

Article

Effect of Biostimulant Formulation on Yield, Quality, and Nitrate Accumulation in *Diplotaxis tenuifolia* Cultivars Under Different Weather Conditions

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Abstract

Perennial wall rocket (*Diplotaxis tenuifolia* L.—DC.) exhibits genotype-dependent responses to biostimulant applications, which have not yet been deeply investigated. A two-year greenhouse factorial experiment was carried out to assess the interactions between five cultivars (Mars, Naples, Tricia, Venice, and Olivetta), three biostimulant formulations (*Cystoseira tamariscifolia* L. extract; a commercial legume-derived protein hydrolysate, “Dynamic”; and *Spirulina platensis* extract) plus an untreated control, and three crop cycles (autumn, autumn–winter, and winter) on leaf yield and dry matter, organic acids, colorimetric parameters, hydrophilic and lipophilic antioxidant activities, nitrate concentration, nitrogen use efficiency, and mineral composition, using a split plot design with three replicates. Protein hydrolysate significantly enhanced yield and nitrogen use efficiency in Mars (+26%), Naples (+25.6%), Tricia (+25%), and Olivetta (+26%) compared to the control, while *Spirulina platensis* increased the mentioned parameters only in Venice (+36.2%). Nitrate accumulation was reduced by biostimulant application just in Venice, indicating genotype-dependent nitrogen metabolism responses. The findings of the present research demonstrate that the biostimulant efficacy in perennial wall rocket is mainly ruled by genotypic factors, and the appropriate combinations between the two mentioned experimental factors allow for optimization of leaf yield and quality while maintaining nitrate concentration under the regulation thresholds.

Keywords: perennial wall rocket; *Spirulina platensis* L.; protein hydrolysate; *Cystoseira tamariscifolia* L.; crop cycle; antioxidants; leaf mineral elements; nitrogen use efficiency (NUE)



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1. Introduction

Perennial wall rocket (*Diplotaxis tenuifolia* L.—DC.) belongs to Brassicaceae family and is very widespread in the Campania region (southern Italy), accounting for about the 65% of the total national production. This species has remarkable nutritional and anticancer properties, the latter due to the high concentration of glucosinolates (glucoerucin, 4-methoxyglucobrassicin) that generate bioactive isothiocyanates [1]. The mineral profile is particularly rich, containing potassium (3500 mg 100 g⁻¹ d.w.), calcium (1200–1800 mg 100 g⁻¹ d.w.), and iron (15–25 mg 100 g⁻¹ d.w.) at significantly higher levels than conventional leafy vegetables [2]. Vitamin C content ranges from 50 to 80 mg 100 g⁻¹ f.w., complemented by substantial folate levels and carotenoids including β -carotene and lutein [3]. Total phenolic compounds (150–300 mg GAE 100 g⁻¹ f.w.), predominantly quercetin and kaempferol derivatives, contribute to great antioxidant activity as also observed in other species [4,5]. The synergy among prebiotic fiber, low caloric density (25 kcal 100 g⁻¹ f.w.), and bioactive compounds makes *D. tenuifolia* a functional food with important potential for cardiovascular protection, gut microbiota modulation, and metabolic health optimization [6].

Nitrate (NO₃⁻) is the prevailing form of nitrogen available to plants, boosting their growth and development, and an essential component, particularly in the biosynthesis of proteins, nucleic acids, and other nitrogen-containing compounds [6,7]. Plants accumulate NO₃ in cellular vacuoles, where they participate in metabolic processes and contribute to osmotic regulation, especially under conditions of limited availability of other osmoregulatory compounds [6].

The physiological dynamics of nitrate accumulation in plant tissues are governed by multiple factors, including species characteristics, cell differentiation, fertilization regimes, and light intensity [3,8]. Leafy vegetables demonstrate pronounced tendency toward NO₃⁻ accumulation and, particularly, rocket (*Diplotaxis tenuifolia* L.—DC.) is a hyperaccumulator species, with concentrations even exceeding 9000 mg·kg⁻¹ fresh [9].

Based on the mentioned implications of nitrate accumulation in leafy vegetables, the European Commission, through the Regulation No. 1258/2011, established the maximum permissible levels of NO₃ in rocket at 6000 mg·kg⁻¹ fresh weight in spring–summer and 7000 mg·kg⁻¹ fresh weight in autumn–winter [10].

Significant genotypic changes in nitrate accumulation have been observed both within and between cultivated and wild rocket [7,11], with the latter type frequently displaying higher NO₃ concentrations [11,12]. However, considering the novelty of wild rocket compared to the historically cultivated *Eruca sativa* L., its predominant diffusion in Mediterranean areas, and the recent (since 2011) nitrate restrictions imposed by the European Union, the application of biostimulants for nitrate reduction represents a recent interesting research topic for this species.

The mentioned differences mainly stem from variations in nitrate uptake mechanisms rather than in reduction capacity, considering that no significant correlation between nitrate accumulation and NO₃ reductase activity has been reported between high- and low-nitrate-accumulating genotypes [13,14]. Indeed, these uptake variations likely involve differences in nitrate transporter expression/activity (e.g., NRT1/NRT2 systems) and/or root morphological traits affecting absorption surface area [15].

Biostimulants contain beneficial substances and/or microorganisms and their application is a promising strategy to manage the nitrate accumulation issue in leafy vegetables such as rocket, also enhancing crop yield and quality; they are applied to plants or to the rhizosphere to stimulate natural processes, enhancing nutrient uptake and efficiency, tolerance to abiotic stress, and crop quality, independently, on their mineral element content [16].

Among the various biostimulant categories, those derived from marine algae (macroalgae), microalgae, and protein hydrolysates have demonstrated effectiveness in mitigating nitrate accumulation in rocket and similar leafy greens. In this respect, seaweed-based biostimulants, primarily extracted from brown algae species such as *Ascophyllum nodosum* and *Ecklonia maxima*, have been shown to significantly reduce nitrate content in rocket leaves [17]. Research by Schiattone et al. [18] demonstrated that the application of biostimulants based on brown algae and yeast reduced nitrate accumulation by approximately 10.6% in wild rocket leaves, compared to untreated controls. These extracts contain bioactive compounds, including polysaccharides (alginates, laminarin), phytohormones (auxins, cytokinins), and osmolytes (betaines), that enhance nitrogen metabolism and assimilation [17].

Microalgal extracts similarly contribute to reduced nitrate accumulation through the action of various bioactive constituents and, particularly, *Spirulina* (*Arthrospira platensis*) application reportedly alters nitrogen assimilation patterns, resulting both in lower tissue nitrate concentrations and higher nitrogen use efficiency [19]. The latter effects may be attributed to the complex array of amino acids, vitamins, and phytohormones present in microalgal biomass stimulating the activity of nitrate reductase.

Protein hydrolysates, particularly those derived from leguminous plant species (Fabaceae), represent another valuable class of biostimulants for nitrate management. El-Nakhel et al. [16] reported that the application of protein hydrolysates obtained from enzymatic hydrolysis of Fabaceae tissues resulted in a 24% decrease in nitrate content of wild rocket leaves, compared to untreated controls. The mentioned hydrolysates are a rich source of peptides and amino acids that enhance nitrogen metabolism and nitrate reductase activity, providing alternative nitrogen sources [16].

Some biostimulants were shown to exert similar effects like red light exposure, encouraging nitrate reductase activity in rocket leaves [20] and, additionally, they may increase photosynthetic efficiency, thereby fostering the availability of carbon skeletons necessary for nitrate assimilation [21]. These compounds also modify root architecture and function, potentially altering nitrogen uptake patterns to reduce nitrate accumulation [22].

Following ingestion, dietary nitrates undergo complex transformations within the human digestive system, starting from the reduction to nitrites; then, upon interactions with gastric acid, they can generate nitrogen oxide compounds, directly functioning as signaling molecules or forming more complex compounds, such as N-nitrosamines, with diverse physiological effects [6].

The genotype-dependent responses to biostimulant applications may reveal valuable synergistic combinations optimizing both production and nutritional quality of rocket leaves, while effectively limiting nitrate accumulation.

The present research was conceived to support the development of sustainable rocket production systems meeting regulatory requirements for NO₃ content, while satisfying consumer demand for high-quality produce with optimal health-promoting properties. In this respect, the present study was aimed at assessing the interactions between plant biostimulants, derived from macroalgae, microalgae, and protein hydrolysates, and genetic potential, under different weather conditions, on rocket yield, nutritional profiles, and nitrate accumulation.

2. Materials and Methods

2.1. Growing Conditions and Experimental Protocol

Research was carried out on rocket (*Diplotaxis tenuifolia* L.—DC.) at the Department of Agricultural Sciences, University of Naples Federico II, Portici (Naples), Italy, in 2022 and 2023. The crops were grown under a greenhouse covered with a polyethylene film, in sandy loam soil (75.5% sand, 17.2% silt, 7.3% clay), with a pH of 6.7 and an electrical conductivity

of 512 mS cm⁻¹. During the cropping seasons, the mean monthly temperatures under the greenhouse were 13.3 °C in October, 10.5 °C in November, 6.6 °C in December, 5.1 °C in January, 6.8 °C in February, and 12.2 °C in March, in 2022; 13.0 °C in October, 10.1 °C in November, 6.9 °C in December, 5.4 °C in January, 6.6 °C in February, and 12.5 °C in March, in 2023.

The experimental protocol was based on the factorial combination between five perennial wall rocket cultivars (Mars, Naples, Tricia, Venice, and Olivetta), three biostimulant formulations (the algae *Cystoseira tamariscifolia* extract; the protein hydrolysate, Dynamic, by Hydro Fert, Barletta, Italy; the microalgae *Spirulina platensis* extract (Micoperi Blue Growth, Ortona, Chieti, Italy)), plus an untreated control, and three crop cycles (autumn, autumn–winter, and winter). The extracts were prepared as follows: For *Cystoseira tamariscifolia*, the thalli were harvested and then dehydrated to be finely ground; at each preparation, the powder was added to osmosis water, shaken in the dark for 24 h, and used in nebulization. For spirulina extract, the powder was added to osmosis water at each treatment, shaken for 24 h, and used in nebulization.

The five commercial wall rocket cultivars have the following characteristics: Olivetta (La Semiorto Sementi, Sarno, Italy) has smooth olive-shaped leaves and rapid 20–25 day maturation; Tricia (Enza Zaden, Tarquinia, Italy) features large serrated leaves, fast growth, and downy mildew resistance; Venice and Naples (Levantia Seed, Porto Viro, Italy) have high leaf serration and strong Fusarium resistance; and Marte (Seedium/Blumen Vegetable Seeds, Milan, Italy) is characterized by erect growth habit and long shelf life. The treatments were arranged in the field using a split plot design with three replicates and the experimental unit covered a 4 m² (2 × 2 m) surface area, with 4000 plants. The biostimulants were foliarly applied by spraying a 15 L solution ha⁻¹ at the concentration of 3 g L⁻¹ for *Cystoseira tamariscifolia* and *Spirulina platensis*, or 3 mL L⁻¹ for the protein hydrolysate, three times in each crop cycle at 7-day intervals, starting when the leaves were 2–3 cm long.

The sowing (4 kg of seeds per hectare) was performed on 14 and 13 October in 2022 and 2023, respectively, with 2 cm spacing along the rows which were 5 cm apart. Crop management was performed using sustainable practices, including an organic pre-seeding fertilization with N, P₂O₅, and K₂O (at a rate of 38, 10, and 30 kg ha⁻¹, respectively), and control of pathogens and pests with copper oxychloride and azadirachtin treatments, respectively. During the three growing seasons, drip irrigation was activated when the soil available water at 10 cm depth dropped to 80%, and N, P₂O₅, and K₂O were supplied by fertigation in each crop cycle at a dose of 112, 30, and 90 kg ha⁻¹, respectively. The leaves were harvested when commercially ready (7 to 10 cm long) at 2–5 cm above soil surface. The harvests were conducted on 24 and 22 November, 10 January, and 8 and 10 March, respectively, in 2022 and 2023.

2.2. Yield Determination and Sample Preparation

At each harvest date, the total leaf fresh weight was measured in every plot. Fresh leaf samples were randomly collected in each experimental plot and stored at –80 °C until the extractions and analyses of the antioxidant activity were performed. Other random leaf samples were dried in an air-ventilated oven until constant weight and, then, ground to a powder in a grinder; next, 500 mg samples were used for the extraction and determination of the organic acids, mineral elements, and nitrate content.

2.3. Color Parameters

Leaf color parameters (L*, a*, and b*) were measured on the central area of the upper surface of ten leaves per replicate by means of a Minolta CR-300 Chroma Meter (Minolta Camera Co., Ltd., Osaka, Japan), based on the CIELAB color space system: L* represents

lightness, from black to white (0 to 100); a^* and b^* represent chroma components from -60 to $+60$, from green to red and from blue to yellow, respectively.

2.4. Antioxidant Activity

Based on the method described by Re et al. [23], the 2,2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method was used to determine LAA. The hydrophilic antioxidant activity (HAA) was measured using the N,N-dimethyl-p-phenylenediamine (DMPD) method by extracting 200 mg of lyophilized material in distilled water [24]. A UV-Vis spectrophotometer was used to detect the absorbance reduction by the solutions at 734 and 505 nm wavelength to determine LAA and HAA, respectively. LAA was expressed as mmol Trolox eq. 100 g^{-1} dry weight (d.w.), while HAA was expressed as mmol ascorbic acid eq. 100 g^{-1} d.w.

2.5. Organic Acids and Mineral Elements

The desiccated rocket leaf tissues were ground and used for organic acid and macro-element profile analysis. Organic acids (malic and citric) and mineral elements such as nitrate, chloride, potassium, calcium, sodium, ammonium, magnesium and phosphorus were separated and quantified by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) coupled to a conductivity detector. An IonPac CG12A ($4 \times 250\text{ mm}$, Dionex, Corporation) guard column and IonPac CS12A ($4 \times 250\text{ mm}$, Dionex, Corporation) analytical column were used for the K, Ca, Na, NH_4^+ , and Mg analysis; for NO_3^- , Cl^- , P, and organic acids determination, an IonPac AG11-HC guard ($4 \times 50\text{ mm}$) column and IonPac AS11-HC analytical column ($4 \times 250\text{ mm}$) were adopted.

To prepare the samples, 250 mg of leaf tissue powder suspended in ultrapure water (50 mL) (Milli-Q, Merck Millipore, Darmstadt, Germany) underwent three freeze-thaw cycles in liquid nitrogen and were then shaken in a water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at $80\text{ }^\circ\text{C}$ for 10 min. The resulting mixture was processed according to a previously described procedure [25]. The macro-element and organic acid profiles were expressed in g kg^{-1} d.w.

2.5.1. Nitrogen Use Efficiency (NUE) Related to Yield Calculation

For nitrogen use efficiency (NUE) related to yield, the following formula was used, based on average values referred to the variable examined per level of N treatment:

$$\text{NUE} = \text{Dry weight of crop (kg ha}^{-1}\text{)} / \text{Amount of nitrogen applied (kg ha}^{-1}\text{)}.$$

2.5.2. Statistical Analysis

The data obtained were statistically processed by three-way analysis of variance and Tukey's test was performed for mean separations at a 0.05 probability level ($p < 0.05$), using the SPSS software version 29 (IBM, Armonk, NY, USA). The data expressed as percentages were subjected to angular transformation before processing. All data are reported as means of three replicates. When no significant interactions arose between the three experimental factors, only the data relevant to their main effects were reported.

3. Results

As no significant interactions arose between the research year and the experimental factors applied, only the average values related to 2022 and 2023 are reported for all the variables examined.

Significant interactions arose both between cultivar and biostimulant formulation (Figure 1) and between biostimulant formulation and crop cycle on yield (Figure 2).

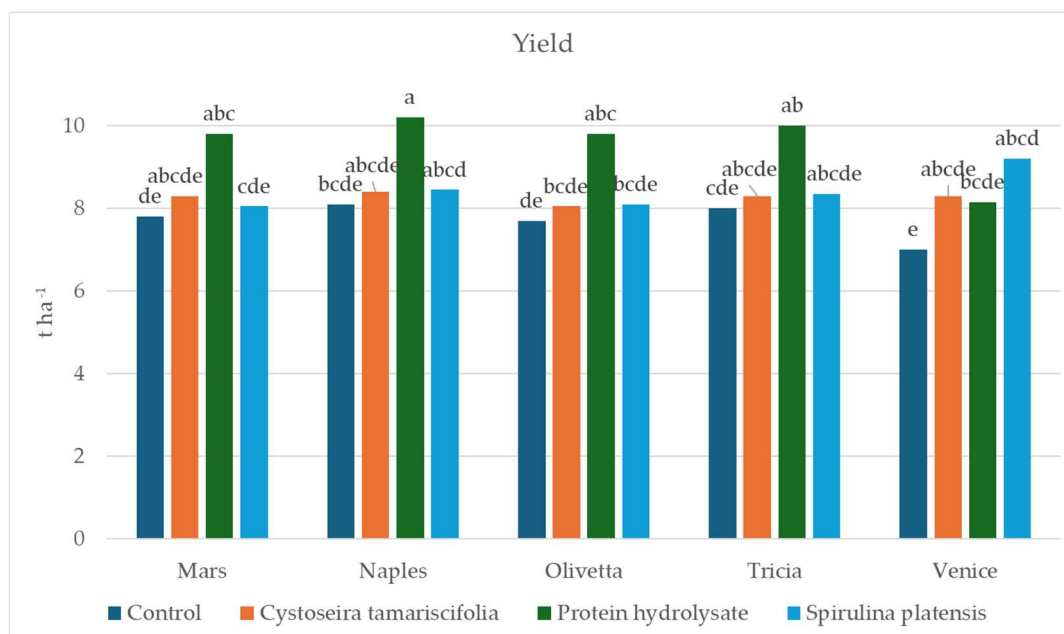


Figure 1. Interaction between cultivar and biostimulant formulation on rocket leaf yield. Values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

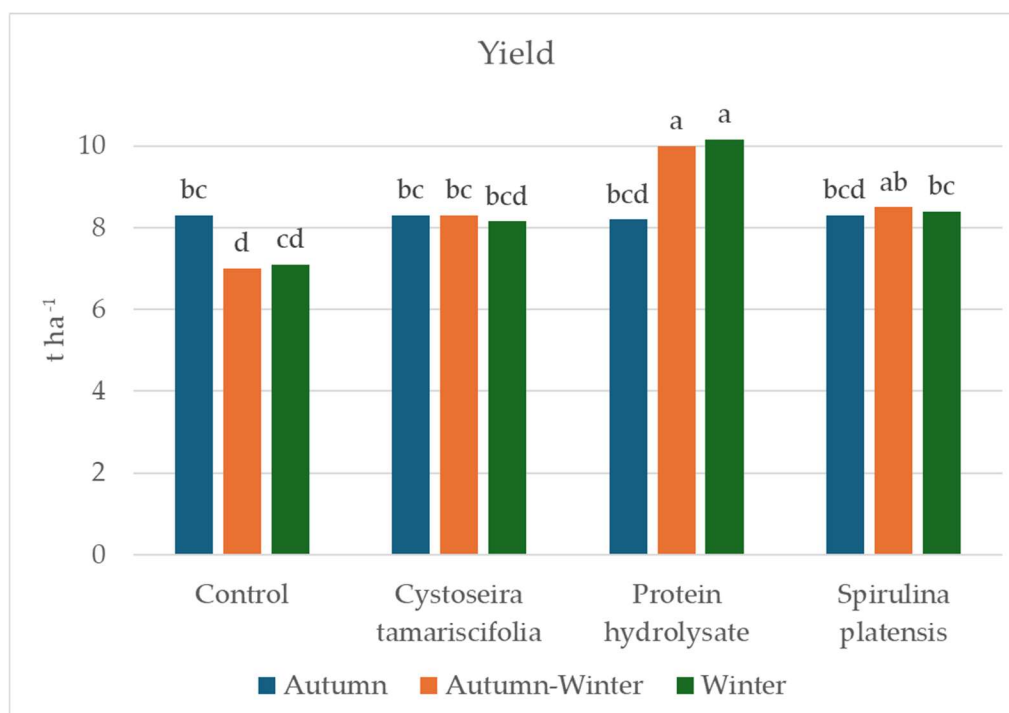


Figure 2. Interaction between biostimulant formulation and crop cycle on rocket leaf yield. Values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

The application of the protein hydrolysate led to a significant yield increase, compared to the control, in four varieties (+26% in Mars, +25.6% in Naples, +26% in Olivetta, +25% in Tricia). The treatment with *Spirulina platensis* resulted in a 36.2% yield increase only in the cultivar Venice, compared to the control which was not significantly different from the application of brown algae *Cystoseira tamariscifolia*. No significant differences arose between the cultivars within each biostimulant formulation.

The interaction between biostimulant formulation and crop cycle on rocket leaf yield is shown in Figure 2. Regarding the untreated control, the autumn crop cycle demonstrated a significantly higher yield compared to the autumn–winter (−18.8%), while the winter cut did not significantly differ from the two earlier ones. The application of brown algae resulted in no significant differences between the three crop cycles, which showed an average yield of 8.5 t ha^{−1}. The use of protein hydrolysate led to 19.3% and 21.4% higher yield in the autumn–winter and winter crop cycle, respectively, compared to the autumn one. The use of *Spirulina platensis* did not result in significant differences between the three crop cycles, with an 8.7 t ha^{−1} average yield. In the first crop cycle, the untreated control led to higher production than the second one but did not significantly differ from the biostimulant treatments. The protein hydrolysate application resulted in the highest yield in the two latest crop cycles, with the untreated control showing the lowest value at the second cut.

Significant interactions “cultivar × biostimulant formulation” and “biostimulant formulation × crop cycle” on the dry matter content in rocket leaves arose.

In Figure 3, we can observe that the application of protein hydrolysate led to the highest dry matter in the cultivars Mars, Olivetta, Tricia, and Naples, though in the latter cultivar it did not significantly differ from *Cystoseira tamariscifolia*; in Venice, no significant differences in dry matter were recorded between the three biostimulants, which showed a better effect than the untreated control.

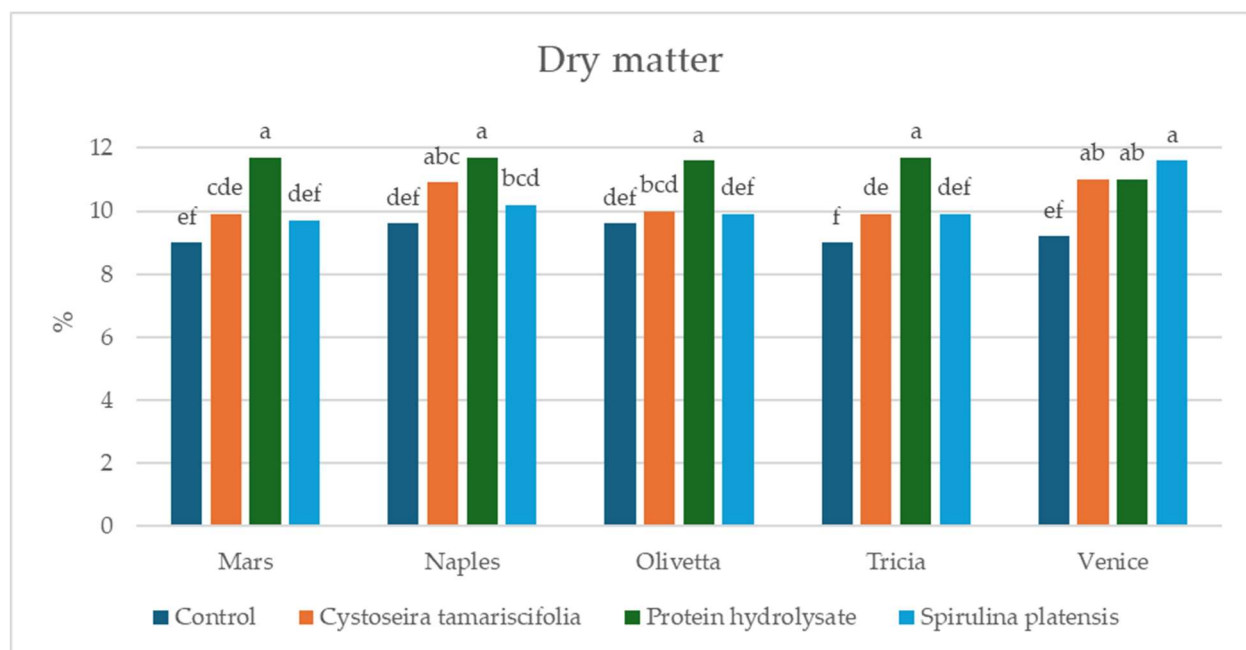


Figure 3. Interaction between cultivar and biostimulant formulation on dry matter in rocket leaves. Values followed by different letters are significantly different at $p < 0.05$ (Tukey’s test).

The interaction between biostimulant formulation and crop cycle (Figure 4) was significant on the dry matter accumulation in rocket leaves. The untreated control showed a 22.4% higher dry matter accumulation in the autumn cycle, compared to the two later ones, whereas the opposite trend was recorded under the protein hydrolysate treatment. The latter application led to the highest dry matter content in rocket leaves in both the second and third crop cycles, whereas the control attained the lowest values.

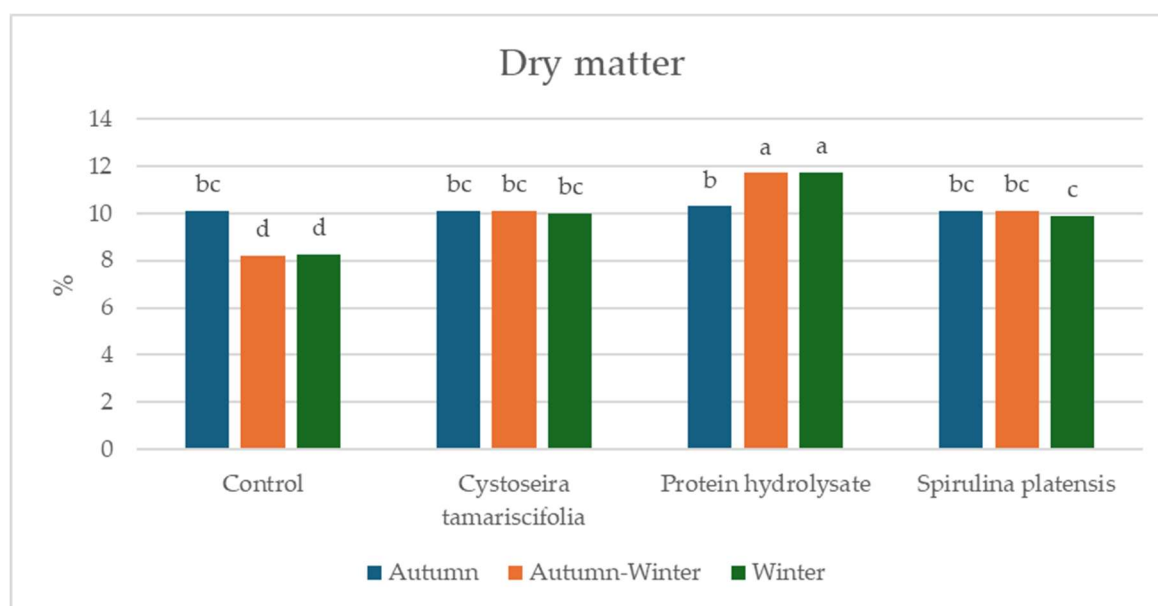


Figure 4. Interaction between biostimulant formulation and crop cycle on dry matter in rocket leaves. The columns represent the average of all cultivars for each biostimulation. Values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

No significant interactions between the experimental factors were recorded on the colorimetric parameters of rocket leaves (L^* , a^* , b^*) and, therefore, only the main effects are presented (Table 1).

Table 1. Colorimetric parameters L , a , and b of rocket leaves as affected by cultivar, biostimulant formulation, and crop cycle.

Experimental Treatment	Leaf Colorimetric Parameters		
	L^*	a^*	b^*
Cultivar (Cv)			
Mars	41.0 a	−14.9	22.0
Naples	41.4 a	−14.6	21.9
Tricia	41.0 a	−14.9	22.1
Venice	38.6 b	−14.7	22.8
Olivetta	40.9 a	−14.9	22.1
		n.s.	n.s.
Biostimulant formulation (B)			
Control	38.7 b	−14.7	22.6
<i>Cystoseira tamariscifolia</i>	39.7 b	−14.8	22.7
Protein hydrolysate	44.0 a	−14.9	21.7
<i>Spirulina platensis</i>	40.0 b	−14.8	21.8
		n.s.	n.s.
Crop cycle (Cy)			
Autumn	40.6	−14.8	22.2
Autumn–winter	40.6	−14.8	22.2
Winter	40.6	−14.8	22.2
	n.s.	n.s.	n.s.
Interaction			
Cv × B	n.s.	n.s.	n.s.
Cv × Cy	n.s.	n.s.	n.s.
B × Cy	n.s.	n.s.	n.s.

n.s. not significant; values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

The L* parameter (brightness) was significantly influenced by both cultivar and biostimulant formulation, but not by crop cycle. Indeed, the variety Venice showed the lowest values, whereas no significant differences were recorded between the other cultivars. The protein hydrolysate application increased the L* parameter by 13.6%, 10.7%, and 9.9%, compared to the untreated control, *Cystoseira tamariscifolia* and *Spirulina platensis*, respectively, which did not significantly differ from each other. The colorimetric parameters “a” and “b” were not affected by the three experimental factors applied.

A significant interaction between cultivar and biostimulant formulation arose on nitrate content (Figure 5).

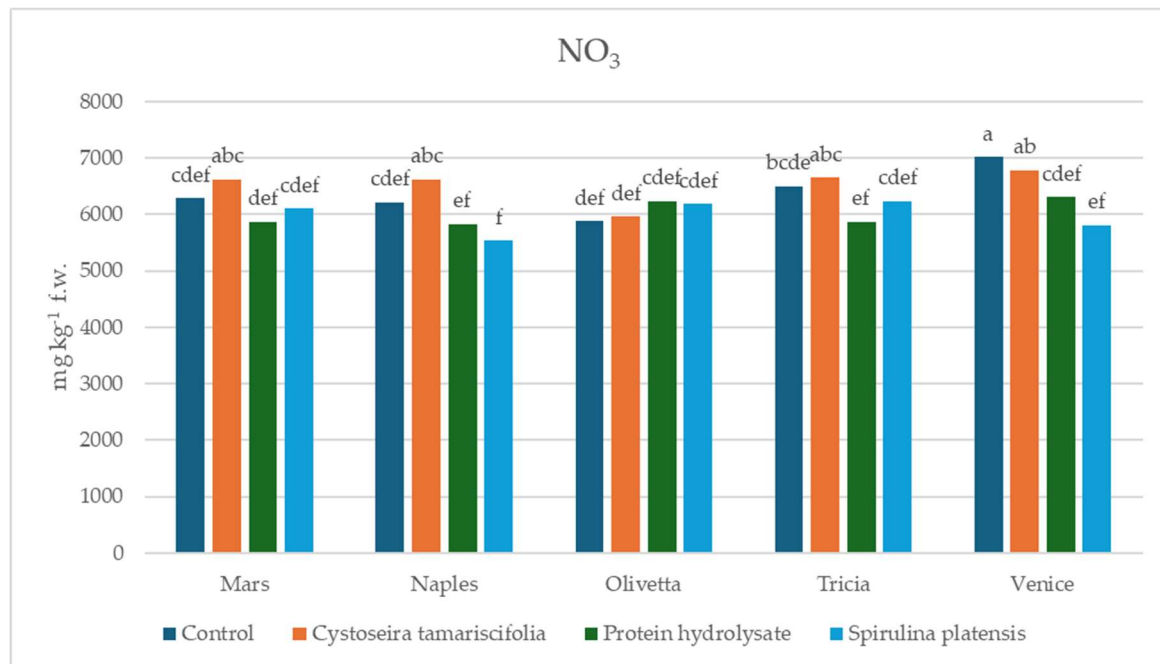


Figure 5. Interaction between cultivar and biostimulant formulation on nitrate content in rocket leaves. Values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

The treatment with *Cystoseira tamariscifolia* caused the highest nitrate accumulation in rocket leaves, though not different from the untreated control, in the cultivars Mars, Naples, Tricia, and Venice, whereas in Olivetta no significant differences were recorded between the biostimulant formulations and the control. The untreated control attained the highest nitrate content in the cultivar Venice; Olivetta leaves showed the lowest nitrate accumulation under the *Cystoseira tamariscifolia* supply; protein hydrolysate and *Spirulina platensis* did not elicit significant differences in nitrate content between the cultivars.

A significant interaction arose between cultivar and biostimulant formulation on nitrogen use efficiency (NUE; Figure 6).

Compared to the untreated control, the NUE was increased by protein hydrolysate in the cultivars Mars, Naples, Tricia, and Olivetta (by 22.6%, 28.0%, 26.8%, and 22.8%, respectively), and *Spirulina platensis* in Venice (by 33.3%). The leaves of the variety Naples showed a higher nitrogen use efficiency than Venice upon the application of the protein hydrolysate biostimulant. No significant differences were recorded between the five cultivars examined under the application of *C. tamariscifolia* and *S. platensis*, as well as in the untreated control.

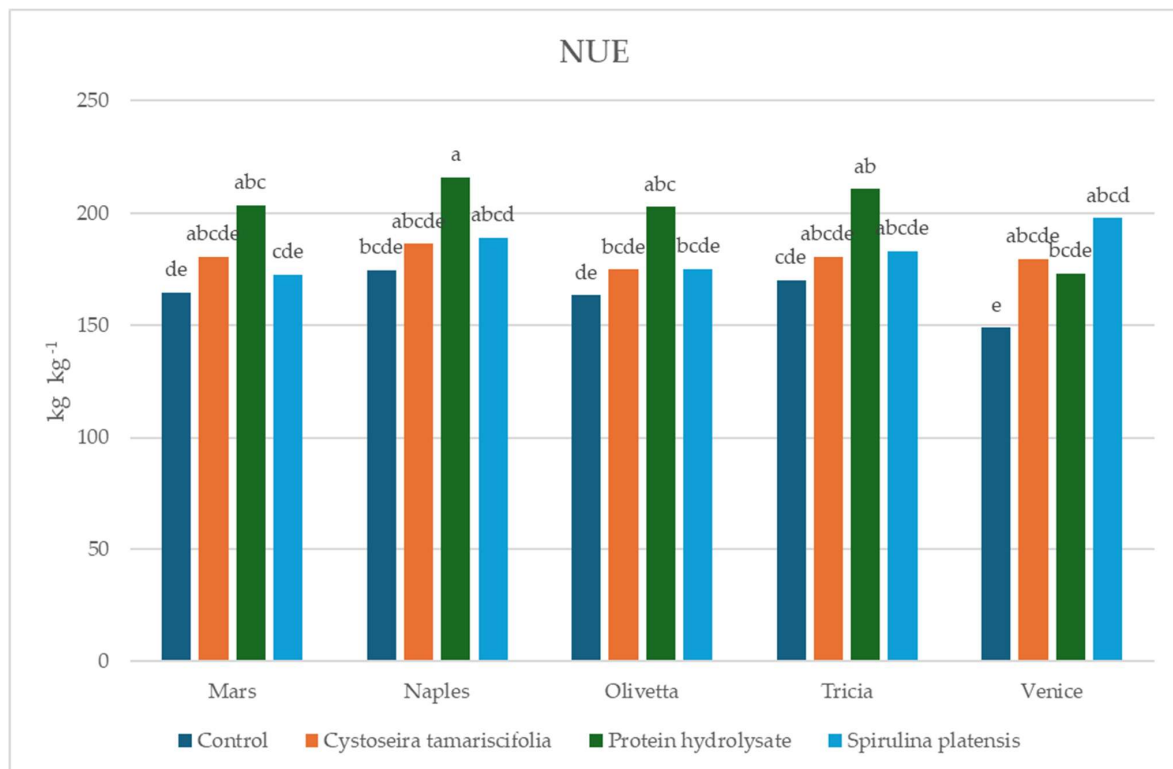


Figure 6. Interaction between cultivar and biostimulant formulation on nitrogen use efficiency in rocket. Values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

The contents of malate and citrate in rocket leaves were significantly influenced by the interaction between biostimulant formulation and crop cycle (Table 2). Indeed, for the untreated control, higher values of malate content were recorded during the autumn–winter crop cycle, followed by the autumn and finally the winter cycle; the brown algae treatment led to higher malate values at the second cycle; under the protein hydrolysate and *Spirulina platensis* applications, the mentioned organic acid showed a similar trend as in the untreated control. In the autumn–winter cycle, the protein hydrolysate and *Spirulina* treatments led to higher malate content than the control, whereas in autumn and winter no significant differences were recorded.

Table 2. Interaction between biostimulant formulation and crop cycle on malate and citrate contents in rocket leaves.

Biostimulant Formulation	Crop Cycle	Malate (g kg ⁻¹ d.w.)	Citrate (g kg ⁻¹ d.w.)
Control	Autumn	9.1 c	23.2 a
	Autumn–winter	24.2 b	10.3 ef
	Winter	6.1 def	15.5 cde
<i>Cystoseira tamariscifolia</i>	Autumn	8.3 cd	20.7 abc
	Autumn–winter	25.6 ab	10.2 ef
	Winter	6.5 cdef	17.8 bcd
Protein hydrolysate	Autumn	7.5 cd	22.2 ab
	Autumn–winter	27.3 a	8.8 f
	Winter	4.6 ef	13.0 def
<i>Spirulina platensis</i>	Autumn	6.8 cde	16.4 cd
	Autumn–winter	28.1 a	10.3 ef
	Winter	4.0 f	11.0 ef

d.w.: dry weight; values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

The citrate content (Table 2) in the untreated control, and in plants supplied with protein hydrolysate and *Spirulina*, was higher in autumn, whereas under *Cystoseira tamariscifolia* application it was significantly lower during the second crop cycle, compared to the other two cycles. Moreover, in the same season the citrate content was lower upon *Spirulina* treatment, compared to the other biostimulants and the untreated control; in winter *Cystoseira tamariscifolia* resulted in higher citrate accumulation than *Spirulina plantensis*; no significant differences between the biostimulant formulations were recorded in autumn–winter.

The hydrophilic antioxidant activity (HAA) was significantly influenced by cultivar and biostimulant formulation (Table 3), whereas only the latter experimental factor showed a significant effect on lipophilic antioxidant activity (LAA). Particularly, the variety Naples showed higher HAA values (0.24 mmol ascorbic acid eq. 100 g⁻¹ f.w.) compared to the other cultivars, which did not differ from each other (0.09 mmol ascorbic acid eq. 100 g⁻¹ f.w., as an average). Regarding the applied biostimulants, *Spirulina platensis* elicited the highest HAA values in rocket leaves (0.2 mmol ascorbic acid eq. 100 g⁻¹ f.w.), whereas no significant differences arose between the other treatments.

Table 3. Effect of cultivar, biostimulant formulation, and crop cycle on the antioxidant activities in rocket leaves.

Experimental Treatment	HAA (mmol Ascorbic Acid eq. 100 g ⁻¹ f.w.)	LAA (mmol Trolox eq. 100 g ⁻¹ f.w.)
Cultivar (Cv)		
Mars	0.09 b	1.95
Naples	0.24 a	1.93
Tricia	0.09 b	1.94
Venice	0.09 b	1.96
Olivetta	0.09 b	1.93
		n.s.
Biostimulant formulation (B)		
Control	0.09 b	2.12 a
<i>Cystoseira tamariscifolia</i>	0.10 b	1.92 ab
Protein hydrolysate	0.10 b	1.90 b
<i>Spirulina platensis</i>	0.19 a	1.82 c
Crop cycle (Cy)		
Autumn	0.12	1.94
Autumn–winter	0.12	1.94
Winter	0.12	1.94
	n.s.	n.s.
Interaction		
Cv × B	n.s.	*
Cv × Cy	n.s.	n.s.
B × Cy	n.s.	n.s.

f.w.: fresh weight; n.s.: not significant; *: significant at $p < 0.05$; values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

The interaction between cultivar and biostimulant formulation was significant on the lipophilic antioxidant activity (LAA; Figure 7).

Rocket leaves in the untreated control showed significantly higher LAA values, compared to the treatment with *Cystoseira tamariscifolia*, in the varieties Mars and Naples (by 19.1% and 16.7%, respectively), while no significant differences arose between the three biostimulant formulations. The untreated control leaves also showed higher values than

the leaves supplied with *Spirulina platensis* in the cultivars Olivetta and Tricia (by 25.0% and 25.4%, respectively).

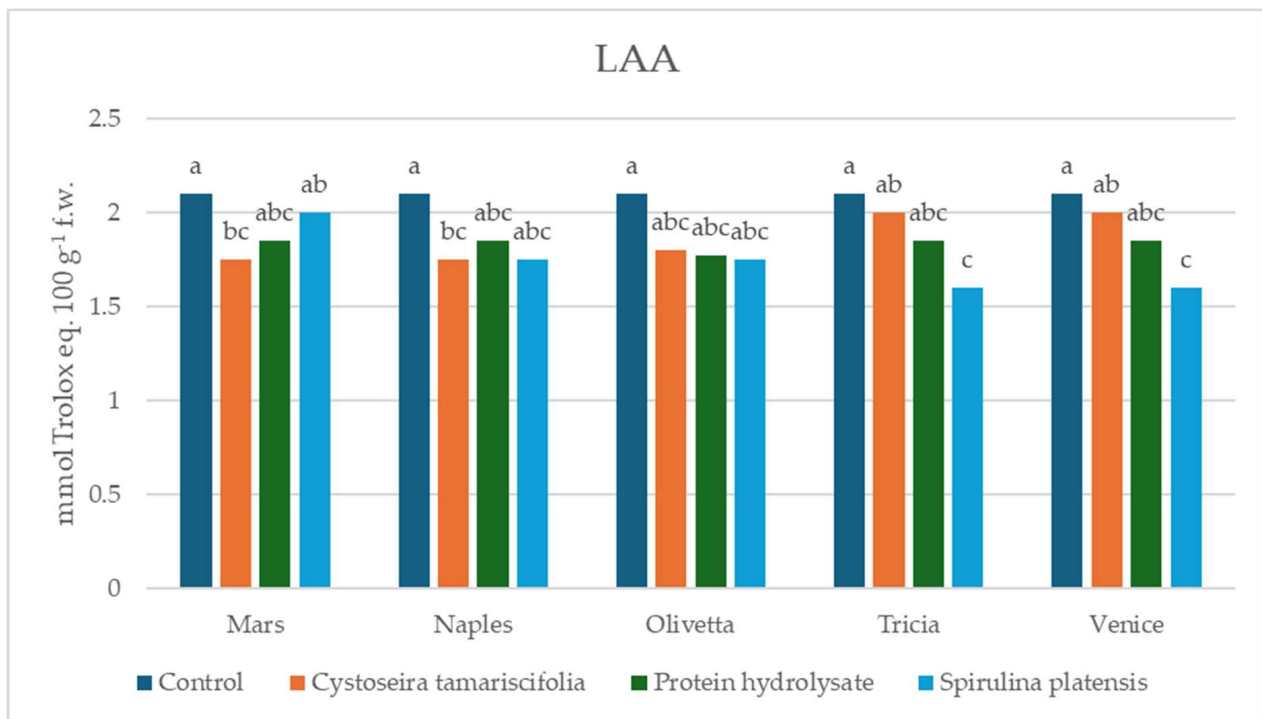


Figure 7. Interaction between cultivar and biostimulant formulation on lipophilic antioxidant activity (LAA) in rocket leaves. Values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

The application of *Spirulina platensis* to the cultivar Mars plants fostered LAA, compared to the cultivars Olivetta and Tricia (+19.1% and +18.34%, respectively), whereas no significant differences were recorded between the cultivars either within each of the two other biostimulants or the untreated control.

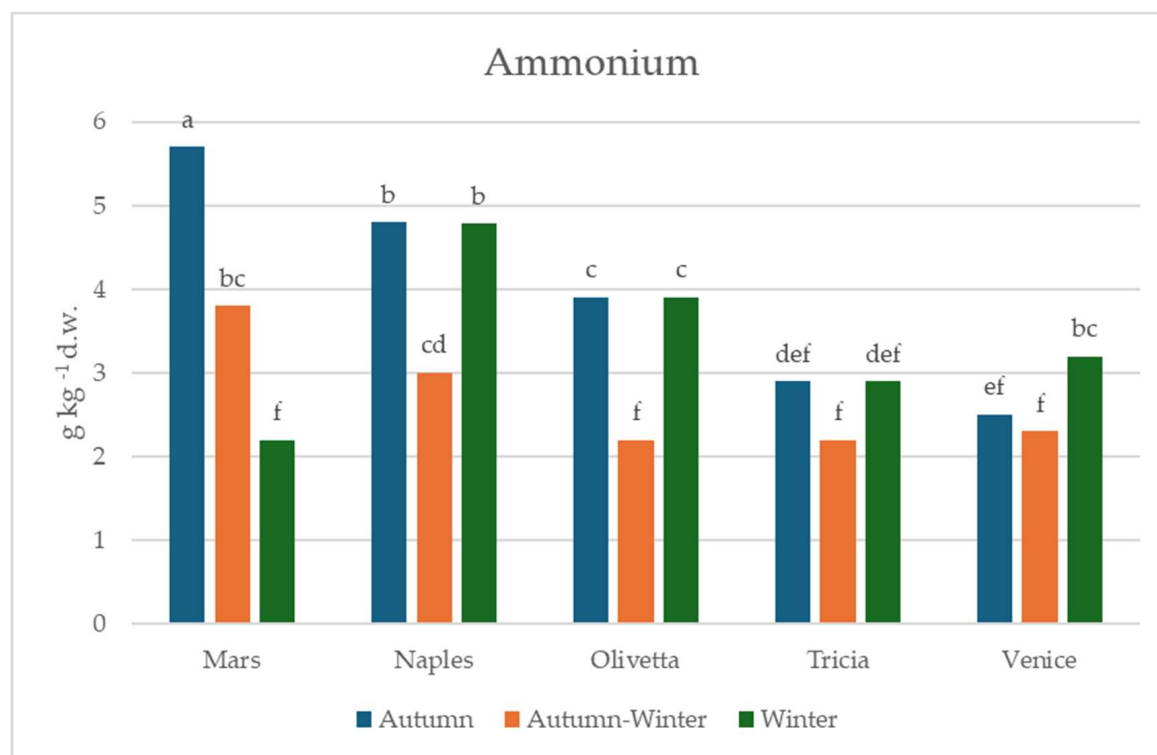
As for the mineral composition of rocket leaves (Table 4), significantly higher chloride content was detected in the cultivars Naples and Olivetta, compared to Mars; Mars and Naples showed higher values of potassium than Venice and Olivetta. The calcium content was higher in Tricia and Venice, compared to Olivetta. Sodium values were the highest in Tricia, although not significantly different from Mars. The cultivar Naples had the highest ammonium accumulation. As for the biostimulant treatments, the untreated control rocket leaves attained higher calcium content than the treatment with *Spirulina platensis*. The crop cycle affected all the mineral elements analyzed, with the rocket leaves grown in the autumn and winter cycles showing the highest contents of chloride and calcium, and those reared in the autumn–winter cycle attaining the highest levels of potassium, sodium, and magnesium.

A significant interaction was recorded between cultivar and crop cycle on ammonium content in rocket leaves (Figure 8). Regarding the comparison between the crop cycles within each variety, the highest ammonium accumulation was recorded in the variety Mars in autumn, in Naples and Olivetta in autumn and winter, and in Venice in winter. No significant differences between the three crop cycles were recorded in the cultivar Tricia.

Table 4. Mean effects of cultivar, biostimulant formulation, and crop cycle on mineral element composition.

Experimental Treatment	Chloride	Potassium	Calcium	Sodium	Ammonium	Magnesium
	(g kg ⁻¹ d.w.)					
Cultivar (Cv)						
Mars	17.6 b	66.1 a	24.3 ab	5.0 ab	3.9 b	4.8
Naples	21.0 a	66.6 a	24.0 ab	4.4 c	4.2 a	4.9
Tricia	18.6 ab	65.0 ab	25.8 a	5.2 a	2.6 d	5.0
Venice	19.0 ab	61.4 b	25.8 a	4.4 c	2.7 d	4.9
Olivetta	20.6 a	62.0 b	22.7 b	4.5 bc	3.4 c	4.7
						n.s.
Biostimulant formulation (B)						
Control	18.6	64.0	25.4 a	4.6	3.1 b	4.9
<i>Cystoseira tamariscifolia</i>	19.3	64.2	24.9 ab	4.8	3.3 ab	4.8
Protein hydrolysate	19.1	63.8	24.5 ab	4.7	3.4 a	4.8
<i>Spirulina platensis</i>	20.5	65.0	23.4 b	4.8	3.6 a	4.7
	n.s.	n.s.		n.s.		n.s.
Crop cycle (Cy)						
Autumn	20.8 a	62.2 b	25.8 a	4.4 b	3.9 a	4.9 b
Autumn–winter	16.8 b	66.6 a	23.0 b	5.3 a	2.6 c	5.1 a
Winter	20.3 a	64.1 ab	24.8 a	4.4 b	3.4 b	4.5 b
Interaction						
Cu × B	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Cu × Cy	n.s.	n.s.	n.s.	n.s.	*	n.s.
B × Cy	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

d.w.: dry weight; n.s.: not significant; *: significant at $p < 0.05$; values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

**Figure 8.** Interaction between biostimulant formulation and crop cycle on the ammonium content in rocket leaves. Values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

As for the comparison between the cultivars within each crop cycle, the highest ammonium content was detected in autumn in the variety Mars, autumn–winter in Mars and Naples, and winter in Naples and Olivetta.

A significant interaction was detected between cultivar and biostimulant formulation on the content of phosphate in rocket leaves (Figure 9). Indeed, in the cultivar Mars, the treatment with *Spirulina platensis* produced a significant decrease in phosphate, compared to the untreated control and the other two biostimulants; no significant differences between the biostimulant formulations, even compared to the control, were detected within the other varieties. As for the comparison between the cultivars within each biostimulant formulation, the highest phosphate content was recorded in the control leaves of the variety Mars, compared to Tricia, Venice, and Olivetta; in Mars and Naples, under *Cystoseira tamariscifolia* application; in Mars, compared to Tricia, Venice, and Olivetta, upon protein hydrolysate treatment; and in Naples, compared to Tricia and Olivetta, under *Spirulina platensis* supply.

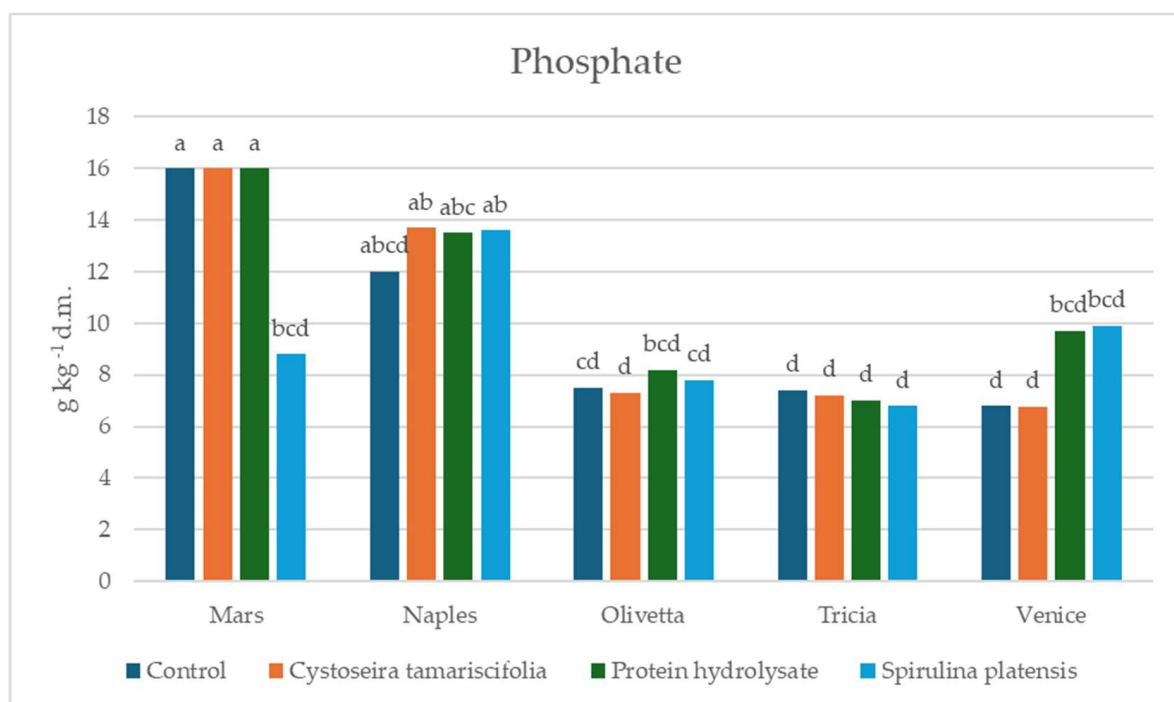


Figure 9. Interaction between cultivar and biostimulant formulation on the phosphate content in rocket leaves. Values followed by different letters are significantly different at $p < 0.05$ (Tukey's test).

The principal component analysis (Figure 10) shows the arrangement of the dependent variables analyzed in this study in relation to the three experimental factors applied. Principal component 1 demonstrates that the crop cycle priority determined the distribution of biostimulant treatment combinations with wild rocket cultivars. The treatments that were most affected by the initial and final experimental conditions (autumn and winter) are placed in the right side, whereas those exhibiting the highest efficacy during the intermediate cycle (autumn–winter) are displayed in the left side.

The variables that were best affected by the experimental treatments were oxalate, malate, sodium, phosphate, magnesium, and potassium, in the intermediate crop cycle, and sulfate, chloride, ammonium, citrate, and calcium in autumn and winter.

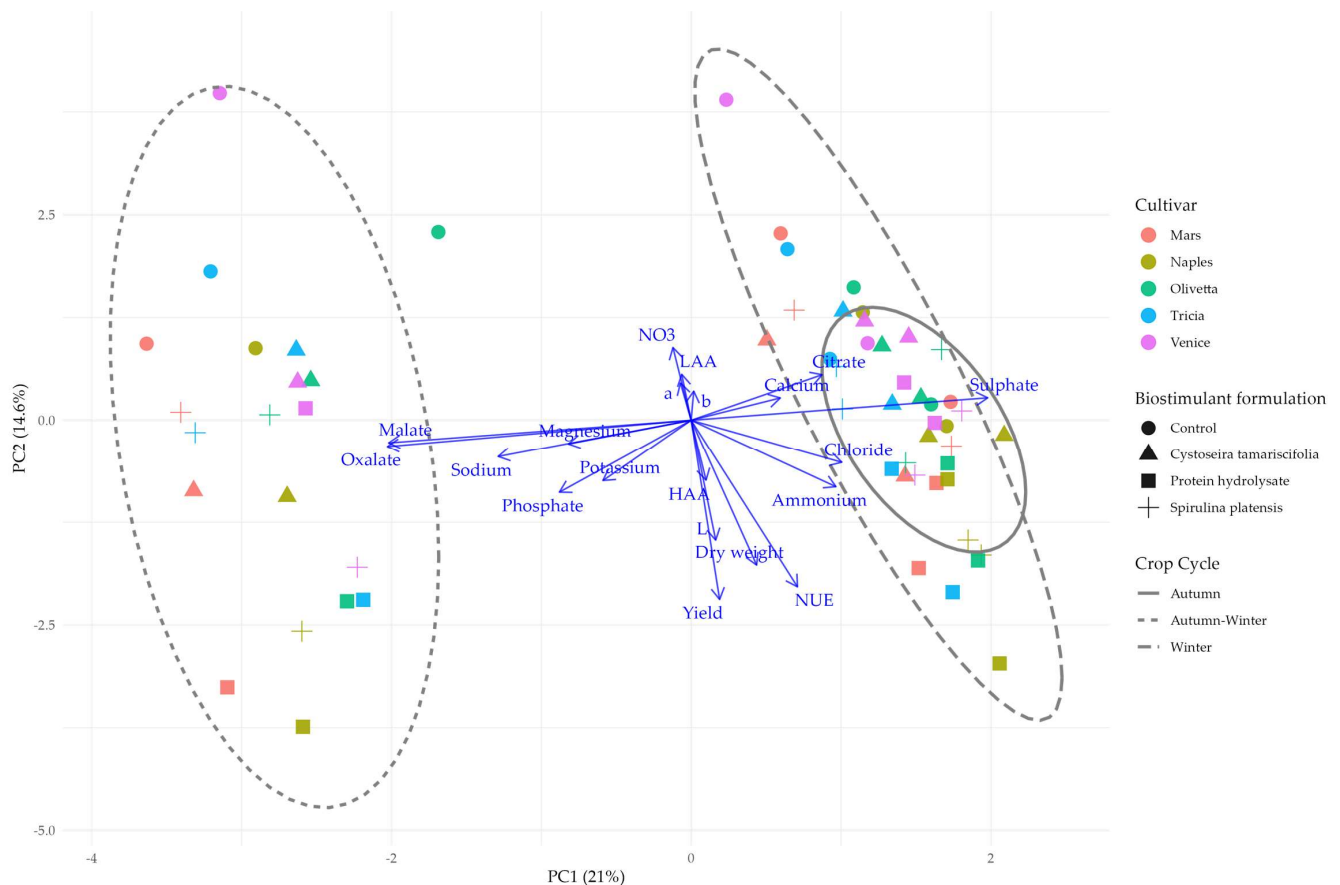


Figure 10. Principal component analysis.

Furthermore, yield, dry weight, and NUE were more influenced by the biostimulant type used and the proprietary protein hydrolysate (indicated by squares in the figure), since a clear decreasing trend on the y-axis is observed. Indeed, control treatments (represented by circles) are mainly located in the upper part of the graph, where NO_3 , LAA, and color components a^* and b^* were the most affected dependent variables.

4. Discussion

4.1. Yield, Quality, Antioxidants, and Mineral Composition

The results from the present two-year research demonstrated that the biostimulant effect on wild rocket (*Diplotaxis tenuifolia* L.) production is strongly genotype dependent. Protein hydrolysate proved to be the most effective biostimulant for the cultivars Mars, Naples, Tricia, and Olivetta, with remarkable yield increase by approximately 25–26%, compared to the untreated control. The latter outcome is consistent with previous findings [26,27] regarding the positive impact of protein hydrolysate on yield and quality in rocket and other leafy vegetables. The obtained results may be due to enhanced nutrient uptake, photosynthetic activity, and nitrogen metabolism of the mentioned varieties [28,29]. However, the rocket cultivar Venice provided a positive response exclusively to *Spirulina platensis* application, with a 36.2% yield increase compared to the untreated control, which is in accordance with the reports from other authors [30,31] concerning other crops. In the latter cases, the treatment with *Spirulina* significantly enhanced growth parameters, photosynthetic pigment concentration, and antioxidant activity, which likely relates to the phytohormones, vitamins, and antioxidant-rich profile of *Spirulina platensis* [32]. The absence of yield response to *Cystoseira tamariscifolia* supply is in contrast with other studies on leafy greens, though it also depends on the biostimulant concentration and/or genotype,

as previously observed in onion varieties [33]. Protein hydrolysate remarkably augmented yield, as also reported by Caruso et al. [26], particularly in the autumn–winter and winter crop cycles (+19.3% and +21.4%, respectively), likely due to the cumulative effects on root development and meristem activity [34].

Protein hydrolysate significantly increased the dry matter content in the cultivars Mars, Tricia, Naples, and Olivetta, confirming its ability to enhance carbon assimilation and structural biomass [26,27]. *Spirulina* significantly encouraged dry matter accumulation in Venice, consistent with previous findings about its positive impact on some metabolic pathways without importantly eliciting the carbohydrate accumulation in different genotypes [31]. The latter results indicate that yield response to sequential harvests in wild rocket is significantly influenced by cultivar. Indeed, in our research some varieties exhibited production increase under biostimulant application, progressing from autumn to winter, whereas other cultivars maintained stable yields or selectively reacted to specific formulations, as also observed in other leafy vegetables [26,27,34].

Leaf brightness (colorimetric L^* value) is a major indicator of visual quality in leafy vegetables and is closely linked to consumer perception of freshness and market value. In our study, the biostimulant application significantly influenced the L^* value, while the a^* and b^* parameters were unaffected. Consistent with our findings, Sherinlincy et al. [35] observed that the application of humic–fulvic acids significantly improved leaf brightness and the overall visual appearance in *Amaranthus tricolor*, emphasizing the genotype-dependent biostimulant effect. Similarly, Zamljen et al. [36] reported a positive influence of brown seaweed-based biostimulants on the L^* value of cucumbers.

The pigment increase plays an important role in influencing light absorption and reflectance, as recorded in lettuce treated with seaweed and protein hydrolysates [37], and the latter effects may be associated to improved leaf turgor, structural density, and altered epidermal surface properties that enhance light scattering.

The higher hydrophilic antioxidant activity (HAA) exhibited by the rocket cultivar Naples is in agreement with previous findings [6] about the genotype-dependent antioxidant responses in leafy vegetables. The significant enhancement of HAA under the *Spirulina platensis* application confirms the enhanced antioxidant capacity recorded in wild rocket treated with microalgae-based biostimulants by El-Nakhel et al. [16].

The contrasting effects of biostimulants on HAA and LAA in the present study highlight their different impact on the antioxidant pathways, as previously reported by Calvo et al. [38] in wall rocket. The reduction in LAA following *Spirulina platensis* treatment, particularly in the cultivars Olivetta and Tricia, suggests that microalgae-derived compounds may prioritize the mechanisms related to water-soluble antioxidants over the lipid-soluble ones, potentially through selective gene expression modulation [22].

The variety-dependent reactions to biostimulant applications, in terms of LAA, reflect previous similar findings in perennial wall rocket [26] and, particularly, the higher values recorded in untreated controls in some cultivars are consistent with the observations of Al-Karaki and Othman [39].

In our research, the lack of significant antioxidant differences between the crop cycles contrasts with previous investigations on wild rocket [16], and this discrepancy may be due to variations in environmental conditions or cultivation systems [40].

The distinct antioxidant profiles induced by different biostimulants suggest the related diversity in biochemical mechanisms, according to the composition of the applied formulation: *Cystoseira tamariscifolia* is rich in polysaccharides and phytohormones [17], whereas protein hydrolysates have a high content of nitrogen-based compounds that enhance protein synthesis [41].

The interaction between cultivar and biostimulant formulation on nitrate accumulation revealed differential responses of the five wild rocket varieties compared. Venice showed the most pronounced reaction to biostimulant application, with both protein hydrolysate and *Spirulina platensis* treatments significantly reducing NO₃ content, compared to the untreated control. This genotype-dependent response agrees with previous research: a 10.6% nitrate content reduction in wild rocket leaves was recorded following biostimulant treatment [18], and an effect on nitrate content in greenhouse-grown spinach was related to the biostimulant formulation used [21].

The contrasting responses of cultivars can be attributed to genotypic variations in nitrogen metabolism pathways, which in lettuce cultivars primarily stem from the change in nitrate uptake mechanisms rather than from differences in nitrate reduction capacity [13]. Reinink et al. [11] reported similar variation in nitrate accumulation in lettuce cultivars, some of which maintained lower nitrate levels regardless of growing conditions.

Interestingly, in the cultivars Mars and Tricia only the application of protein hydrolysate elicited a tendency toward lower nitrate content, compared to *Cystoseira tamariscifolia* treatment, though not significantly different than the untreated control. The latter outcome is consistent with the findings by El-Nakhel et al. [16], who demonstrated a 24% decrease in nitrate content in wild rocket leaves upon the application of protein hydrolysates derived from Fabaceae tissues, like in our study, despite remarkable variations between the genotypes.

No significant differences in nitrate accumulation were observed in the variety Olivetta under the biostimulant treatments, suggesting a genetic stability of nitrate metabolism regardless of biostimulation formulation, as also reported by Escobar-Gutiérrez et al. [12] in lettuce cultivars with rather stable nitrate content under different growing seasons and conditions.

The importance of selecting appropriate biostimulant–genotype combinations to effectively prevent nitrate accumulation in wild rocket was supported by Santamaria [9], who emphasized the need for integrated strategies to manage nitrate content in leafy vegetables, particularly the nitrate hyperaccumulator species like rocket.

The interaction between cultivar and biostimulant formulation was significant on nitrogen use efficiency (NUE) and, indeed, four of the five varieties tested (Mars, Naples, Tricia, and Olivetta) showed significant enhancements in NUE upon protein hydrolysate application, compared to the untreated control (+22.6%, +28.0%, +26.8%, and +22.8%, respectively). The latter increases highlight the potential of legume-derived protein hydrolysates to improve nitrogen utilization in wild rocket, consistent with previous reports [24] about the biostimulant encouragement of nitrogen metabolism and nutrient use efficiency in maize seedlings through modulation of enzyme activities.

The mentioned positive effects of biostimulants are likely connected with their rich array of peptides and amino acids, which can encourage nitrogen metabolism, also fostering nitrate reductase activity, as reported by Carillo et al. [42]; the latter authors found that protein hydrolysate application under suboptimal N fertilization regimes boosted the marketable yield of greenhouse spinach through the enhancement of nutrient uptake and photosynthetic efficiency.

The cultivar Venice showed a different response pattern, with only *Spirulina platensis* treatment leading to higher NUE (+33.3%), compared to the control. Similarly, Siringi et al. [19] observed that *Spirulina* applications altered nitrogen assimilation patterns in lettuce, resulting in enhanced nitrogen use efficiency, while maintaining or reducing tissue nitrate concentrations.

The different NUE responses of wild rocket cultivars to biostimulants recorded in our research are consistent with previous reports [43] regarding yield increases in different leafy

green genotypes, from 11% in Swiss chard to 36% in mustard greens under seaweed extract application. Similarly, Paçuta et al. [44] observed significant interactions between durum wheat varieties and biostimulant formulation on production and resource use efficiency.

The highest performance of protein hydrolysate in improving NUE in different cultivars, connected with its rich amino acid and bioactive peptide profile, has also been reported by Colla et al. [45] in terms of enhancement of nitrogen uptake and assimilation in tomato plants, with genotype-dependent response magnitude. The latter authors attributed the mentioned effects to the ability of protein hydrolysates to modulate root architecture and encourage the activity of key enzymes involved in nitrogen assimilation. Other authors reported variety-dependent reactions to biostimulants in baby rocket [39], the increase of photosynthetic efficiency and, accordingly, of carbon skeleton availability necessary for nitrate assimilation in spinach [21], and the modification of root architecture and functions in *Corchorus olitorius*, potentially altering nitrogen uptake patterns and improving NUE [46].

Consistent with our findings, Burns et al. [7] emphasized that genotype is a primary determinant of nitrate accumulation in leafy vegetables, along with environmental and management factors, including biostimulant application serving as modulators of genetic predisposition.

In the present study, the different reactions of the cultivar Venice to *Spirulina* application, compared to the other cultivars, agrees with the reports of Fan et al. [47], who demonstrated that the enhancement of antioxidant levels and metabolic processes, elicited by seaweed extracts in spinach, varied with the genotype.

All the biostimulant treatments elicited the peak of malate accumulation in autumn–winter, with the protein hydrolysate and *Spirulina platensis* showing a better effect than the untreated control in the mentioned crop cycle. The latter biostimulant-induced amplification of malate synthesis suggests the modulation of carbon metabolism enzymes, consistent with the reports about the effect of protein hydrolysates in increasing the activity of TCA cycle enzymes [22]; in contrast, citrate showed the highest levels in the autumn crop cycle under all the treatments. In this respect, the biostimulant modulation of the related metabolic paths is reportedly associated with the change in cellular pH regulation [47,48].

The mineral content analysis revealed significant genotypic variations, with Naples and Olivetta accumulating the highest chloride levels, while Mars and Naples showed the highest potassium accumulation. Calcium content was the highest in the cultivars Tricia and Venice and was negatively affected by *Spirulina platensis* application, compared to the untreated control. The latter negative effect contrasts with previous studies reporting enhanced calcium uptake under different biostimulant applications [26], suggesting the biostimulant-dependent effects on mineral transport mechanisms.

The crop cycle significantly influenced mineral accumulation patterns, with the leaves grown in autumn–winter showing top values of potassium, sodium, and magnesium, which likely reflect changes in nutrient requirements and transport mechanisms. The biostimulant-induced modifications of mineral profiles are consistent with the observations by Colla et al. [48], who reported that protein hydrolysates can alter nutrient uptake through modulation of root architecture and transporter expression.

The trends in ammonium content as a function of cultivar and crop cycle reflect the genotype-dependent regulation of nitrogen metabolism throughout plant development, confirming the different nitrogen use efficiency among leafy vegetable cultivars [16].

The different nutrient assimilation efficiency among leafy vegetable cultivars recorded by Burns et al. [7] reflects the genotype-dependent effect of biostimulant formulations on phosphate content in the rocket cultivar leaves in our research.

4.2. Economic Feasibility

According to priority in the present study, the economic feasibility of biostimulant applications in *Diplotaxis tenuifolia* L. has been evaluated as a risk mitigation strategy against regulatory non-compliance rather than solely as a yield enhancement tool. Biostimulants can provide secondary benefits through a small increase in yield [49], but their primary economic value lies in their ability to reduce leaf nitrate concentrations by 10–33%, thereby lowering the probability of exceeding the EU regulatory thresholds [50–52].

The investment becomes economically sustainable when the cost of biostimulant application is counterbalanced by the reduced risk of losing the total crop because of nitrate threshold violations. This is particularly valuable when nitrate levels are likely to approach the regulation limits [8] consequent to greenhouse growing in winter when nitrate accumulation is usually higher [49], even more upon intense nitrogen fertilization [53].

However, the biostimulant employment may not be sustainable in extensive cultivation systems with naturally low nitrate levels [27], or low market value when the crop loss risk is minimal. The decision framework should consider the probability-weighted expected yield loss against the annual cost of biostimulant applications.

5. Conclusions

The results of the present two-year research demonstrate that the efficacy of biostimulants on perennial wall rocket (*Diplotaxis tenuifolia* L.—DC.) is strongly cultivar dependent. Protein hydrolysate showed the most remarkable effect, increasing yield and nitrogen use efficiency in four varieties (Mars, Naples, Olivetta, and Tricia), while *Spirulina platensis* significantly improved the mentioned parameters only in the cultivar Venice. Protein hydrolysate also enhanced the dry matter accumulation and leaf brightness, while *Spirulina* increased the hydrophilic antioxidant activity. Matching the protein hydrolysate with the varieties Mars, Naples, Olivetta, and Tricia, and *Spirulina* with Venice, would lead to potential environmental and economic benefits related to reduced nitrogen fertilizer use.

The findings of the present investigation highlight the importance of adapting the biostimulant formulation to the cultivar and provide an important contribution toward a sustainable approach to plant nutrition.

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