to Tukey's HSD test at $p = 0.05$. SW: algae extracts + macronutrients + amino acids; HF: humic + fulvic acids; SiC: Si + Ca; Si: Si; VP: plant proteins + amino acids; C_{NB}: without addition of biostimulants; RF: rain-fed plants; IR.1: 50% of field capacity; IR.2: 100% of field capacity.

The observed WUE values, especially those of Batavia lettuce, are in the same range recorded by Kuslu et al. [68] who examined the effect of deficit irrigation on a curly lettuce genotype. Moreover, based on our results, it could be suggested that specific biostimulants may beneficially affect the water relation of plants and improve its efficient use under water scarcity or full irrigation by increasing total plant weight and leaf weight and consequently crop performance [69]. However, apart from the best WUE values, the total plant weight and leaf weight also have to be considered in order to identify the irrigation conditions that allow profitable yields and rational use of water resources. In this scenario, the two lettuce types tested responded differently with the Romaine type being more resilient to water deficit than the Batavia type, thus allowing for higher yields under water deficit (I1 treatment) for most of the biostimulants tested. Similarly to our study, Lin et al. [70] suggested that betaine and chitin significantly increased WUE in lettuce plants subjected to regulated deficit irrigation and its application protected plants under water stress conditions. The results of Taha et al. [71] were in the same line, since pollen grain extracts significantly improved WUE values in basil plants grown under water stress, while pyroglutamic acid also had beneficial effects on lettuce plants grown under deficit irrigation [34]. Taha et al. [71] also proposed, as potential mechanisms of action, the prevention of water loss due to osmoprotection or the induction of the antioxidant mechanisms of plants [71]. Moreover, Ors and Suarez suggested that the combined application of water and salinity stress may significantly increase WUE values of spinach plants, although a little effect of water stress was recorded. The increasing salinity also resulted in increased WUE values of spinach plants [72]. In contrast to our study, Rouphael et al. [62] did not record a significant impact of biostimulant application on the WUE of zucchini plants, while the authors suggested a significant decrease under saline conditions. Moreover, Vetrano et al. [26] reported a lack of effect on the WUE of lettuce plants for the application of bacterial inoculum, while Pokluda et al. [73] did not report any effects of biostimulants on the WUE of spinach plants grown under chilling stress. On the other hand, Balestrini et al. [74] and Begum et al. [75] associated improved WUE with arbuscular mycorrhizal symbiosis. In the study of Choi et al. [76] who tested two application forms of the same biostimulant (foliar spray and root drench of protein hydrolysates) on lettuce leaves, only root application differed from the untreated plants, while no significant differences were recorded between the two application forms. All these results from literature reports suggest there is a crop-specific response to particular biostimulants which may variably regulate plant water relations and improve WUE of vegetable crops without compromising the yield and the farmer's income.

3.2. Chemical Composition of Leaves

The chemical composition of Romaine- and Batavia-type lettuce plants leaves is presented in Tables 5 and 6, respectively. In the case of Romaine lettuce, free proline content showed a variable response to irrigation regime and biostimulant application (Table 5). In particular, the highest proline content under rain-fed was detected for the plants that were not treated with biostimulants (C_{NB}), whereas the VP treatment resulted in the lowest overall proline content. Deficit irrigation had a variable effect on proline content depending on the biostimulant product, thus showing an increase (C_{NB}, Si, VP), a decrease (SiC, SW), or no effect (HF) compared with the rain-fed conditions. Moreover, the proline content under full irrigation also showed a varied response and either increased (HF, Si, VP) or decreased (C_{NB}, SiC, SW) compared with rain-fed conditions. Similar results were recorded in Batavia-type lettuce plants, where proline content was lower under full irrigation condition for almost all the tested biostimulants (except for Si and VP treatments where proline content increased compared with rain-fed conditions but decreased over the deficit irrigation regime; Table 6). Considering that free proline content is associated with the non-enzymatic antioxidant defense system of plants, our findings indicate that specific biostimulants may alleviate water-stress effects by inducing the accumulation of proline which acts as an osmoprotectant under stress conditions, modulates the activities of antioxidant enzymes and the subcellular functions, or acts as a cleansing molecule of reactive oxygen species [34,77]. Moreover, according to Malejane et al. [53] and Adhikari et al. [54], a variable response to water stress should be expected between different lettuce genotypes, which is in agreement with our results since the tested genotypes showed a variable content of free proline concerning the irrigation regime and biostimulant application.

Table 5. Content of free proline, chlorophylls (chlorophyll a, b, and total chlorophylls), and carotenoids of Romaine-type lettuce plants in relation to irrigation regime and biostimulant application (means ± SD).

Biostimulant	Irrigation Treatment	Free Proline mg/g f.w.	Chlorophyll a mg/gr f.w.	Chlorophyll b mg/gr f.w.	Total Chlorophylls mg/gr f.w.	Carotenoids mg/gr f.w.
C_{NB}	RF	3.42 ± 0.005 Ba	0.016 ± 0.0002 Cc	0.008 ± 0.0000 Bb	0.024 ± 0.0002 Bd	0.021 ± 0.0004 Bd
	I1	4.28 ± 0.02 Ab	0.023 ± 0.0005 Bc	0.011 ± 0.0000 Ab	0.034 ± 0.0005 Ab	0.030 ± 0.0002 Aa
	I2	0.50 ± 0.002 Cf	0.0308 ± 0.0001 Ac	0.008 ± 0.0004 Bb	0.039 ± 0.0004 Ab	0.023 ± 0.0000 Bc
SiC	RF	2.47 ± 0.01 Ae	0.023 ± 0.0001 Bb	0.015 ± 0.0002 Aa	0.038 ± 0.0001 Bb	0.028 ± 0.0001 Ab
	I1	1.39 ± 0.003 Cf	0.035 ± 0.0002 Aa	0.011 ± 0.0003 Bb	0.046 ± 0.0001 Aa	0.032 ± 0.0000 Aa
	I2	1.65 ± 0.01 Bd	0.017 ± 0.0001 Ce	0.005 ± 0.0004 Cc	0.022 ± 0.0004 Cd	0.015 ± 0.0001 Bd
HF	RF	2.74 ± 0.04 Bc	0.026 ± 0.003 Aab	0.007 ± 0.0006 Bb	0.033 ± 0.0030 Bc	0.024 ± 0.0001 Cc
	I1	2.81 ± 0.007 Bd	0.027 ± 0.0002 Ab	0.018 ± 0.0002 Aa	0.045 ± 0.0004 Aa	0.029 ± 0.0001 Ba
	I2	6.29 ± 0.07 Ab	0.014 ± 0.001 Bf	0.004 ± 0.0007 Bc	0.018 ± 0.0011 Ce	0.039 ± 0.0026 Aa
SW	RF	2.65 ± 0.009 Ad	0.013 ± 0.0001 Bd	0.007 ± 0.0003 Bb	0.020 ± 0.0002 Bd	0.015 ± 0.0001 Be
	I1	1.73 ± 0.004 Be	0.023 ± 0.0004 Ac	0.012 ± 0.0003 Ab	0.035 ± 0.0001 Ab	0.029 ± 0.0001 Aa
	I2	1.54 ± 0.06 Ce	0.023 ± 0.0004 Ae	0.012 ± 0.0003 Aa	0.035 ± 0.0001 Ac	0.029 ± 0.0001 Ab
Si	RF	2.96 ± 0.005 Cb	0.027 ± 0.0002 Ca	0.014 ± 0.0004 Aa	0.042 ± 0.0001 Ba	0.034 ± 0.0002 Aa
	I1	5.54 ± 0.03 Ba	0.034 ± 0.0005 Ba	0.011 ± 0.0016 Ab	0.045 ± 0.0017 Ba	0.031 ± 0.0004 Aba
	I2	8.29 ± 0.03 Aa	0.040 ± 0.0002 Aa	0.013 ± 0.0003 Aa	0.054 ± 0.0004 Aa	0.028 ± 0.000 Bb
VP	RF	1.89 ± 0.006 Cf	0.016 ± 0.0003 Cc	0.006 ± 0.0003 Ab	0.021 ± 0.0005 Bd	0.017 ± 0.0001 B
	I1	3.86 ± 0.01 Ac	0.028 ± 0.0001 Bb	0.001 ± 0.0001 Bc	0.038 ± 0.0001 Ab	0.024 ± 0.0001 Ab
	I2	2.04 ± 0.003 Bc	0.035 ± 0.0036 B A	0.002 ± 0.0108 Bd	0.037 ± 0.0260 Abc	0.022 ± 0.0002 Ac

Means in the same column of the same biostimulant treatment followed by different capital letters are significantly different according to Tukey's HSD test at p = 0.05. Means in the same column of the same irrigation treatment followed by different lowercase letters are significantly different according to Tukey's HSD test at p = 0.05. SW: algae extracts + macronutrients + amino acids; HF: humic + fulvic acids; SiC: Si + Ca; Si: Si; VP: plant proteins + amino acids; C_{NB}: without addition of biostimulants; RF: rain-fed plants; I1: 50% of field capacity; I2: 100% of field capacity.

Apart from free proline content, chlorophyll content can be also used as a stress indicator since it reflects the photosynthetic activity of plants [78,79]. The results of chlorophyll a, chlorophyll b, and total chlorophyll content in Romaine-type lettuce plants are presented in Table 5. A variable response to the irrigation regime and biostimulant application was recorded, although total and or individual chlorophyll content decreased under rain-fed conditions for most of the biostimulants tested (except for the SiC and HF treatments where the full irrigation showed the highest content). In the case of Batavia-type lettuce plants, the same trend was recorded only for the C_{NB} and SW treatments, whereas the combinations of Si × I1 and VP × I2 showed the highest and lowest overall values of individual and total chlorophyll content (Table 6). The literature reports suggest contrasting results regarding the chlorophyll content under stress conditions, with some reports indicating an increase in chlorophyll content due to the increased number of chloroplasts in the leaves of stressed plants [80], while others report a decrease in the content of chlorophyll due to cell oxidative damage and the deterioration of metabolic processes [81,82]. Goni et al. [83], who examined three commercial biostimulatory products that contained *Ascophyllum nodosum* extracts in a tomato plant pot experiment under irrigation stress, suggested that two of the tested formulations showed significantly higher chlorophyll content under reduced irrigation compared with the untreated plants. Moreover, Hernandez et al. [57] indicated that humates did not impact chlorophyll content and also suggested that morphological responses of lettuce plants to biostimulant application should be attributed to physiological responses. Additionally, the usage of fertilizers with peptides and amino acids or protein hydrolysates considerably enhanced crop production and chlorophyll content due to their stimulating effects on the phyllosphere's plant growth-promoting bacteria, which in turn affect plant growth [84].

Table 6. Content of free proline, chlorophylls (chlorophyll a, b, and total chlorophylls), and carotenoids of Batavia-type lettuce plants in relation to irrigation regime and biostimulant application (means ± SD).

Biostimulant	Irrigation Treatment	Free Proline mg/g f.w.	Chlorophyll a mg/gr f.w.	Chlorophyll b mg/gr f.w.	Total Chlorophylls mg/gr f.w.	Free Proline mg/g f.w.
C_{NB}	RF	3.02 ± 0.003 Ac	0.026 ± 0.0004 Bc	0.007 ± 0.0001 Bc	0.033 ± 0.0003 Bc	0.029 ± 0.0002 Bb
	I1	2.69 ± 0.03 Be	0.025 ± 0.0008 Bd	0.008 ± 0.0004 Bb	0.033 ± 0.001 Bd	0.018 ± 0.0001 Cd
	I2	2.09 ± 0.006 Cd	0.037 ± 0.0007 Aa	0.020 ± 0.0002 Aa	0.057 ± 0.0005 Aa	0.042 ± 0.0023 Aa
SiC	RF	4.17 ± 0.01 Aa	0.040 ± 0.0003 Aa	0.019 ± 0.0001 Aa	0.059 ± 0.0004 Aa	0.045 ± 0.0001 Aa
	I1	0.71 ± 0.03 Bf	0.035 ± 0.0009 Bb	0.008 ± 0.0006 Bb	0.043 ± 0.001 Bb	0.012 ± 0.0004 Ce
	I2	0.63 ± 0.007 Be	0.015 ± 0.001 Cc	0.004 ± 0.0003 Cd	0.019 ± 0.001 Cd	0.036 ± 0.0001 Bb
HF	RF	3.72 ± 0.08 Ab	0.036 ± 0.001 Ab	0.011 ± 0.0002 Bb	0.047 ± 0.002 Ab	0.025 ± 0.0006 Bc
	I1	3.15 ± 0.02 Bc	0.030 ± 0.0008 Bc	0.008 ± 0.0002 Bb	0.037 ± 0.0009 Bc	0.021 ± 0.0001 Bc
	I2	2.24 ± 0.01 Cc	0.031 ± 0.0002 Bb	0.016 ± 0.0002 Ab	0.047 ± 0.0001 Ab	0.032 ± 0.0001 Ac
SW	RF	2.60 ± 0.006 Bd	0.019 ± 0.0002 Bd	0.012 ± 0.0003 Ab	0.031 ± 0.0002 Bc	0.025 ± 0.0002 Ac
	I1	2.91 ± 0.003 Ad	0.020 ± 0.0001 Be	0.008 ± 0.0002 Ab	0.028 ± 0.0004 Be	0.022 ± 0.0001 Ac
	I2	2.28 ± 0.009 Cc	0.031 ± 0.002 Ab	0.009 ± 0.0002 Ac	0.040 ± 0.002 Ac	0.025 ± 0.0009 Ad
Si	RF	2.09 ± 0.02 Ce	0.012 ± 0.0003 Be	0.003 ± 0.0002 Bd	0.015 ± 0.0005 Be	0.021 ± 0.002 Bd
	I1	5.58 ± 0.18 Aa	0.059 ± 0.0002 Aa	0.027 ± 0.0002 Aa	0.086 ± 0.0004 Aa	0.055 ± 0.0001 Aa
	I2	2.58 ± 0.005 Bb	0.014 ± 0.0001 Bc	0.007 ± 0.0003 Bc	0.020 ± 0.0002 Bd	0.018 ± 0.0001 Be
VP	RF	2.96 ± 0.02 Cc	0.021 ± 0.0008 Ad	0.005 ± 0.0003 Be	0.026 ± 0.0006 Bd	0.015 ± 0.0002 Ce
	I1	3.83 ± 0.003 Ab	0.022 ± 0.0002 Ae	0.009 ± 0.0002 Ab	0.031 ± 0.0002 Ad	0.028 ± 0.0001 Ab
	I2	3.10 ± 0.02 Ba	0.009 ± 0.0001 Bd	0.003 ± 0.0004 Bd	0.012 ± 0.0005 Ce	0.023 ± 0.008 Bd

Means in the same column of the same biostimulant treatment followed by different capital letters are significantly different according to Tukey's HSD test at $p = 0.05$. Means in the same column of the same irrigation treatment followed by different lowercase letters are significantly different according to Tukey's HSD test at $p = 0.05$. SW: algae extracts + macronutrients + amino acids; HF: humic + fulvic acids; SiC: Si + Ca; Si: Si; VP: plant proteins + amino acids; C_{NB}: without addition of biostimulants; RF: rain-fed plants; I1: 50% of field capacity; I2: 100% of field capacity.

Total carotenoid content of Romaine- and Batavia-type lettuce plants is presented in Tables 5 and 6, respectively. A variable response was detected depending on the irrigation regime and biostimulant treatment although no specific trend was observed. In the case of Romaine-type lettuce plants, the higher amounts of total carotenoids were recorded for I1 and/or I2 treatments, regardless of the biostimulant treatments, while the highest values were measured for the HF × I2 treatment and the lowest ones for the treatments of SiC × I2 and SW × RF (rain-fed conditions; Table 5). In contrast, the carotenoid content in Batavia-type lettuce was lower under deficit irrigation (I1) compared with the rest of the irrigation treatments for almost all the tested biostimulants, except for Si and VP treatments where the highest content was recorded (Table 6). Moreover, considering the effects of the tested irrigation and biostimulant treatments on total plant weight, there is no correlation of total plant weight and total carotenoid content since the treatments where high total weight values were recorded (see Tables 3 and 4) did not correspond to high total carotenoid content. Therefore, considering that carotenoids are light-harvesting pigments which stabilize the membranes of chloroplasts, they can contribute to stress mitigation and photosynthesis regulation, thus allowing for high biomass yield [85]. However, although Sarker et al. [86] suggested an increase in carotenoid content in *Amaranthus tricolor* with increasing salinity, Singh and Tiwari [87] reported that total carotenoids in wheat increased up to a level of salinity (100 mM of NaCl) and then showed a decrease. This finding indicates that depending on the crop, the protective role of carotenoids is effective up to a specific stress level above which the antioxidant mechanism is disrupted followed by a decrease in total carotenoid content. Based on this, it could be assumed that the specific biostimulants may induce the osmoprotective mechanisms of plants which along with the non-enzymatic antioxidant mechanism contribute to the overall response to water-stress conditions tested in our study. However, the inconsistent results of our study indicate that more research is needed to reveal the actual protective mechanisms of biostimulants in combination with the antioxidant compounds content.

4. Conclusions

The ongoing climate crisis and the lack of irrigation water availability necessitate the redesign of current farming practices since water shortage combined with other biotic and abiotic pressures is detrimental to plant productivity and product quality, especially in open-field and protected vegetable cropping systems. For this purpose, the integration of ecofriendly techniques such as deficit irrigation and biostimulant application are pivotal for the sustainability of agroecosystems and the viability of the cropping sector. The results of our study indicate a variable response to deficit irrigation for the measured parameters depending on the genotype (lettuce type) and the biostimulant product composition. In general, HF, SW, and Si biostimulants benefited yield parameters under deficit irrigation (I1) conditions for Romaine-type lettuce plants, whereas Batavia-type plants were more susceptible to water stress and the highest yield was recorded under full irrigation (I2), regardless of the biostimulant product. Regarding water use efficiency, the same biostimulants (e.g., HF, SW, and Si) recorded the highest values under deficit irrigation (Batavia type) and rain-fed conditions (Romaine type). On the other hand, SiC and VP or SiC and Si increased WUE values under rain-fed or deficit irrigation in the case of Batavia and Romaine type lettuce plants, respectively. These findings indicate the viability of this agronomic practice in commercial conditions when water shortage is evidenced, thus allowing the best possible crop performance under limiting conditions as well the most efficient use of natural resources (e.g., irrigation water). In conclusion, the tested biostimulant products may act differently depending on the irrigation conditions as well as on the tested type of plants. However, despite the variable effect, the observed trends indicate the beneficial effects of specific biostimulant products that contain humic and fulvic acids, seaweed extracts, and Si on crop yield and water use efficiency. Therefore, further research is needed regarding the application of deficit irrigation in combination with biostimulant application to provide useful information for the improvement of water use efficiency of leafy vegetable crops such as lettuce and the alleviation of the severe effects of water shortage on crop productivity. Moreover, future research should focus on revealing the possible mechanism of action that allow specific biostimulant products to alleviate the negative effects of water shortage on crop yield through the improved and more efficient use of the available water.

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