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Biostimulants of Different Origins Increase Mineral Content and Yield of Wild Rocket While Reducing Nitrate Content through Successive Harvests

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Abstract: Nowadays, biostimulant application is a sustainable practice with which to reduce inputs while maintaining crop yield and quality. Furthermore, the successive harvesting technique is also adopted to increase overall yield and reduce production costs in leafy vegetables. Therefore, a greenhouse experiment was performed to compare four different biostimulants, (i) two from enzymatic hydrolysate of Fabaceae species, (ii) one made from betaine, alginic acid and caidrin, (iii) and another one made from alfalfa extract, algae and molasses rich in low-molecular-weight amino acids, in order to verify their ability to limit nitrate accumulation in wild rocket leaves while boosting yield and quantitative and qualitative components through successive harvests. Successive harvests increased the marketable yield of wild rocket by 41% on average compared to the first harvest, whereas biostimulants treatments increased the yield by 38% on average compared to the control. The SPAD index was increased due to successive harvesting and biostimulant application. While biostimulant application resulted in a 24% decrease in nitrates, it also caused a considerable increase in mineral content in wild rocket leaves. Both biostimulant application and successive harvesting showed promising results, and they could be suggested in leafy vegetable cultivation due to the boosted yield and quality.

Keywords: by-products; triacontanol; amino acids; protein hydrolysate; *Ascophyllum nodosum*; vegetal origin; Fabaceae; *Diplotaxis tenuifolia* L. DC

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1. Introduction

In the present day, innovative farming techniques in agriculture are shifting towards eco-friendly and sustainable schemes, where environment unfriendly inputs are diminished while production and quality are maintained [1,2], production cost is reduced and plant resilience is boosted [3]. Such a balance between low inputs and maintained production/quality could be achieved through breeding programs that unfortunately are time-consuming and not applicable for all the species [1]. Therefore, the involvement of novel and eco-friendly tools such as the use of natural products as biostimulants in boosting plants performance/quality has become a common practice [1,4,5]. Indeed, during the last years, an increase in biostimulant research and utilization as a horticultural practice with which to boost plant nutrition while reducing the dependence on fertilizers has been recorded [2,3]. As pointed out by Kivarga et al. [2], several research reports have demonstrated that biostimulants present reduced biological toxicity, accelerated reduction in their ingredients in the environment, mediocre mobility in food and low application dose, which ensure no risks to the environment or to human health. Such facts are increasing the commercial significance of biostimulants, which have become an important segment of sustainable green economics; therefore, more emphasis is being placed on validating advanced scientific protocols aiming to have innovative products with clear action mechanisms [6]. De Diego and Spíchal [6] mentioned that the industry of biostimulants has evolved in the last 10 years, with big global companies taking part at this point, in addition to an approximately 12% compound annual growth rate (CAGR) regarding the global market size of biostimulants for the next five years (USD 5 billion by 2027).

Biostimulants are mostly made of natural compounds from aquatic and terrestrial ecosystems, and metabolites from microbial or plant origin and thus considered eco-friendly in the framework of sustainable agriculture [7]. A major sustainable practice is the implementation of by-products to develop biostimulants such as hydrolyzates or extracts [8,9]. Moreover, natural products include humic acids and fulvic acids, sea weed extracts, amino acids, gelatin mixtures, non-protein substances, phenols and salicylic acid [2,3,10,11]. Biostimulant production comprises diverse technologies such as cultivation, processing, cell-rupture treatment, fermentation, hydrolysis and extraction, while biostimulant composition can be mono- or multi-component [2,7]. Additionally, the final product is dictated by the raw material source and the technology used for the process [7].

The positive effect of biostimulants on horticultural crops is due to the bioactive compounds present such as amino acids and phytohormones that stimulate plant growth by altering the primary metabolism, in addition to modulating the secondary metabolism of plants, through the improvement of nutrient uptake and triggering phytochemical synthesis [2,3,12]. Furthermore, biostimulants can increase pigment levels in leafy vegetables, as well as their antioxidant capacity [1], improve leaf color [13] and reduce nitrate content by boosting nitrogen metabolism [14]. Nitrate is mainly consumed through vegetables [15,16]; therefore, consumers are concerned about its concentration, notwithstanding the fact that these vegetables are endowed with functional qualities. Considering the negative impact of nitrate intake on consumer health (e.g., the formation of carcinogenic N-nitroso compounds and the impairment of oxygen delivery due to the formation of methaemoglobin), special care is needed to reduce its accumulation during cultivation to provide safe products [17–19]. Nevertheless, the positive impact of biostimulants is closely related to a myriad of conditions such as the abiotic conditions, application dose, time of application, species and even the cultivars of the treated crops [13].

Baby leaf vegetable production is increasing as a response to market demand; therefore, to satisfy these higher demands, crops are cultivated in controlled environments such as greenhouses and more growing cycles/successive harvests are being adopted [13,20,21], leading to earlier production and less labor costs [15,20,22]. On the other hand, successive harvesting is a common practice in various leafy greens, and according to the literature it may significantly increase the overall yield when compared to single harvesting practices [15,20,21,23]. Briefly, this practice refers to the application of multiple harvests within the same growing period, which is possible when the main apex remains intact after collecting the leaves or if regrowth takes place from auxiliary buds. The number of harvests depends on the genotype and the growing conditions since not all the species and growing periods are suitable for this technique due to the susceptibility to inflorescence formation under specific temperature and photoperiod conditions [22]. Moreover, wild rocket is considered a hyper-accumulator of nitrate, but since the market demands high standards, adequate techniques should be adopted such as the implementation of biostimulants or the application of multiple harvests [24].

Considering the importance of increasing yields and maintaining the quality of the edible products in leafy vegetable crops, the aim of this research was to compare four different biostimulants of diverse origins, in order to verify their ability to limit nitrate accumulation in wild rocket leaves while boosting yield and its quantitative and qualitative components. Although several studies have been conducted to evaluate the effect of biostimulant application on leafy vegetable yield parameters and chemical compositions, the combinatory effect of biostimulants and successive harvesting is scarcely studied. Therefore, the novelty of our study lies in the evaluation of the combined effect of biostimulant application and successive harvesting on the performance and chemical profile of wild rocket plants.

2. Materials and Methods

2.1. Experimental Design, Biostimulants Characteristics, Plant Material, and Crop Management

2.1.1. Plant Material

The experiment was carried out during the 2022 winter–spring season in a plastic greenhouse at Gussone Park, the experimental station of the Department of Agricultural Sciences (University of Naples "Federico II") located in Portici (40°48.870′ N; 14° 20.821′ E; 70 m a.s.l.). The greenhouse was covered by a diffused-light film with a UV-B transmission range (150 microns thick).

Seedlings of wild rocket (*Diplotaxis tenuifolia* L. DC), cv. "Reset" (Maraldi Sementi Srl, Cesena, Italy), were transplanted directly in soil on 1 February 2022; each plot was 1.00 × 1.00 m (area 1.00 m^2) and 20 seedlings per plot were used. The used cultivar is characterized by green leaves with medium-sized lobes, high potential yield, great tolerance against *Fusarium*, and overall appreciable crop flexibility, which makes it suitable for production throughout the year. Plants were harvested 5 times throughout the growing season starting from 22 March to 9 June 2022; each harvest defined a production cycle, referred to as I, II, III, IV, and V, in chronological order.

2.1.2. Experimental Design

Four different biostimulants were tested while an untreated control (NB) was also used. The four biostimulants were Carrier Top® (Bio1); Actiwave® (Bio2); StimoloMo® (Bio3); Ilsastim+® (Bio4). All biostimulants were applied three times per growing cycle at doses indicated by the manufacturers, namely 2.5 g L^{-1}, 2 mL m^{-2}, 3 mL L^{-1}, and 3 mL L^{-1}, for Bio1, Bio2, Bio3, and Bio4, respectively. The solutions were sprayed on rocket leaves, and simultaneously, control plants were sprayed with water. All the treatments were replicated three times, accounting for a total of 15 plots (300 plants in total).

2.1.3. Biostimulant Characteristics

Carrier Top®, produced by Dom Terry Agrisolutions srl (Milano, Italy), is an innovative product based on the enzymatic hydrolysate of Fabaceae species, with 15% total amino acids and 5% free amino acids, making it able to improve the assimilation of fertilizers and systemic herbicides (https://domterryagrisolutions.com/carrier-top, accessed on 20 January 2022).

Actiwave®, produced by Valagro SpA (Atessa, Italy), contains betaine, alginic acid, and caidrin, a vitamin K1 derivative, which assures correct and balanced nutrition, and increases the plants' ability to absorb nutrient uptake; these components allow an increase in yield, reduction in nitrate content in green leafy vegetables, and increase in plants' ability to overcome abiotic stress. The chemical composition of the product includes the following: 3% total nitrogen (N), of which 1% is organic N and 2% is ureic N, 12% organic carbon, 7% K_2O and 0.5% Fe (https://www.valagro.com/italy/it/prodotti/farm/biostimolanti/actiwave, accessed on 20 January 2022).

StimoloMo®, produced by Fertenia Srl (Bellizzi, Italy), is a plant-based biostimulant product obtained from alfalfa extract, algae (*Ecklonia* spp.) and molasses rich in low-molecular-weight amino acids; it also contains potassium and betaine. It is a growth promoter and has a carrier effect if it is combined with herbicides, fungicides, insecticides, and fertilizers. Its chemical composition includes the following: 5% organic N, 3% Mo, 0.1% Zn (EDTA), 15% organic carbon, 33% total amino acids and 6% free amino acids (https://www.fertenia.it, accessed on 20 January 2022).

Ilsastim+®, produced by ILSA S.p.A. (Arzignano, Italy), is an enzymatic hydrolysate obtained from Fabaceae species tissues (peas, beans, soybeans, alfalfa, etc.); it contains 1%

total N, 6% total amino acids, of which 1.5% are free amino acids, 10% organic C, sulfurated compounds, and 8 mg kg^{-1} of natural triacontanol (TRIA; https://www.ilsagroup.com/it/prodotti/prodotto/87/ilsastim.htm -accessed on 20 January 2022). TRIA is a compound normally present in Fabaceae, that acts as a plant growth regulator [25], and also has a beneficial effect on the physiological and biochemical characteristics of several plant species grown under abiotic stress (i.e., salt stress) [26].

2.1.4. Crop Management

Regarding the agricultural practices implemented, 18 kg ha^{-1} of fertilizer (calcium nitrate 22%) was added after each harvest. Plots were also regularly watered with volumes of water equal to the evapotranspiration (ET) calculated using the Hargreaves formula [27]. No pesticide applications were carried out.

The duration of production cycles and the timing of fertilizer and biostimulant applications are reported in Table 1.

Table 1. Timing of fertilizer and biostimulant applications, and cycle duration.

Cycle	Fertilization	Biostimulant Applications (DAT/DAPH *)			Cycle Duration
	(DAT/DAPH *)	Application 1	Application 2	Application 3	(Days)
I	17	22	31	40	49
II	3	6	11	16	22
III	3	5	10	15	21
IV	2	4	9	14	19
V	2	3	8	13	17

* DAT: days after transplant (refers to Cycle I); DAPH: days after previous harvest (refers to successive cycles, e.g., Cycle II-V).

2.2. Crop Growth Conditions: Soil and Temperature

Soil Chemical Analyses

The soil was loamy sand with a high content of phosphorus and potassium and a good content of organic matter. The detailed information is shown in Table 2.

Table 2. Physical and chemical properties of the experimental soil.

Soil Properties	Units	Mean Values
Coarse sand	%	33.2
Fine sand	%	42.3
Silt	%	17.0
Clay	%	7.5
N-total (Kjeldahl method)	%	0.112
P_2O_5 (Olsen method)	ppm	209.9
K_2O (tetraphenylborate method)	ppm	1718.2
Organic matter (bichromate method)	%	2.39
NO_3-N	ppm	20.32
NH_4-N	ppm	11.23
pH		7.67
Electrical conductivity	dS m^{-1}	0.15

Before the analytical determination, the soil samples were oven-dried at 40 °C. Regarding the nitrogen (N) content, organic N content was determined via the Kjeldahl method [28]. On the other hand, the nitrate-(N-NO_3) and ammonia-nitrogen (N-NH_4) contents were measured on the soil water extract based on the cadmium reduction method proposed by Sah [29], where the absorbance of solutions at wavelengths of 500 and 425 nm, respectively, was recorded with a spectrophotometer, Hach DR 2000 (Hach Co., Loveland, CO, USA). The organic matter was determined following the bichromate method proposed by Walkley and Black [30]. The determination of available phosphorus was assessed via the Olsen method [31], which is a colorimetric method. The determination of potassium was carried out via spectrophotometry at 650 nm, after the extraction of dried soil samples with sodium bicarbonate, and the successive addition of tetraphenylborate [32].

During the whole crop cycle, the air temperature was continuously monitored by a weather station (Vantage Pro, Davis, Hayward, CA, USA) placed within the greenhouse, and the values are illustrated in Figure 1. The data are reported as ten-day means of the maximum (Tmax) and minimum (Tmin) temperatures.

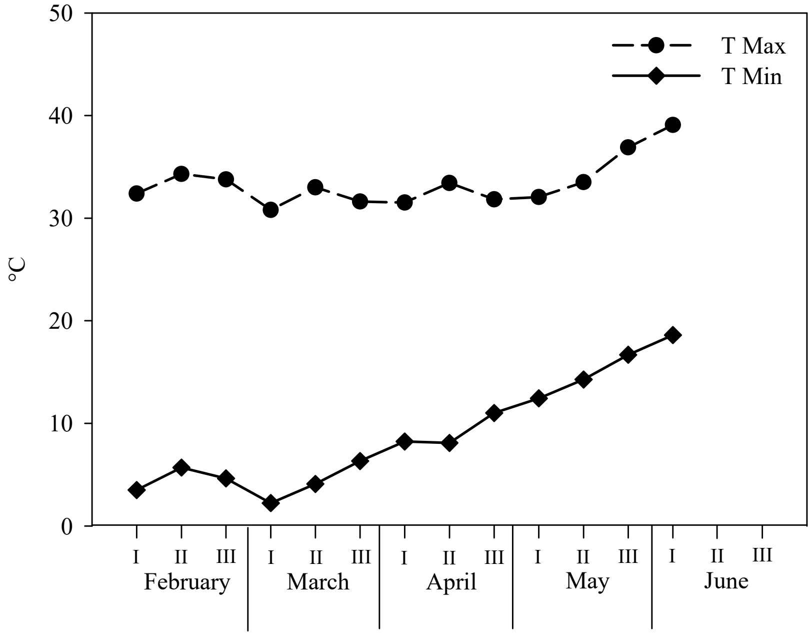

Figure 1. Maximum and minimum air temperature during the growing period of rocket. Roman numerals (I–III) refer to the three ten-day periods of each month.

2.3. *Yield Measurements, SPAD Index and Color Parameters*

At each harvest, all plants of each plot were cut and weighed in order to determine the fresh yield, which was expressed in kg m^{-2} of fresh weight; in addition, the number of leaves m^{-2} and the average leaf weight were also recorded.

Moreover, at each harvest, the SPAD index was measured with a chlorophyll meter (SPAD-502, Konica Minolta, Tokyo, Japan) in ten young fully expanded leaves per replicate, in the middle part of each leaf, avoiding the midrib.

Finally, the CIELAB color parameters L* (lightness), a* (green/red), and b* (blue/yellow) were also determined on the same ten leaves with a colorimeter (Minolta CR-300 Chroma Meter, Minolta Camera Co., Ltd., Osaka, Japan) and reported as an average for each treatment.

One sample of 100 g of fresh leaves per replicate was collected and oven-dried at 60 °C to reach a constant weight in order to determine the dry weight and consequently dry matter content. Then, the dry samples were ground using an IKA mill (IKA-Werke, Staufen, Germany) and sieved through a 2 mm sieve.

2.4. *Nitrate Content and Mineral Analysis*

A sub-sample of 0.5 g dw from each replicate was used to measure the leaves' nitrate content via the Foss FIAstar 5000 (FOSS Italia S.r.l., Padova, Italy) continuous flow analyzer. The method is described in detail in the study of Di Mola et al. [13]. The results are expressed in mg kg^{-1} fw. As for the mineral analysis, 0.250 g dw from each replicate was used for the extraction of minerals and subsequently analyzed via ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) as described by Rouphael et al. and coworkers in their study [33]. The results are expressed in g kg^{-1} dw.

2.5. Statistical Analysis

The experiment was laid out according to the completely randomized design (CRD) with three replicates (n = 3). All data were subjected to an analysis of variance (ANOVA) with the SPSS software package (SPSS version 22, Chicago, IL, USA), using harvesting time (H) and biostimulant product (B) as the main factors. The means were separated using Tukey's HSD test, at $p \leq 0.05$.

3. Results and Discussion

The results of the two-way ANOVA for all the analyzed parameters are reported in Tables 3 and 4. No significant interaction between the number of harvests (H) and the biostimulants application (B) was registered; therefore, only the main effects of the tested factors were considered. All parameters were affected by both the number of harvests and biostimulant applications, except for color parameters (L*, a*, b*, chroma, and hue values), average leaf weight, and calcium content, which were affected only by the number of harvests.

Table 3. Analysis of variance of yield and its parameters and color parameters; significance of main factors and interaction.

Significance	Yield	DM	Leaf No	ALW	L*	a*	b*	Chroma	Hue
Harvest (H)	0.001	0.001	0.001	0.001	0.001	0.01	0.001	0.001	0.001
Biostimulant (B)	0.001	0.01	0.01	ns	ns	ns	ns	ns	ns
H × B	ns	ns	ns	ns	ns	ns	ns	ns	ns

DM: dry matter; No: number; ALW: average leaf weight; L* = brightness; a* = green/red; b* = blue/yellow ns: non-significant. Significant differences at $p \leq 0.01$ and 0.001 (Tukey's HSD test).

Table 4. Analysis of variance of SPAD index and mineral content; significance of main factors and interaction.

Significance	SPAD	Nitrate	Na	K	Mg	Ca	P	Cl	S
Harvest (H)	0.001	0.01	0.001	0.001	0.001	0.05	0.05	0.001	0.001
Biostimulant (B)	0.001	0.001	0.001	0.05	0.001	ns	0.001	0.05	0.001
H × B	ns	ns	ns	ns	ns	ns	ns	ns	ns

DM: dry matter; Nb: number; ALW: average leaf weight; ns: non-significant; differences at $p \leq 0.05$, 0.01 and 0.001 (Tukey's HSD test).

3.1. Marketable Yield and Its Components

The marketable yield of rocket was significantly affected by the number of harvests (Figure 2). Notably, after the second harvest, no significant differences were recorded, since only the first harvest was significantly lower than the successive ones (1.00 vs. 1.41 kg m^{-2}, mean value of II–V harvests) (Figure 1). Our results indicate that rocket plants could be subjected to multiple harvests considering the short growth cycle of the plant and the short time interval between harvests, thus allowing prolonged growing periods and increased total fresh yields. The low fresh yield recorded in the first cutting of our study could be attributed to the low temperatures that prevailed in this period (beginning of February to mid-March) which resulted in a slow growth rate, as indicated by the long growth cycle of the first cutting (49 days compared to 17–22 for the rest of the harvests). The increasing temperatures throughout the rest of the growing period, especially the increase in Tmin, resulted in faster plant growth and higher fresh biomass production. In the study of Petropoulos et al. [21], it was also suggested that the fresh yields of separate cuttings may significantly differ from each other, while the growing period (autumn–winter and winter–spring) may also affect the fresh yield of the different cuttings due to different temperature and light (light intensity and photoperiod) regimes. Furthermore, Abdalla [34], who also applied multiple harvest in rocket plants grown in a floating system during the January–March growing period, suggested that five cuttings were possible. However, when compared to the practice in which plants were replaced with new ones after harvest, the authors did not record any difference in the total fresh yield between the two cropping systems despite the different number of growth cycles implemented (three growth cycles in the single harvest system and five growth cycles in the multiple harvest system) [34]. In contrast to our study, Corrado et al. [23] and Carillo et al. [35] suggested that the first harvest yield was higher than the second one for basil (*Ocimum basilicum*) and lettuce (*Lactuca sativa*) plants, respectively, while Di Mola et al. [36] reported similar results for wild rocket plants cultivated between April and June. This contradiction could be partly attributed to different growing periods and consequently to different air temperatures throughout the experiments, as well as to differences in the growth cycle duration and harvesting stage between the species [15,37]. The effect of growing conditions on fresh yield was also noted in the study of Di Mola et al. [38], who performed a similar experiment with six successive harvests in wild rocket plants during the autumn–spring period (November to May) and suggested higher yields at the later harvests conducted in April and May, regardless of the biostimulant treatment.

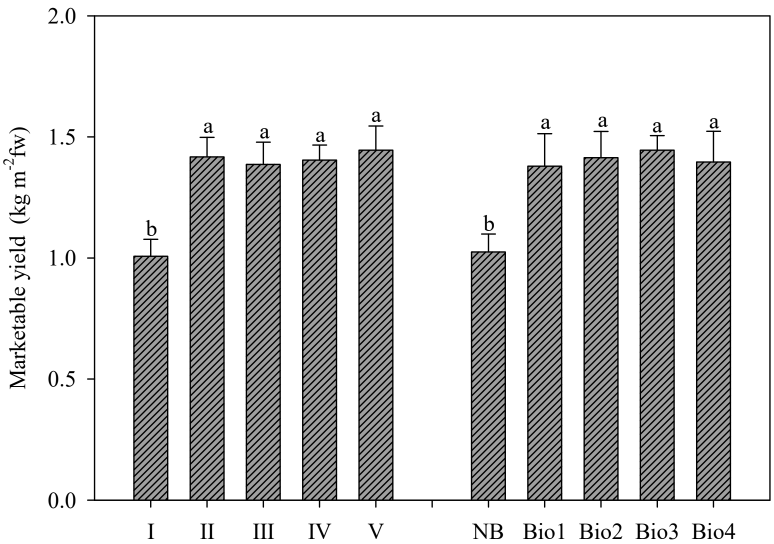

Figure 2. Rocket yield as affected by the different harvests (I to V) and biostimulant applications (NB: untreated plants; Bio1: Carrier Top; Bio2: Actiwave; Bio3: StimoloMo; Bio4: Ilstastim+). Different letters above the vertical bars indicate significant differences according to Tukey's HSD test ($p < 0.001$) for each of the tested factors. Vertical lines above each bar indicate mean values ± standard error (n = 3).

On the other hand, the application of biostimulants had a profound effect on the fresh yield, since all the tested products elicited an increase compared to the untreated control (+37.5% on average), but no significant differences were observed among the various biostimulants implemented on rocket plants (Figure 2). The positive effects of biostimulants on the growth and yield of rocket plants have been previously reported in the literature, which revealed that biostimulatory products of varied composition had a beneficial effect. For example, Candido et al. [24] suggested that the use of an azoxystrobin-based biostimulant may increase the marketable yield of wild rocket plants (*Diplotaxis tenuifolia* L. DC) after the first crop cycle, while positive effects on yield were also recorded for biostimulants that contained protein hydrolysates obtained from legumes, tropical extracts, *Trichoderma harzianum*, *Moringa oleifera* extracts and Azoxystrobin [14,39–43]. Additionally, Di Mola et al. [38] suggested a significant increase in fresh yield in wild rocket plants treated with a biostimulant that contained seaweed and alfalfa extracts and amino acids, especially for harvests performed in late spring. Apart from the biostimulant product composition, the application dose may also have an effect on plant growth and yield, suggesting that further research is needed in order to fine-tune this practice for commercial application [34].

The results regarding the dry matter (DM) content are presented in Table 5. After the third harvest, the leaf dry matter showed lower values than the first two harvests did, with the lowest value of 8.1% being registered for the third harvest. The second harvest was characterized by the highest DM value of 10.9% and was significantly higher than all the other harvests (Table 5). Moreover, the leaf DM was not significantly different among the different biostimulant treatments, except for the case of Bio4 which exhibited significantly lower DM compared to the untreated control where the highest overall value was recorded (Table 5). The differences in the DM content of leaves between different harvestings has been also reported in leafy vegetables such as basil [23], lettuce [35] and rocket [42]. According to the literature, the application of biostimulants may have a varied effect on leaf dry matter content depending on the biostimulatory product. For example, Di Mola et al. [38] suggested that the application of a biostimulant that contained alfalfa and seaweed extracts and amino acids resulted in a decrease in the DM of rocket leaves compared to that of the untreated plants, regardless of the nitrogen application rate, while they also recorded significant differences between successive harvests. In another study, the application of legume-derived protein hydrolysates and/or tropical plant extracts on baby rocket leaves increased the amount of water per cm^2 of leaf area and consequently decreased the DM content [13,36]. In contrast, Giordano et al. [42] suggested that the effect of biostimulants on DM content of perennial wall rocket leaves depends on the harvesting date when successive harvests are implemented and that biostimulants may increase DM content after the first harvesting. Similar results were reported by Caruso et al. [40], who also suggested a positive effect of biostimulants on the DM content of perennial wall rocket, while they also detected a significant effect of the growing period on the same parameter. Therefore, it should be noted that such inconsistencies in literature reports could be associated not only with the varied composition of biostimulants but also with differences in the growing conditions, since according to Tuncay et al. [44], dry matter content in rocket leaves may be significantly affected by the growing month.

Table 5. Effect of harvest (I to V) and biostimulant application (NB: untreated; Bio1: Carrier Top; Bio2: Actiwave; Bio3: StimoloMo; Bio4: Istastim+) on dry matter, number, and average weight of rocket leaves.

Treatments	Leaves		
	Dry Matter (%)	**N° m^{-2}**	**ALW (g)**
Harvest			
I	10.0 ± 0.24 b	4018.6 ± 315.7 c	0.25 ± 0.01 c
II	10.9 ± 0.51 a	4223.5 ± 326.5 bc	0.34 ± 0.02 ab
III	8.1 ± 0.54 d	5199.5 ± 544.9 ab	0.27 ± 0.02 bc
IV	9.0 ± 0.23 c	5591.8 ± 671.2 a	0.26 ± 0.03 c
V	9.5 ± 0.15 bc	3768.3 ± 726.8 c	0.41 ± 0.08 a
Biostimulants			
NB	9.9 ± 0.42 a	3730.7 ± 493.8 b	0.29 ± 0.04
Bio1	9.7 ± 0.35 ab	5106.7 ± 676.9 a	0.28 ± 0.03
Bio2	9.4 ± 0.18 ab	4676.4 ± 353.7 ab	0.32 ± 0.03
Bio3	9.3 ± 0.29 ab	4827.7 ± 610.6 a	0.32 ± 0.04
Bio4	9.1 ± 0.42 b	4460.3 ± 450.0 ab	0.32 ± 0.03

Different letters within each column indicate significant differences according to Tukey's HSD test; $p < 0.01$ and 0.001. ALW: average leaf weight. $n = 3$.

The number of leaves m^{-2} showed a parabolic trend; thus, it increased until the fourth harvest and then a rapid decrease was recorded. The latter was significantly higher than the first two and the fifth harvests, while no significant differences were recorded from the third harvest (Table 5). This finding indicates that growing conditions may affect the number of leaves, since the increasing temperatures and the improvement in lighting conditions after the first harvests were associated with a higher number of leaves until the fourth harvest. It is interesting to point out that the lower number of leaves m^{-2} in the fifth harvest was counterweighed by the higher average leaf weight, thus achieving a yield equal to that of the other harvests (see Table 5 and Figure 2). This difference in leaf morphology at the last harvest (less and larger leaves) compared to the preceding ones could be also attributed to the high temperatures and long photoperiods which may induce flowering and the formation of larger and longer leaves [45]. This finding is in agreement with the results of Corrado et al. [23], who also reported that the higher number of leaves was not associated with the highest fresh leaf weight and specific leaf area in basil plants that were harvested twice. Additionally, Petropoulos et al. [21] suggested that the number of leaves in *Cichorium spinosum* plants grown in the winter–spring period may increase after the first harvest and then show a decrease in subsequent harvests, while the same trends were observed in three Genovese basil cultivars [20] and lettuce plants [35,46]. However, the authors associated the higher number of leaves with higher yields, which was not the case in our study, probably due to the different species tested. On the other hand, the different biostimulants elicited a 27.8% mean increase in leaf number compared to the NB plants, but only Bio1 and Bio3 were significantly different from the control (NB) treatment (Table 5). As for the average leaf weight (ALW), it was only influenced by the successive harvests, with biostimulants showing no significant effects compared to the control treatment (Table 5). In particular, the average leaf weight was higher in the second and fifth harvest than in the first and fourth harvests, whereas no significant differences were recorded between the second and third harvest. According to the literature reports, the application of biostimulants is associated with an increased number of leaves without any significant effects on total yield or the average leaf weight [38,39]

3.2. Color Parameters, SPAD Index, and Mineral Content of Rocket Leaves

Differences in the color parameters of rocket leaves in this experiment were only dictated by successive harvests and not by the different biostimulant applicationa (Table 6). The lightness (L*) of rocket leaves showed a mean value of 42.1, where the significant lowest value was recorded in the second harvest, and the highest ones were recorded at the last two harvests and at the first one. The green intensity was lower (higher a* parameter values) at the second and third harvest (Table 6), being not significantly different from the values recorded at the first and fourth harvest, whereas the lowest overall value was recorded at the last harvest. Similarly, the b* parameter reached the highest value (yellowish color) at the fifth harvest, although it was not significantly different from that at the fourth harvest. Finally, the hue parameter showed the lowest value at the last harvest, being not significantly different from that of the fourth harvest, whereas Chroma was the highest at the last harvest, but not significantly different from that at the first and fourth harvest. (Table 6). Our results indicate that for most of the color parameters (except for lightness), the last harvest was clearly discriminated from the rest of the harvest, showing either the highest or the lowest overall values. Di Mola et al. [38] also reported a significant effect of the harvesting date on the green intensity (a*) of wall rocket leaves, whereas lightness (L*) and b* parameters were not affected. Moreover, Ciriello et al. [47] recorded significant differences between two harvestings for most of the color parameters (except for b* and Chroma values) in Genovese basil genotypes. In contrast, Bantis et al. [15], who studied the effect of multiple harvests in spinach and rocket plants, did not observe significant differences in the color parameters of both species when compared to the control plants of which a single harvest was carried out. These contradictions could be attributed to the different growing conditions throughout the experiment and especially to the increasing temperatures.

Regarding the biostimulant application effect, our results are in agreement with those reported by Visconti et al. [48], who suggested that the application of *Trichoderma* did not affect color parameters in rocket leaves, while Consentino et al. [49] reported that only the green intensity increased (lower a* values) in the leaves of celery plants due to animal- or plant-derived protein hydrolysates. Similarly, Giordano et al. [42] and Caruso et al. [39] did not observe significant effects on the color parameters of wall rocket after the application of tropical plant extracts and legume-derived protein hydrolysates. In contrast to our study, Caruso et al. [40] suggested that the single and/or combined application of biostimulants affected the leaf color of rocket plants, especially the combined application of *Trichoderma* and protein hydrolysates which significantly improved all the tested parameters (L*, a*, and b*). Additionally, Di Mola et al. [13] reported a significant effect of seaweed extracts and protein hydrolysates on the color parameters of baby leaf lettuce. These contradictions in the literature suggest that biostimulant composition, growing conditions and the genotype are important for defining their effects on the visual quality of leafy vegetables, as indicated by color parameters.

Table 6. Effect of harvest (I to V) and biostimulant applications (NB: untreated; Bio1: Carrier Top; Bio2: Actiwave; Bio3: StimoloMo; Bio4: Ilstastim+) on L*, a*, and b* color parameters of rocket leaves.

Treatments	L*	a*	b*	Chroma	Hue
Harvest					
I	42.4 ± *0.44* ab	−13.6 ± *0.48* ab	20.2 ± *0.77* b	24.3 ± *0.90* ab	124.0 ± *0.19* a
II	40.2 ± *0.50* c	−12.9 ± *0.43* a	19.3± 0.92 b	23.2± *0.98* b	123.7 ± *0.63* a
III	42.2 ± 0.56 b	−12.9 ± *0.47* a	19.2 ± *0.90* b	23.1 ± *1.01* b	124.0 ± *0.35* a
IV	43.4 ± 0.57 a	−13.4 ± *0.35* ab	20.6 ± *0.83* ab	24.6 ± *0.85* ab	123.2 ± *0.63* ab
V	42.3 ± *0.65* ab	−14.0 ± *0.60* b	22.2 ± *1.13* a	26.2± 1.43 a	122.3 ± 0.79 b
Biostimulants					
NB	42.6 ± *0.30*	−13.6 ± *0.50*	20.7 ± *0.92*	24.8 ± *1.12*	123.4 ± *0.43*
Bio1	41.8 ± *0.72*	−13.1 ± *0.35*	20.2 ± *0.66*	24.1 ± *0.72*	123.2 ± *0.54*
Bio2	41.8 ± *0.74*	−13.3 ± *0.51*	20.3 ± *0.97*	24.3 ± *1.05*	123.4 ± *0.57*
Bio3	42.2 ± *0.54*	−13.3 ± *0.56*	20.2 ± *1.07*	24.2 ± *1.28*	123.5 ± *0.62*
Bio4	42.1 ± *0.42*	−13.3 ± *0.42*	20.0 ± *0.93*	24.1 ± *1.00*	123.7 ± *0.44*

Different letters within each column indicate significant differences according to Tukey's HSD test; $p < 0.01$ and 0.001. L* = brightness; a* = green/red; b* = blue/yellow. $n = 3$.

The SPAD index ranged from 37.8 at the first harvest to 42.2 at the last harvest, without significant differences between the successive harvests after the first one (a mean value of 41.2; Figure 3). Similarly to the marketable yield, the SPAD index was higher in all treated plants with biostimulants with an average increase of 14.4% compared to the NB plants, and no significant difference between the biostimulant treatments (Figure 3). The increasing trends in the SPAD index when successive harvesting is implemented have been also confirmed by Alexopoulos et al. [50] in wild leafy vegetables. Similarly, several studies have recorded a positive effect of biostimulants on the SPAD index values of rocket and other leafy vegetables compared to those of the untreated ones, while they also associated the increased SPAD index with increased crop yields due to the more efficient function of the photosynthetic apparatus [12,13,38,39,42,51,52]. In contrast, Formisano et al. [20] did not record any differences in SPAD values when basil plants were subjected to multiple harvests, while Visconti et al. [48] did not suggest any effects for *Trichoderma* application in lettuce and spinach plants. Considering that literature reports have associated the SPAD index value in leafy vegetables with various agronomic practices (e.g., fertilization, irrigation regime, saline irrigation, etc. [47,53,54], it could be that such inconsistencies in research data could be attributed to differences in growing conditions that may conceal the effects of biostimulant application.

Nitrate content in rocket leaves showed a decreasing trend after the second harvest, with the third harvest recording the lowest overall value and then an increase was observed until the last harvest. The highest nitrate content was measured in the first harvest, being significantly higher only from the third harvest. In any case, nitrate content was 20.80% higher in the first harvest compared to the average values of the successive harvests (2929 vs. 3538 mg kg^{-1}; Figure 4). The observed trends could be attributed to increasing temperatures, which may increase the uptake and assimilation of nutrients and thus result in decreased nitrate content (up to the third harvest), whereas the increasing nitrate content after the third harvest could be explained by the involvement of nitrates in osmoregulation mechanisms due to the increased water requirements of plants associated with high temperatures [21]. According to Bonasia et al. [17], nitrate content fluctuation in wild rocket plants could be expected depending on the genotype, the cultivation system and the growing period, while successive harvesting in basil plants resulted in higher total nitrogen and nitrate content in the second cutting (mid-June) compared to the first one (mid-April) [23]. Similarly, Ciriello et al. [47] reported a contrasting effect of successive harvesting on nitrate content in basil leaves depending on the cultivar, thus highlighting the genotypic differences in the nitrogen assimilation of the species, while the harvesting time within the day may also affect the measured nitrate content [16]. The genotypic effect on nitrate accumulation was also noted by Bantis et al. [15], who suggested that successive harvesting may increase nitrate content in rocket leaves, while no effects were recorded in the case of spinach. Furthermore, Masclaux-Daubresse et al. [55] suggested that the formation of new leaves may induce the remobilization of nitrates to expanding leaves and therefore result in increasing nitrate content, as observed in our study after the third harvest where despite the increasing temperatures nitrate accumulation was observed.

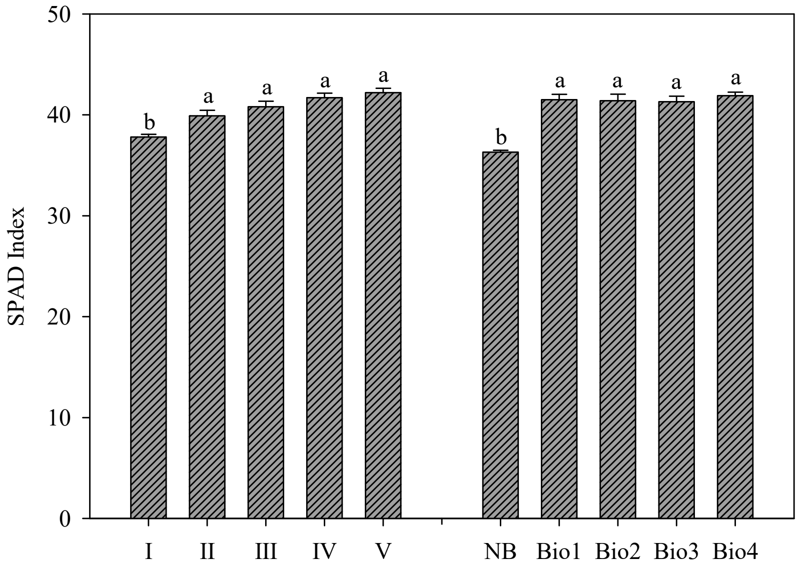

Figure 3. SPAD index of rocket leaves affected by the different harvests (I to V) and biostimulant applications (NB: untreated; Bio1: Carrier Top; Bio2: Actiwave; Bio3: StimoloMo; Bio4: Ilstastim+). Different letters above vertical bars indicate significant differences according to Tukey's HSD test ($p < 0.001$) for each one of the tested factors. Vertical lines above each bar indicate mean values ± standard error ($n = 3$).

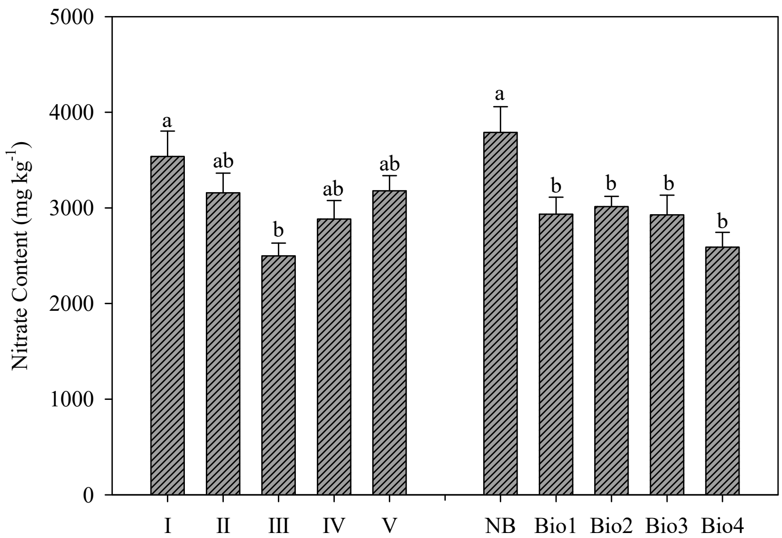

Figure 4. Nitrate content of rocket leaves affected by the different harvests (I to V) and biostimulant applications (NB: untreated; Bio1: Carrier Top; Bio2: Actiwave; Bio3: StimoloMo; Bio4: Istastim+). Different letters above each bar indicate significant differences according to Tukey's HSD test ($p < 0.001$) for each of the tested factors. Vertical lines above each bar indicate mean values ± standard error; $n = 3$. Nitrate content is expressed in mg kg^{-1} of fresh weight.

As suggested by the manufacturers, all biostimulants limited the nitrate accumulation in rocket leaves. In particular, all the used biostimulants did not exhibit significant differences between them and registered a mean value of 2866 mg kg^{-1}, which was about 24.3% lower compared to the nitrate content measured in the untreated (NB) plants (Figure 4). According to Ertani et al. [56], the use of biostimulants that contain protein hydrolysates may increase nitrogen assimilation and decrease nitrate accumulation, while similar results were reported for caidrine-based biostimulants [57]. However, contrasting results regarding the effects of biostimulants on nitrate accumulation are also reported in the literature. Therefore, Caruso et al. [36] noted that the application of *Trichoderma* and protein hydrolysates on wall rocket resulted in increased nitrate content compared to the control treatment, while Rouphael et al. [12] suggested a variable effect depending on the biostimulant composition with seaweed and herbal extracts and vegetal oils inducing nitrate accumulation in spinach plants compared to the untreated plants or plants treated with legume-derived protein hydrolysates. In contrast, El-Nakhel et al. [4] recorded an increase in the nitrate content of spinach plants for protein hydrolysates obtained from legumes, a difference that could be attributed to the different sowing dates, genotypes tested and growing systems compared the study of Rouphael et al. [12]. Similar increasing trends were also recorded by Di Mola et al. [13] in the case of lettuce plants treated with seaweed extracts and protein hydrolysates, as well as by Di Mola et al. [36] for baby rocket leaves subjected to tropical plant extracts and protein hydrolysates. All of these results highlight the variable response of crops to biostimulant application since they indicate that several other factors are implicated including, the genotype, the growing conditions, the cultivation practices and so forth.

The harvesting time and the application of biostimulants had a significant effect on leaf mineral content (Table 7). All the mineral elements studied in this experiment (K, Mg, Ca, P, Mg and S) exhibited a concentration decrease in the second and third harvest, while the highest overall value was recorded in the last (fifth) harvest (except for the case of P). In particular, Mg concentration decreased significantly at the third harvest compared with the first harvest, while S concentration decreased significantly at the second and third harvest compared with the first harvest. After the fourth harvest, the content of all the elements increased again and reached similar or higher values than the first harvest values. Moreover, the biostimulant applications elicited an increase in the concentration of all the minerals, except for the case of Ca where the application of biostimulants had no significant effect (Table 7). The observed increase was significant for K, Mg and P content, only when biostimulants Bio2, Bio3 and Bio4 were applied, whereas Bio1 only significantly increased P concentration compared to the control. In addition, S content was significantly increased only by the Bio2 and Bio4 treatments.

Table 7. Effect of harvest (I to V) and biostimulant applications (NB: untreated; Bio1: Carrier Top; Bio2: Actiwave; Bio3: StimoloMo; Bio4: Ilstastim+) on K, Mg, Ca, P, and S content of rocket leaves.

Treatments	K	Mg	Ca	P	S
	g kg^{-1} dw	g kg^{-1} dw	g kg^{-1} dw	g kg^{-1} dw	g kg^{-1} dw
Harvest					
I	47.1 ± 3.5 ab	4.34 ± 0.30 ab	26.3 ± 2.9 ab	2.59 ± 0.17 ab	8.61 ± 0.79 ab
II	43.2 ± 2.1 b	4.05 ± 0.13 b	24.2 ± 1.5 b	2.48 ± 0.22 b	6.70 ± 0.45 cd
III	43.5 ± 1.0 b	3.53 ± 0.11 c	25.0 ± 0.9 ab	2.77 ± 0.19 ab	5.62 ± 0.88 d
IV	47.8 ± 2.3 ab	4.68 ± 0.19 a	26.7 ± 2.1 ab	3.11 ± 0.21 a	7.59 ± 1.11 bc
V	50.0 ± 2.1 a	4.77 ± 0.28 a	27.3 ± 1.1 a	2.78 ± 0.38 ab	9.67 ± 1.13 a
Biostimulant					
NB	43.5 ± 2.5 b	3.87 ± 0.26 b	27.2 ± 2.2	2.20 ± 0.25 b	6.39 ± 1.16 b
Bio1	46.0 ± 1.5 ab	4.27 ± 0.24 ab	24.4 ± 1.5	2.85± 0.16 a	7.54 ± 0.62 ab
Bio2	47.5 ± 2.4 a	4.34 ± 0.18 a	25.6 ± 1.2	2.81 ± 0.42 a	8.62 ± 0.73 a
Bio3	47.0 ± 2.6 a	4.45 ± 0.12 a	26.3 ± 1.6	2.90 ± 0.22 a	7.49 ± 0.87 ab
Bio4	47.6 ± 2.1 a	4.43 ± 0.21 a	26.0 ± 1.9	2.97 ± 0.12 a	8.14 ± 0.99 a

Different letters within each column indicate significant differences according to Tukey's multiple-range test ($p < 0.05$) for the same factor.

Similarly to our study, Giordano et al. [42] highlighted the significant single and combinatory effect of biostimulant products on the mineral composition of rocket plants harvested at different dates, although these authors did not study the effect of the harvesting date. On the other hand, Formisano et al. [20] recorded significant differences in the P and Mg content of basil leaves subjected to successive harvesting, whereas K content remained unaffected. Additionally, Caruso et al. [39] suggested the significant effect of the growing period and biostimulant application on the mineral composition of rocket plants, with the growing period having an impact on K, S, Mg and Ca content, while biostimulant application increased P and Ca content compared to the control (untreated plants). According to the literature, biostimulant application may induce mineral accumulation in leafy vegetables, as in the case of K and Mg [12,49] or P, K, Mg, Ca and S [51] in spinach and celery leaves, P and Mg in rocket leaves [40], K, Ca and Mg in perennial wall rocket leaves [12], and P in lettuce plants [52]. Rouphael et al. [12] suggested that such positive effects could be associated with hormonal-like effects, including improved root architecture, which facilitates mineral uptake and translocation, and improved transportation of macronutrients through cell membranes. Moreover, Fiorentino et al. [41] highlighted the varied response of different species (lettuce and rocket) to the same *Trichoderma*-based biostimulants in terms of mineral composition, a finding which indicates crop specificity.

4. Conclusions

Our results indicate the varied effect of biostimulant applications and multiple harvesting regimes on the yield and quality parameters of rocket plants depending on the particular combinations of the tested treatments. Most importantly, both of the tested agronomic practices were associated with a higher marketable yield compared to the control plants, suggesting its use as innovative and ecofriendly techniques with which to increase farmers' income without increasing chemical inputs and resources. On the other hand, biostimulant application was associated with improved quality of the marketable product, as indicated by the decreased nitrate content and the increased mineral content. In conclusion, both biostimulant application and successive harvesting showed promising results and they could be suggested in leafy vegetable cultivation. In addition, the results of our study could be useful for leafy vegetable growers in terms of yield and quality improvement. However, further research is needed regarding the growing conditions and the biostimulant's composition in order to reveal the synergisms that may improve the yield and quality of the final product.

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