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Foliar Application of Protein Hydrolysates on Baby-Leaf Spinach Grown at Different N Levels

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Abstract: Surpluses of N are associated with environmental and health problems. To optimise N use and reduce nitrate accumulation in leafy species like spinach, the application of biostimulants is suggested. An experiment in controlled conditions (growth chamber/soilless) evaluated baby-spinach responses to two protein hydrolysates (PHs) from plant (legume, Trainer[®]) and animal (meat, Isabion[®]) sources, combined with three N rates: 2 (N2, deficient), 8 (N8, sub-optimal), and 14 (N14, optimal) mM of N. Biometrical and morphological traits of shoots and roots as well as the physio-metabolic (gas exchange, N assimilation, and NUtE), physical, mineral, and antioxidant profiles of leaves were assessed. The legume-PH boosts growth and yield only at the highest N conditions, while there was no effect at lower N rates. The legume-PH modulates root architecture and chlorophylls has positive responses only at optimal N availability, such as an increase in N uptake, leaf expansion, and photosynthetic activity at the canopy level. The PHs do not improve NUtE, leaf colour, consistency, cations, or antioxidants. Neither do PHs have any effect on reducing nitrate accumulation. Legume-PH improves N assimilation only at optimal N availability, while meat-PH does not, reaching the highest nitrate value at the highest N rate (2677 mg kg⁻¹ fw), even if this value is under the EC limits for fresh spinach.

Keywords: root morphology; leaf expansion; N uptake; chlorophylls; nitrate; photosynthetic activity; gas exchange; biostimulants



Citation: Bonasia, A.; Conversa, G.; Lazzizzera, C.; Elia, A. Foliar Application of Protein Hydrolysates on Baby-Leaf Spinach Grown at Different N Levels. *Agronomy* **2022**, *12*, 36. <https://doi.org/10.3390/agronomy12010036>

Academic Editor: Essaid Ait Barka

Received: 6 November 2021

Accepted: 20 December 2021

Published: 24 December 2021

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1. Introduction

Spinach (*Spinacia oleracea* L.) is an important leafy green vegetable, highly appreciated all around the world for its nutritional properties, year-round availability, and different uses as frozen food and ready-to-eat product. Spinach leaves are an accessible source of natural antioxidants having protective properties for human health [1,2], but they also represent a source of anti-nutritional compounds such as nitrate and oxalate [3]. According to Biemond et al. [4], spinach plants have low efficiency in terms of N uptake and use, thus requiring high rates of fertiliser use to grow well and reach a higher leaf quality (standard green colour of leaves) [5]. The reduction of N inputs in leafy species, including spinach, is a crucial issue, both for limiting the nitrate accumulation in leaves and for reducing the economic and environmental impacts of fertilisation.

In the context of more sustainable agriculture, biostimulants are considered valid tools. They are products defined as novel, environmentally-friendly, and capable of contributing to the achievement of high growth, yield performance, and good-quality products [6].

Among the biostimulant categories, the protein hydrolysates (PHs) are nitrogen (N)-containing compounds, obtained by thermal and/or chemical and/or enzymatic hydrolysis of raw materials (of plant or animal origin), able to improve the plant nutritional status, by increasing the efficiency of the nutrient acquisition process, regardless of the nutrient levels supplied with the biostimulant itself [7,8].

The extensive literature on PHs shows them to be very effective in enhancing crop performances through the increase in plant nutrient availability, nutrient uptake and

metabolic use, abiotic stress resistance, and the expression of quality traits of leafy and fruit vegetables [9–12]. The use of PHs has recently been considered as an environmentally friendly strategy to reduce inputs and to improve nitrogen use efficiency (NUE) [8,13]. According to Colla et al. [14], the PHs have been assessed to reduce nitrate accumulation in leafy vegetables.

Among the plant-derived PHs, the legume-derived PH Trainer[®] has been widely tested both on fruit and leafy vegetables. The experiments assessed its efficiency to boost growth, and the productive and qualitative features of vegetables along with its potential to overcome induced abiotic stresses (such as salt, water, oxygen, and nutrient deficiency) [11,15–26]. In particular, the legume-PH Trainer[®] has been found to improve growth and/or yield even under deficient and/or sub-optimal N conditions in several greenhouse leafy vegetables, such as spinach, wild rocket, lettuce, and lamb's lettuce [23–26].

Little information related to the capacity to improve the NUE and/or reduce nitrate accumulation in leafy species by the use of different PHs from the legume-PH-Trainer[®] or by animal-derived PHs is available. In addition, despite the number of scientific works focused on unravelling the mechanism of action elicited by the application of PHs/Trainer[®] in leafy species, none of them showed a complete appraisal of plant physiology (chlorophyll concentration; leaf gas exchange) and N metabolism (N indices, N assimilation pattern, and NUE) in response to different N levels applied in controlled conditions (growth chamber) and supplied in the nutrient solution in a soilless cultivation system (thus, without any soil N interference).

Taking into account all the previous considerations, an experiment was carried out to verify the possibility to reduce the N supply and nitrate accumulation in spinach crop grown with two PH biostimulants from different origins (plant and animal) under variable nitrogen (N) conditions, in controlled conditions (growth chamber and soilless cultivation technique). The biometrical (growth, yield), morphological (shoot and root), and physio-metabolic responses of spinach under N*B treatments, along with the physical (colour and thickness of leaves) and antioxidant profiles (vitamin C, phenols, and carotenoids) have also been investigated.

Along with the already and widely-tested (plant-derived) Trainer[®], in the present study another PH, derived from an animal source (Isabion[®]) and consequently provided with a different aminogram, has been examined. To the best of our knowledge, Isabion[®] has never been experimentally tested on (leafy) vegetables before this work.

2. Material and Methods

2.1. Plant Growing Conditions, Treatments, and Experimental Design

The experiment was carried out at the vegetable growing facilities of the Department of Agriculture, Food, Natural resources and Engineering (DAFNE) of the University of Foggia (Italy).

Seeds of spinach (*Spinacia oleracea* L.) ('El Prado', Syngenta, Basel, Switzerland), previously hydro-primed by soaking at 20 °C for 24 h in distilled water, were hand-sown on 23 March 2019 (948 plants per m²), in polystyrene trays (35 cm × 53 cm × 5 cm) filled with Brill 3 TYPical peat (Gebr. Brill Substrate GmbH & Co. KG, Georgsdorf, NI, Germany).

Trays were placed in a custom-made growth chamber (Piardi Technologie del Freddo s.r.l., Castenedolo, BS, Italy) (dark, 20 °C, 80% relative humidity—RH). Germination took place after 5 days.

After emergence, the trays were grown under the following conditions: RH, 70%; photoperiod and temperature, 10/14 h and 18/15 °C day/night; Photosynthetic Photon Flux Density (PPFD), and 200 μmol·m⁻² s⁻¹. Illumination was supplied by Fluora 36 W fluorescent lights (Ledvance GmbH, Garching, Germany).

Spinach plants were grown using the ebb and flow soilless cultivation system. The growing set-up consisted of (9) aluminium benches (120 cm long, 103 cm wide, with a 5-cm high border), connected through a 12-V Johnson Marine Pump (Spx Flow Inc., Charlotte,

NC, USA) to a tank (40 L) positioned below, which was used for nutrient solution (NS) replenishment.

The polystyrene trays were laid on the benches (6 per bench) and were sub-irrigated 2 times a day (at 9:00 AM and 3:30 PM) by a 2-min flow of NS through the benches at the base of the trays. A total of 30 L of NS was always maintained in the NS tanks throughout the cycle by replenishment with new NS.

The treatments assessed were: (a) Three nitrogen (N) fertilisation levels in the NS, namely 2 mM (N2), 8 mM (N8), and 14 mM (N14) of N; and (b) two commercial biostimulants namely, Trainer[®] (Italpollina s.p.a, Rivoli Veronese, VR, Italy) (Bt) and Isabion[®] (Syngenta, Basel, Switzerland) (Bi) applied as a foliar spray. The water spray represented the control (B0).

A split-plot experimental design was adopted with three replications: N treatment in the main plot (bench) and B treatment in the sub-plot (tray). The experimental unit was represented by 2 trays (0.36 m⁻²). The whole trial consisted of 54 polystyrene trays (Table S1).

The biostimulants, belonging to the protein hydrolysate category, from a plant (Trainer[®]) and animal (Isabion[®]) source, were applied at the dose recommended by the manufacturer: 2 and 3 L ha⁻¹ for Trainer[®] and Isabion[®], respectively, diluted in 600 L of water. In detail, 3.3 mL L⁻¹ for Trainer[®] and 5.0 mL L⁻¹ for Isabion[®].

The treated plants were uniformly sprayed two times during the growing cycle: At 28 (at the 2nd true leaf stage) and 38 days after sowing. The composition of each biostimulant is reported in Supplementary Table S2.

The NS was prepared by dissolving soluble salts in tap water to achieve the composition for the application rates of N2, N8, and N14 with the appropriate amounts for each nutrient solution: 1.2, 6.0, 2.0, and 3.5 mmol L⁻¹ of P, K, Mg, and Ca, respectively. Micronutrients were supplied according to Johnson et al. [27]. The EC and pH of the NS were measured daily using a portable conductivity meter and pH meter (Hanna Instruments Italia s.r.l., Villafranca, PD, Italy). The EC of the NS was approximately 2.0 ± 0.2 dS m⁻¹. Whenever necessary, the pH was maintained at 5.5–6.5 using 1 M HCl.

Harvest was on 7 May 2019, 45 days after sowing.

2.2. Sampling and Measurements

The harvest of baby spinach leaves was performed by cutting 1 cm above the collar. The fresh and dry weight, area, number, height, main colour indices of leaves and fresh and dry weight, total length, and mean diameter of roots were determined on 15 plants sampled from the experimental unit.

2.2.1. Bio-Morphological Features of Spinach Leaves

The fresh yield was calculated by harvesting the whole experimental unit.

In order to determine the dry weight of leaves, a portion of fresh material was dried in a thermo-ventilated oven at 70 °C until it reached a constant mass. The dry matter (DM) concentration was calculated as [(dry weight/fresh weight)*100].

Leaf area was measured using LI-COR 3100 (LI-COR Inc., Lincoln, NE, USA).

Colour indices measurements based on the Ciel*a*b* scale 1976—L* (lightness), a* (green to red), and b* (yellow to blue) were performed on the images of fresh leaves. In addition, the hue angle (h°), as a derived parameter ($\tan^{-1} 148 (b^*/a^*)/6.2832$)*360), was calculated. Images were taken using the image acquisition station created by Immagini & Computer s.n.c. (Bareggio, MI, Italy), equipped with: 4 white Tornado ESaver (23 W) lamps (Philips s.p.a., Milano, MI, Italy); a Nikon D5200 camera (Nikon Corporation, Tokyo, Japan); and Image Pro Plus 7.0 software (Media Cybernetics Inc., Rockville, MD, USA).

The Specific Leaf Area (SLA), indicative of leaf texture consistency, was calculated as a leaf area/leaf dry weight ratio (cm² g⁻¹).

2.2.2. Bio-Morphological Features of Spinach Roots

Fresh roots were carefully washed with distilled water after harvest, made free from any substrate particles, and then paper dried. The dry weight and DM concentration of roots were determined as described for leaves.

To determine root morphological features (total length and average diameter), roots were spread out on a transparent tray filled with water and scanned at 360 dpi with an Epson Perfection V750 PRO scanner (Epson Italia s.p.a., Cinisello Balsamo, MI, Italy). The captured images were then processed using WinRHIZO Pro image analysis software (Regent Instruments Inc., Québec, QC, Canada).

2.3. Chemical Analyses

Chemical analyses of leaves were performed on lyophilized samples, except for the determination of vitamin C. The plant material was dried using a CoolSafe Scanvac freeze-dryer (LaboGene ApS, Vassingerød, Denmark), successively powdered, packed in hermetic jars, and then stored at $-20\text{ }^{\circ}\text{C}$ until the analyses were carried out. All samples were analysed in triplicate.

2.3.1. Inorganic Ion and Nitrogen Concentration

Nitrogen leaf concentration was determined by the Dumas method: Plant lyophilized material (0.5 g) was combusted, and the volume of N_2 gas given off by the sample was used to calculate the total plant N concentration [28] using the FP-582 Analyser (Leco Italy s.r.l, Cassina de Pecchi, MI, Italy).

Inorganic cations were extracted from lyophilized samples (1 g), previously ashed at $550\text{ }^{\circ}\text{C}$ for 6 h in the Z1200 muffle furnace (Zetalab s.r.l., Padova, PD, Italy) and acid digested (20 mL of 1 mol L^{-1} HCl in boiling water for 30 min), before injection into the ion chromatography system. For inorganic anions, the lyophilized samples (0.5 g) were extracted with 50 mL of eluent solution (3.5 mM sodium-carbonate and 1.0 mM sodium-bicarbonate) in a shaking water bath at room temperature for 30 min. The mixture was filtered through Whatman n. 2 paper. The filtrates were filtered again through a $0.22\text{-}\mu\text{m}$ Millipore filter, before injection into the ion chromatography system. The ion chromatography system was equipped with: An isocratic pump, conductivity detector; a model AS-DV auto-sampler; a self-generating ERS-500 suppressor (4 mm), an Ion-Pac AS23 analytical column ($4\text{ mm} \times 250\text{ mm}$, particle size $6\text{ }\mu\text{m}$), and an eluent solution (3.5 mM sodium-carbonate and 1.0 mM sodium-bicarbonate) at a flow rate of 1 mL min^{-1} (Dionex—Thermo Fisher Scientific, Waltham, MA, USA) (specifically, for anion analysis); a self-generating DRS-600 suppressor (4 mm); an IonPac CS12A analytical column ($4 \times 250\text{ mm}$, $5\text{ }\mu\text{m}$); and an eluent solution (20 mM methanesulfonic acid) at a flow rate of 1 mL min^{-1} (Dionex—Thermo Fisher Scientific, Waltham, MA, USA) (specifically, for cation analysis).

2.3.2. Concentration of Phenols

The total phenols were extracted in lyophilized samples (0.1 g) using the modified method reported by Gil-Izquierdo et al. [29]. Briefly, samples were double extracted in water/methanol (20:80, *v/v*) solution and centrifuged. Total phenol (TP) content was determined by mixing the methanolic extracts with the Folin–Ciocalteu reagent and the absorbance was read at 750 nm. The results are expressed as gallic acid equivalents (g.a.e.) using a calibration curve.

2.3.3. Ascorbic Acid, De-Hydro-Ascorbic Acid, and Vitamin C Concentration

L-ascorbic acid (AA), L-de-hydro-ascorbic acid (DHAA), and vitamin C (AA + DHA) contents were determined as described by Zapata and Dufour [30] with some modifications.

Fresh material (10 g) was homogenised with a homogeniser for 2 min with 10 mL of methanol/water (5:95), plus citric acid (21 g L^{-1}), EDTA (0.5 g L^{-1}), and NaF (0.168 g L^{-1}). The homogenate was filtered through two layers of cheese-cloth and the pH was adjusted to 2.2–2.4 by addition of 6 mol L^{-1} HCl.

After centrifugation at $12,000 \times g$ for 5 min at 4°C , the supernatant was filtered through a C18, 500-mg, Sep-Pak cartridge (Agilent Technologies Italia s.p.a, Cernusco sul Naviglio, MI, Italy) and then through a $0.2\text{-}\mu\text{m}$ cellulose acetate filter.

The HPLC analysis was carried out after the derivatisation of DHAA into the fluorophore 3-(1,2-dihydroxyethyl) furo[3,4-b]quinoxaline-1-one (DFQ), with 1,2-phenylenediamine di-hydro-chloride (OPDA). Samples of $20\ \mu\text{L}$ were analysed with an HPLC (Dionex ICS 3000, Dionex-Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with: An isocratic pump, a $10\text{-}\mu\text{L}$ injection loop, and an AS-DV auto-sampler. Separations of DFQ and AA were achieved on a Synergi 4u Hydro-RP 80A C18 column ($250\ \text{mm} \times 4.60\ \text{mm}$) (Phenomenex Inc., Castel Maggiore, BO, Italy), maintained at 25°C combined with an Ulti-Mate 3000 Diode Array Detector (DAD) (Dionex—Thermo Fisher Scientific Inc., Waltham, MA, USA). The mobile phase was $5\ \text{mmol L}^{-1}\ \text{KH}_2\text{PO}_4$ at pH 4.5. The flow rate was $1\ \text{mL min}^{-1}$. The detector wavelengths were $348\ \text{nm}$ for DHAA and $251\ \text{nm}$ for AA.

2.3.4. Concentration of Total Chlorophylls and Carotenoids

The concentration of total chlorophylls and carotenoids was determined by using the Sumanta et al. [31] method with some modifications.

Lyophilized samples ($0.05\ \text{g}$) were mixed with $1.5\ \text{mL}$, 80% ethanol (ethanol:water = 80:20), containing 0.1% hydrochloric acid in a 10-mL tube with a screw cap. The mixture was treated with ultrasonic power using a DU-32 digital ultrasonic cleaner (Argo Lab s.r.l., Carpi, MO, Italy) at room temperature for 30 min.

After sonication, the samples were centrifuged in a refrigerated Coulter Allegra TM 25 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA) (4°C , 15 min, $4000 \times g$) and then the supernatant was collected.

The above steps were repeated twice and supernatants were combined. Finally, the extract was filtered by using $0.22\text{-}\mu\text{m}$, reinforced nylon membrane filters.

The extract solution was measured with an Evolution 201 UV-Visible spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 664, 649, and 470 nm.

Pigment concentration was estimated using the following equation:

Total chlorophylls = $17.32\ (\text{A}_{649}) + 7.18\ (\text{A}_{664})$; Chl a = $13.36\ (\text{A}_{664}) - 5.19\ (\text{A}_{649})$; Chl b = $27.43\ (\text{A}_{649}) - 8.12\ (\text{A}_{664})$; Total carotenoids = $(1000\ (\text{A}_{470}) - 2.13\ \text{Chl a} - 97.63\ \text{Chl b})/209$.

2.4. Indices of Nitrogen Nutrition Status and Nitrogen Use Efficiency

The Nitrogen Use Efficiency was calculated as Nitrogen Utilisation Efficiency (NUE) (g g^{-1}) as the unit shoot dry weight per unit of up-taken N, as suggested by Moll et al. [32].

Indices of nitrogen (N) status were calculated as follows. The N uptake (Nupt) (g m^{-2}) was expressed as N units per area unit. The reduced-N ($\text{g } 100\ \text{g}^{-1}$ of Nupt) was expressed as the Nrid to Nupt ratio, Nrid being calculated as the difference between total N and N-nitrate concentration. The Specific Leaf Nitrogen (SLN) (g m^{-2}) was calculated as leaf N content per unit leaf area.

2.5. Gas Exchange Measurements

Two days before harvesting, the leaf exchange measurements were carried out. Photosynthetic rate (An), stomatal conductance to H_2O (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) were measured using the portable photosynthesis systems for gas exchange measurements Li-6400XTF (LI-COR Inc., Lincoln, NE, USA) on a fully expanded single leaf of 6 randomly selected plants per experimental unit. The photo-synthetically active radiation was kept at $1500\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$, the leaf temperature at 20.0°C , and the CO_2 concentration at $400\ \mu\text{mol mol}^{-1}$ of air.

2.6. Statistical Analysis

A two-way analysis of variance was performed using the SAS/STAT® Statistical Analysis Software (SAS, Cary, NC, USA). The least significant difference (LSD) test ($p = 0.05$) was used to establish differences between means.

3. Results

3.1. Yield, Growth, and Morphology of Spinach Leaves

The yield, growth, and morphology of spinach leaves affected by nitrogen fertilisation rate (N) and biostimulant application (B) are reported in Table 1.

The fresh weight of leaves, consistently with the yield, increased with increasing N in interaction with the B treatment (Figure 1A). Specifically, at the N14-fertiliser rate, the legume-PH-treated plants showed the highest value of the fresh weight/yield of leaves (3.28 g per plant; 3.10 kg m^{-2}), which was +16% higher than in the control and in the meat-PH-treated plants; at the N8 level, the meat-PH-treated plants reached a fresh weight of leaves +19% higher than in the untreated and legume-PH-treated plants; and in the N2 treatment, a lower leaf fresh weight was found in the meat-PH-treated plants than in the legume-PH plants (Figure 1A).

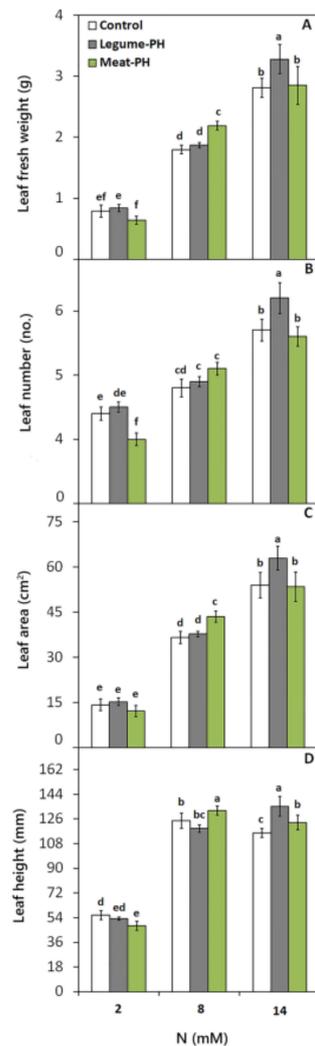


Figure 1. Effect of nitrogen doses and foliar application of biostimulants on fresh weight (A), number (B), area (C), and height (D) of spinach leaves. Vertical bars (standard error) ($n = 6$) with different letters are significantly different according to the LSD test ($p = 0.05$).

Table 1. Yield, growth, and bio-morphological features of spinach leaves as affected by nitrogen nutrition and the foliar application of biostimulants.

Treatment	Yield (kg m ⁻²)	Fresh Weight (g)	Dry Weight (g)	Number (no.)	Area (cm ²)	Height (mm)	Dry Matter (g kg ⁻¹)	Specific Leaf Area (cm ² g ⁻¹ dw)	h° (-)	L* (-)	Chlorophyll			
											a	b	Total	
												(µg g ⁻¹ dw)		
Nitrogen level (N)														
N2	0.71 c ⁽²⁾	0.75 c	0.07 c	4.3 c	13.8 c	52.40 b	101.5 a	181.2 c	113.5 b	70.8 a	431.9 b	100.3 b	532.7 b	
N8	1.85 b	1.95 b	0.16 b	5.0 b	39.2 b	125.3 a	82.7 b	246.4 a	124.3 a	67.1 b	675.2 a	186.9 a	857.6 a	
N14	2.82 a	2.98 a	0.26 a	5.9 a	56.7 a	124.8 a	86.8 b	222.5 b	127.7 a	66.1 b	714.7 a	170.8 a	885.3 a	
Biostimulant (B)														
Control	1.70 a	1.79 a	0.16 a	5.0 ab	34.9 a	98.8 a	92.0 a	211.3 a	122.3 a	67.7 a	597.8 a	142.0 b	739.9 b	
Legume-PH	1.89 a	2.00 a	0.17 a	5.2 a	38.6 a	102.5 a	86.9 a	225.3 a	121.7 a	67.9 a	638.1 a	172.8 a	807.4 a	
Meat-PH	1.79 a	1.89 a	0.16 a	4.9 b	36.3 a	101.2 a	92.2 a	213.6 a	121.4 a	68.4 a	585.9 a	143.2 b	728.4 b	
Significance ⁽¹⁾														
N	***	***	***	***	***	***	***	***	*	***	***	***	***	
B	ns	ns	ns	*	ns	ns	Ns	ns	ns	ns	ns	*	*	
N * B	*	*	*	*	*	**	Ns	ns	ns	ns	ns	ns	ns	

⁽¹⁾ n.s., *, ** and *** not significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. ⁽²⁾ Different letters within the column indicate significant differences at $p = 0.05$.

As a whole, the number of leaves per plant (Figure 1B) and the leaf area (Figure 1C) increased from the N2 to the N14 rate, in interaction with the B treatment. The height of leaves was also affected by N*B (Figure 1D). Specifically, in N14, the legume-PH-plants showed the highest number of leaves, leaf area, and height; in N8 fertilised-plants, meat-PH caused an increase in leaf area and height in comparison with untreated and legume-PH plants; and at the N2 level, the number of leaves (Figure 1B) and height (Figure 1D) sprayed with the meat-PH biostimulant was reduced in comparison with control and legume-PH-plants.

The dry weight of leaves increased with increasing N in interaction with the applied treatments (N*B) (Figure 2A). In the N14-fertiliser rate, the legume-PH-treated plants showed the highest leaf dry weight (0.28 g per plant), a value higher (+16%) in comparison with the control and meat-PH-treated plants. At the N8 level, the meat-PH-treated plants reached a dry weight of leaves (0.18 g per plant) higher (+21%) in comparison with untreated and legume-treated plants; and in N2 treatment, no effect of B emerged (Figure 2A)

3.2. Growth and Morphology of Spinach Roots

The growth and morphological traits of spinach roots are reported in Table 2.

Table 2. Growth and morphology of spinach roots as affected by nitrogen nutrition and by the foliar application of biostimulants.

Treatment	Fresh Weight (g)	Dry Weight (g)	Dry Matter (g kg ⁻¹)	Length (cm)	Diameter (mm)	Root/Shoot (-)
Nitrogen level (N)						
N2	0.31 ab ⁽²⁾	0.02 a	66.8 a	230.8 a	0.38 b	0.29 a
N8	0.26 b	0.02 a	72.6 a	190.8 b	0.44 ab	0.12 b
N14	0.36 a	0.02 a	68.4 a	166.2 b	0.45 a	0.09 b
Biostimulant (B)						
Control	0.33 a	0.02 a	68.8 a	190.4 ab	0.44 a	0.16 a
Legume-PH	0.32 a	0.02 a	69.6 a	210.9 a	0.41 b	0.18 a
Meat-PH	0.29 a	0.02 a	69.4 a	186.6 b	0.43 a	0.17 a
Significance ⁽¹⁾						
N	*	ns	ns	**	*	***
B	ns	ns	ns	*	*	ns
N * B	ns	*	ns	ns	ns	ns

⁽¹⁾ n.s., *, **, and *** not significant or significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. ⁽²⁾ Different letters within the column indicate significant differences at $p = 0.05$.

Fresh weight of roots was only affected by the N level: N14-plants showed the highest, N8-plants showed the lowest, and N2-plants showed an intermediate value.

The dry weight of roots was affected by the N*B interaction (Figure 2B). Specifically, the two PHs determined a higher root dry weight in comparison with the control, both at the N8- (+24%) and N2- (+21%) rates, while, at the N14-fertiliser rate, the meat-treated plants showed a root dry weight -27% lower than the control and the legume-treated plants (Figure 2B).

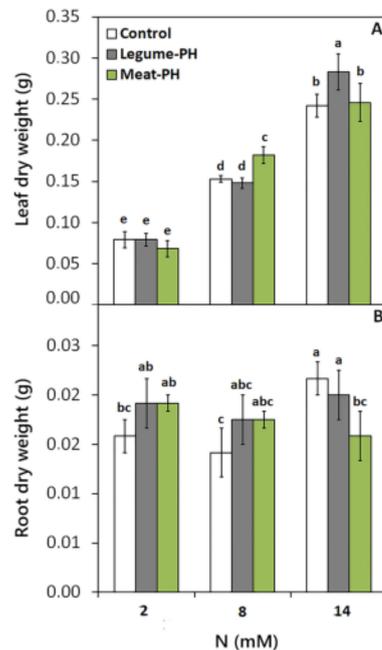


Figure 2. Effect of nitrogen doses and foliar application of biostimulants on the dry weight of leaves (A) and roots (B) of spinach leaves. Vertical bars (standard error) ($n = 6$) with different letters are significantly different according to the LSD test ($p = 0.05$).

The dry matter concentration of roots was not affected by the applied treatments, 69.3 g kg^{-1} on average.

The N2-plants had the longest roots, while the N8 and N14 had the shortest ones. The N14-roots had the greatest diameter, the N2-roots had the lowest, while the N8-ones had an intermediate value.

The meat- and legume-PH caused the shortest and longest roots, respectively. The legume-PH sprayed plants showed the thinnest roots, while the untreated ones and those treated by the meat-PH had the greatest root diameter.

The root/shoot ratio (on a dry basis) was affected only by the N rate: The N2-plants showed the highest, while the higher N rates had the lowest values.

3.3. Biophysical Features and Gas Exchange Measurements of Spinach Leaves

The biophysical features are shown in Table 1. The main indices of leaf appearance (h° , the hue angle; L^* , the lightness index), dry matter concentration (DM), and the specific leaf area (SLA) were only influenced by the N fertilisation levels.

Plants grown at the lowest N level developed leaves with the highest L^* and lowest h° values, while the plants fed with higher N levels showed the opposite. The leaf DM was the highest with the lowest N rate (N2) and the lowest with both higher N doses (on average, 85 g kg^{-1}). N2-leaves had the lowest, while N8-ones had the highest SLA.

For the concentration of total chlorophylls, chlorophyll a and b were affected by N, with a greater value in the plants fed by the higher N-levels, while the lowest was found in N2-plants. The total chlorophylls and chlorophyll b were also affected by B application; the legume-PH-treated plants had a concentration of chlorophyll b (+20%) and total chlorophylls (+10%) higher than those of meat-PH-treated-plants and the control plants.

All parameters of leaf gas exchange are shown in Table 3.

Table 3. Leaf gas exchange of spinach as affected by nitrogen nutrition and by foliar application of biostimulants.

Treatment	Photosynthetic Rate (An)	Stomatal Conductance to H ₂ O (gs)	Intercellular [CO ₂] (Ci)	Transpiration Rate (E)
	($\mu\text{mol m}^{-2} \text{s}^{-1} \text{CO}_2$)	($\text{mol m}^{-2} \text{s}^{-1}$)	($\mu\text{mol mol}^{-1}$)	($\text{mmol m}^{-2} \text{s}^{-1}$)
Nitrogen level (N)				
N2	5.25 b ⁽²⁾	0.16 b	328.8 a	1.52 c
N8	9.67 a	0.22 a	304.2 b	3.05 a
N14	10.63 a	0.22 a	280.9 c	1.89 b
Biostimulant (B)				
Control	8.57 a	0.19 a	301.9 a	2.05 a
Legume-PH	8.69 a	0.20 a	300.4 a	2.16 a
Meat-PH	8.30 a	0.21 a	311.6 a	2.24 a
Significance ⁽¹⁾				
N	***	**	***	***
B	ns	ns	ns	ns
N * B	*	ns	ns	ns

⁽¹⁾ n.s., *, **, and *** not significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. ⁽²⁾ Different letters within the column indicate significant differences at $p = 0.05$.

The effect of N*B on the photosynthetic rate (An) shows an increasing trend from 5.25 in the lowest N level to 10.15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of CO₂ in the highest N-fertilised treatments, on average. In particular, legume PH application improved An at the lowest N rate (+51%) in comparison with the meat-PH-plants and control-plants; with no difference found with B in N8; while, legume-PH showed a decrease in comparison with the meat-PH-plants (−13%) and the control- (−21%) plants (Figure 3). The stomatal conductance (gs), the concentration of CO₂ in intra-cellular spaces (Ci), and the transpiration rate (E) were only affected by the N level. The value of gs was the lowest in N2-plants and the highest in N14 (on average, 0.22 $\text{mol m}^{-2} \text{s}^{-1}$ of H₂O), E showed the lowest value in N2 and the highest in N8, Ci decreased with increasing N rates.

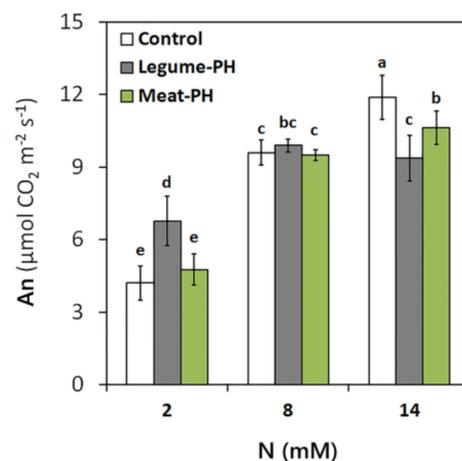


Figure 3. Effect of nitrogen doses and foliar application of biostimulants on the photosynthetic rate in spinach leaves. Vertical bars (standard error) ($n = 18$) with different letters are significantly different according to the LSD test ($p = 0.05$).

3.4. Nitrogen Nutritional Status and Nitrogen Use Efficiency of Spinach Leaves

The indices of the N nutritional status and N use efficiency in spinach plants are reported in Table 4.

Table 4. Indices of nitrogen nutrition status and of nitrogen utilisation efficiency (NUE) of spinach leaves as affected by nitrogen nutrition and by foliar application of biostimulants.

Treatment	N concentration (g kg ⁻¹ dw)	N Uptake (g m ⁻²)	Reduced-N (g 100 g ⁻¹ Nupt)	Specific Leaf Nitrogen (SLN) (g m ⁻² Leaf Area)	NUE (g dw g ⁻¹ Nupt)
Nitrogen level (N)					
N2	20.4 c	1.5 c ⁽²⁾	99.8 a	1.1 c	50.6 a
N8	39.5 b	6.0 b	95.5 b	1.6 b	25.5 b
N14	46.7 a	11.3 a	86.3 c	2.1 a	21.5 b
Biostimulant (B)					
Control	35.1 a	6.0 a	94.6 a	1.6 a	33.2 a
Legume-PH	36.2 a	6.6 a	94.4 a	1.6 a	31.2 a
Meat-PH	35.2 a	6.3 a	92.7 b	1.6 a	33.2 a
Significance ⁽¹⁾					
N	***	***	***	**	**
B	ns	ns	***	ns	ns
N * B	ns	*	**	ns	ns

⁽¹⁾ n.s., *, **, and *** not significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. ⁽²⁾ Different letters within the column indicate significant differences at $p = 0.05$.

N concentration, specific leaf nitrogen (SLN), and N uptake increased linearly with increasing N rates, this latter in interaction with B treatments (Figure 4A). Specifically, at the highest N rate (N14), plants sprayed with the legume-PH showed the highest value (12.5 g N m⁻²). On the contrary, at the N8 dose, plants treated with meat-PH (6.8 g m⁻² of N) showed a higher value of N uptake than that of untreated plants and those treated with legume-PH; no effect of B emerged at the N2 rate (Figure 4A).

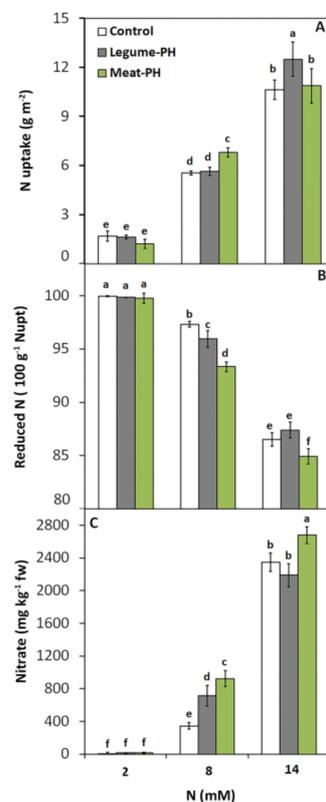


Figure 4. Effect of nitrogen doses and foliar application of biostimulants on N uptake (A), reduced-N (B), and nitrate concentration (C) of spinach leaves. Vertical bars (standard error) ($n = 9$) with different letters are significantly different according to the LSD test ($p = 0.05$).

The reduced-N content decreased with increasing N levels, in interaction with B treatments (Figure 4B). In particular, the meat-PH application determined a lower value of the reduced-N in comparison with the legume-PH-plants and especially the untreated ones, at both the N8 and N14 rates (Figure 4B).

N2-plants showed the highest NUtE, while the highest N-fertilised plants showed the lowest (on average, 23.5 g dw g⁻¹ Nupt).

3.5. The Mineral Status of Spinach Leaves

Anion and cation concentrations as individual and total are reported in Table 5.

The leaf concentration of total anions and all individual anions were reduced by increasing the N fertilisation rate. The accumulation of Cl and PO₄, the most abundant anions, followed a rising trend from the N2- to N14-rates, whereas the highest accumulation of SO₄ occurred in N8-plants. The SO₄ leaf concentration was higher (+14%) in plants treated with meat-PH than untreated plants and legume-PH-ones.

The NO₃ concentration increased with increasing the N rates, in interaction with B treatments (Figure 4C). In particular, the meat-PH-treated plants accumulated a higher value in comparison with the legume-PH plants and especially the un-treated ones, both at the N8 and N14 rates (Figure 4C).

The leaf concentration of K decreased, while Na and Mg increased, with the increase in the N dose. The Ca concentration was affected by the applied treatments. The N2-plants showed the highest value of Ca, while the N8- and N14-levels were the lowest. The plants sprayed with the legume-PH had a lower value in comparison with untreated and meat-PH-treated plants.

Neither the N fertilisation rate nor PH-biostimulant application or their interaction had a significant effect on the total cation concentration, being on average 122 g kg⁻¹.

Table 5. Mineral status of spinach leaves as affected by nitrogen nutrition and by foliar application of biostimulants.

Treatment	Anions					Cations				
	Total	Cl	PO ₄	SO ₄	NO ₃	Total	Na	K	Mg	Ca
(g kg ⁻¹ dw)										
Nitrogen level (N)										
N2	185.6 a ⁽²⁾	117.7 a	54.4 a	13.4 b	0.2 c	125.0 a	16.5 c	89.9 a	6.1 c	14.2 a
N8	103.4 b	51.9 b	25.9 b	16.7 a	8.8 b	121.9 a	19.8 b	81.6 b	8.2 b	12.3 b
N14	82.3 c	26.6 c	17.6 c	9.7 c	28.3 a	119.6 a	34.9 a	62.0 c	10.8 a	11.9 b
Biostimulant (B)										
Control	123.1 a	67.4 a	32.0 a	13.0 b	10.8 c	123.0 a	23.7 a	77.9 a	8.5 a	13.0 a
Legume-PH	121.7 a	62.4 a	33.0 a	12.2 b	12.5 b	120.3 a	23.2 a	76.7 a	8.8 a	12.0 b
Meat-PH	128.5 a	66.5 a	33.0 a	14.6 a	14.4 a	124.9 a	24.3 a	79.0 a	8.3 a	13.4 a
Significance ⁽¹⁾										
N	***	***	***	***	***	ns	***	***	***	***
B	ns	ns	ns	**	***	ns	ns	ns	ns	*
N * B	ns	ns	ns	ns	**	ns	ns	ns	ns	ns

⁽¹⁾ n.s., *, **, and *** not significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. ⁽²⁾ Different letters within the column indicate significant differences at $p = 0.05$.

3.6. Antioxidant Compounds in Spinach Leaves

The concentration of phenols, carotenoids, vitamin C, and its components, ascorbic (AA) and the de-hydro-ascorbic (DHAA) acids, in spinach leaves, are reported in Table 6.

Table 6. The concentration of phenols, carotenoids, vitamin C, and its components—ascorbic (AA) and de-hydro-ascorbic (DHAA) acids—in spinach leaves as affected by nitrogen nutrition and by foliar application of biostimulants.

Treatment	Phenols ⁽³⁾	Carotenoids	Vitamin C	DHAA	AA
	(g.a.e. mg 100 g ⁻¹ dw)	(mg 100 g ⁻¹ dw)	(mg 100 g ⁻¹ fw)		
Nitrogen level (N)					
N2	1674 a ⁽²⁾	51.9 c	26.6 a	22.5 a	4.1 a
N8	1139 b	58.5 b	23.7 a	21.8 a	1.9 b
N14	605 c	66.2 a	21.5 a	19.6 a	1.9 b
Biostimulant (B)					
Control	1152 a	58.4 a	25.3 a	22.3 a	2.9 a
Legume-PH	1103 a	59.7 a	23.7 a	21.5 a	2.2 a
Meat-PH	1163 a	58.5 a	22.0 a	20.1 a	2.7 a
Significance ⁽¹⁾					
N	***	***	ns	ns	**
B	ns	ns	ns	ns	ns
N * B	*	ns	ns	ns	ns

⁽¹⁾ n.s., *, **, and *** not significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. ⁽²⁾ Different letters within the column indicate significant differences at $p = 0.05$. ⁽³⁾ gallic acid equivalent.

As a whole, the concentration of phenols decreased with increasing the fertilisation rate, in interaction with B (Figure 5). Specifically, in an N2 fertilisation level, the plants sprayed with the legume-PH-biostimulant showed a reduction (−8%), while the meat-PH-treated plants showed an increase in the phenolic concentration (+9%) in comparison with the untreated ones (Figure 5).

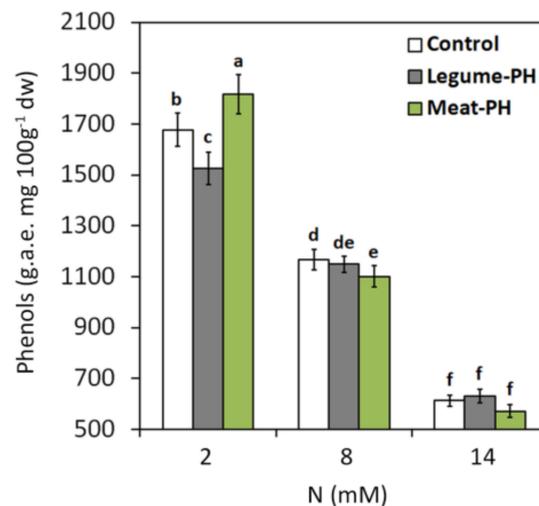


Figure 5. Effect of nitrogen doses and foliar application of biostimulants on phenols of spinach leaves. Vertical bars (standard error) ($n = 9$) with different letters are significantly different according to the LSD test ($p = 0.05$).

The concentration of total carotenoids was only affected by N treatment, which increased with increasing the N rates. Vitamin C and its components were not affected by treatments, except for the AA concentration which was highest in N2 plants.

4. Discussion

4.1. Effect of Foliar Application of Biostimulants and Nitrogen Nutrition on Growth and Yield

Biomass accumulation in plants is a remarkably stable function of light intercepted by the canopy [33] and CO₂ transformation into dry matter via photosynthesis [34]. Without any light intercepted gradients by the canopy, the leaf area expansion along with canopy

architecture plays an important role in determining biomass accumulation [35,36]. The relationship between N supply and biomass accumulation relies on the interregulation of multiple plant physiological processes as well as C and N assimilation, and N uptake [37]. The effect of N uptake on growth passes through canopy expansion development and photosynthesis activity [38,39].

The spinach growth increased with increasing N supply concomitantly with an increase in N uptake, N concentration (Table 4), and leaf expansion (Table 1) as well as the enhancement of the photosynthetic rate at canopy level, due to the increase in photosynthetically-active enzymes (i.e., the RuBisCo), as shown by the specific leaf nitrogen (SLN) (Table 4). The SLN, which is the amount of N per leaf area unit, is well known to be correlated to the content of photosynthetic pigments, the activity of photosynthetic enzymes, and photosynthetic rate [40–42].

In light of the following evidence, it can be clearly assumed that N2 is a deficient N level as inferred from the highest root:shoot ratio (Table 2), indicative of a convey of the assimilates to root growth to the detriment of shoot growth. Moreover, the N2 rate reduced growth, yield, CHLs, and leaf expansion (Table 1), along with the N concentration, N uptake, and SLN (Table 4). The deficient N condition compromised the photosynthetic CO₂ assimilation (A_n), with a lower gas exchange rate (g_s) (Table 3) and enhanced CO₂ concentration in intracellular spaces (C_i; Table 3), indicative of the lower demand for CO₂. The N14 rate could be considered as an optimal N level. It induced an optimal N nutrition by enhancing SLN, N concentration, and N uptake (Table 4), despite maintaining unchanged the NUtE compared with N8. As a consequence, the promoted A_n (Table 3), CHLs and leaf expansion (Table 1), resulted in improved growth and yield of N14 plants (Table 1). The N8 treatment has to be considered as a sub-optimal N level since it showed growth and yield lower than those of N14, accumulating lower nitrate and higher N-reduced percentage (Tables 4 and 5).

In this research, the effect of the protein hydrolysates (PHs) on fresh weight/yield was strictly related to N fertilisation (Figure 2), specifically in optimal (N14), sub-optimal (N8), and deficient (N2) conditions. The best fresh performance of spinach was in plants treated by the legume-PH when fed with the optimal N rate (N14) (Figure 1A), strictly in line with the leaf morphological characteristics (number, area, and height) and the related leaf expansion (Figure 1B–D). This latter is certainly pivotal in determining the increase in the photosynthetic activity at the canopy level and consequently of the dry matter accumulation.

The response of A_n with the legume-PH applied at the N14 level is unexpected and ‘paradoxical’ (Figure 3), since the dry mass accumulation is expected to be strictly correlated to the photosynthetic rate [43,44]. Considering that canopy photosynthetic assimilation is evaluated as a result of the compounding effect of the total leaf area (‘sink size’ CO₂) and photosynthetic rate per unit surface (‘sink intensity’ for CO₂) [45,46], in our case the instrumental observations made at the leaf scale (‘sink intensity’) do not allow for the easy extrapolation of unique information on the photosynthetic capacity of the entire canopy. Beyond the above-cited ‘paradoxical’ discrepancy, we can associate the performance in terms of dry weight accumulation (Figure 2A) to the enhancing of the photosynthetic rate by the legume-PH at the N14-rate, since the leaf expansion (‘sink size’) was significantly stimulated by that N*B combination (Figure 1B–D). This necessarily means that the CO₂ assimilation rate of the entire canopy was greatly favoured as well.

According to dry biomass partitioning, it seems that the meat-derived-PH provoked an inhibition of root growth (Figure 2B), which could have contributed to reducing its potential growth performance in comparison with the legume-derived-PH (Figure 2A). The response of the meat-PH on the root architecture (Table 2) could also help to explain the lack of positive growth performance of plants treated with this PH at N14 (Figure 2A). The meat-PH tends to reduce root elongation (Table 2), attributable to a higher concentration in glycine, which is the most prevalent amino acid (25% of the total free amino acids

and peptides) in the meat-PH (Table S2). Accordingly, a recent study carried out by Han et al. [47] proved the inhibitory effect of exogenous glycine on root elongation in pakchoi.

At sub-optimal N conditions (N8), the meat-PH sprayed plants had the highest fresh yield (Figure 1A), imputable to larger leaf expansion along with a greater height (Figure 1D), and area of leaves (Figure 1C). In general, the response N*B on growth (Figure 2A) and yield (Figure 1A) of spinach closely overlaps that of N uptake (Figure 4A). Specifically, in the case of the meat-PH-application in sub-optimal N conditions, the positive response on growth (Figure 2A) and yield (Figure 1A) is strictly imputable to an increase in N uptake (Figure 4A), leaf expansion (Figure 1C,D), and photosynthetic activity at the canopy level.

Under deficient N conditions, our study found no (legume-PH) or positive (meat-PH) effects on the PHs on the fresh (Figure 1A) or dry (Figure 2A) biomass of spinach leaves. These findings were quite different from those previously reported by several Authors, which affirmed that the PHs, in particular the legume-PH-Trainer[®], boosted the growth and/or yield even in deficient and/or sub-optimal N conditions in spinach and several greenhouse leafy vegetables, such as wild rocket, lettuce, and lamb's lettuce [23–26]. Specifically, the application of legume-PH-Trainer[®] on winter-spring-spinach grown for 60 days under variable N rates (0, 15, 30, and 45 kg ha⁻¹) boosted the growth (15 kg ha⁻¹) and productive response at low N availability (0, 15, and 30 kg ha⁻¹) [23]. In winter spinach, grown for 60 days in large sandy soil pots, the legume-derived-PH elicited a significant increase in yield at all N levels (0, 2.25, and 4.5 g m⁻² of N) [26]. Trainer[®] has also been reported to have high performance in other leafy species [28,29]. It is quite difficult to find the reason for these different results because many other variables come into play when comparing our conditions with those reported in the above literature (indoor vs. greenhouse, soil vs. soilless, form, and concentration of soil native N, form and concentration of N in the nutrient solution, and dose and the number of applications of PHs). Thus, we can only suppose that our growing conditions and/or cycle duration do not allow the examined PHs, and in particular, the legume-PH, to bring out their positive actions on the growth/yield of spinach in very limited N conditions.

The meat-PH-Isabion[®], chosen for its different aminogram from that of legume-PH-Trainer[®], has never been experimentally tested on vegetables before this work. Up to now, the only scientific data available are on a cut flower species (*Zinnia elegans* L.) [48], highlighting the well-known general capacity of the amino acids/peptides present in the PHs to stimulate plant growth by regulating the physiological activities of the plant.

The ability of PHs to affect root architecture is already well known [49,50]. In our case, the root architecture was affected by B, but to the extent to which it was strictly PH-type dependent, probably according to a diverse amino-acid profile (Table S2).

The positive effect of the legume-PH on root apparatus as well as the longest and thinnest roots (Table 2) could be ascribed to its richness in root hair promoting peptides (RHPPs) having a hormone-like, in particular, an auxin-like-activity. Among these RHPPs, tryptophan is the most prevalent (Table S2) and it -3-acetic acid (IAA), the phytohormone auxin involved in the root growth process (length, size, and density), usually promoting is the primary precursor in the biosynthesis of indole root elongation [51,52].

Our findings agree with previous papers reporting root morphological changes induced by Trainer[®] in several species, in terms of increase in root length [9,11,16].

The greatest root elongation along with the highest root thinning, experienced by the legume-PH-plants (Table 2) because of the tryptophan amino-acid (Table S2), implies a greater exposed surface area of roots, ensuring better access to nutrients (minerals) including N, as affirmed by a vast literature [10,53–55]

In our case, no increase in mineral (cation) uptake occurred in the presence of the tested PHs (Table 5), but an interesting increase in N uptake was observed according to a N*B-dependent pattern (Figure 4A).

A positive auxinic effect of the legume-PH on root morphology, although independent of the applied N dose (Table 2), clearly resulted in increased N uptake, especially at the N14 rate (Figure 4A). Thus, only in the case of the highest N availability, the root apparatus

of legume-PH-plants clearly was the most efficient at N uptake (Figure 4A), inducing high leaf expansion (Figure 1B–D), and probably also in the light interception and canopy photosynthetic activity. Consequently, the leaf dry (Figure 2A) and fresh (Figure 1A) yields were greatly favoured by this specific N*B combination. The B treatment effect emerged on the chlorophylls (CHLs). Their increase could induce better photosynthetic efficiency and lead to better plant performance. In our case, the foliar application of PH-legume in comparison with the meat-PH-biostimulant significantly improved the concentration of total CHLs, especially the secondary chlorophyll, CHL b (Table 1). This higher accumulation of CHLs in legume-derived-PH leaves is probably attributable to a higher content of primary amino acids as well as glutamate (Table S2). It should also be noted that glutamate is the precursor for chlorophyll synthesis in developing leaves [56].

The increase in the concentration of CHLs in the legume-PH-treated plants, despite occurring irrespective of the applied N dose (Table 1), resulted in an improvement in photosynthetic efficiency of the entire canopy, favoured only by the highest N availability (N14), thus contributing to boosting growth (Figure 2A) and yield (Figure 1A) in this specific N*B combination. Similarly, an increase in CHLs, at all N doses, was observed in legume-PH sprayed lettuce [24], lamb's lettuce [26], and wild rocket [25] baby leaves, and was seen to be strictly related to the boosting of yield, according to a supposed increase in photosynthetic activity.

The root architecture, which was positively regulated by the legume-PH application (Table 2), prompted N uptake in optimal N conditions (Figure 4A), which consequently stimulated the leaf expansion (Figure 1B–D). All these factors resulted in a general increase in light interception along with higher photosynthetic activity at the canopy level, thus in an increase in growth and yield (Figures 1A and 2A). In addition, the CHLs elicited by the legume-PH application (Table 1) contributed to the stimulation of light interception as well as to the increase in the photosynthesis process, growth, and yield in the highest N availability conditions (Figures 1A and 2A).

4.2. Effect of Foliar Application of Biostimulants and Nitrogen Nutrition on Mineral Profile, Indices of N Status and N Use Efficiency

The legume-PH sprayed in spinach [20,23] as in other leafy [21] and fruit [22] vegetable species has been proven to promote mineral uptake, especially of cations (Ca, K, and Mg). The so-called 'mineral acquisition response' of Trainer[®] has been proved in winter-spring-spinach at all N rates applied (0, 15, 30, and 45 kg ha⁻¹) (in the case of Ca) or at the highest N rate (in the case of Mg) [23].

Differently, in our study, the PHs appear to reduce the mineral (cation) uptake, thus the response of the legume-PH on Ca concentration or the absence of response to other cations (Table 5) turned out to be quite surprising. Likely, the growing conditions set in our experimentation did not allow the tested PHs, and notably, the legume-PH, to perform its well-known 'cation acquisition response', at any N rate. The high availability and readiness of nutrients in the NS supplied in our soilless growing system could probably have nullified the above-mentioned positive effect of PHs/Trainer[®].

In relation to the B effect on nitrate concentration, this study assesses the high general capacity to accumulate nitrate induced by the PHs/legume-PH-Trainer[®] and any ability to reduce nitrate (Table 5; Figure 4C). According to the specific literature on several leafy species, the application of the PH-Trainer[®], in any growing condition (cultivation system: soil, lysimeter, or soil pot; cultivation periods; and N levels) has been demonstrated to be ineffective in reducing the nitrate concentration, both in 60-day-winter-spring- [23] and in 30-day-spring [20] spinach, as well as in wild rocket [25] and lettuce [24] baby-leaves. The application of the legume-PH-Trainer[®] in several cases even determined an increase in nitrates in comparison with untreated plants, independently of the N dose supplied, in 60-days-winter-spinach [26], and other leafy species grown as baby-leaves, spring-rocket [25].

An overview of N*B response on N uptake (Figure 4A), reduced-N (Figure 4B), which represents the assimilation into organic forms of the inorganic N uptake, and nitrate accumulation (Figure 4C) can be useful in clarifying the N assimilation pattern. This latter proved to be B-dependent especially at higher N doses (N8 and N14) (Figure 4).

Significant inhibition of N assimilation occurred with the meat-PH, since a lower percentage of reduced N and a higher nitrate accumulation occurred in the meat-PH sprayed plants than the unsprayed ones (Figure 4). This was evident both in the case of a higher (N8) or similar (N14) amount of N taken up in comparison with the control (Figure 4A). Scientific evidence in root tips of cotton [57] indicated an inhibition action of glycine on the nitrate reductase activity, a key enzyme in the N/nitrate assimilation pathway of plants. Glycine, an abundant amino acid in meat-PH (Table S2), could have provoked an inhibition of the N assimilation pattern, regardless of the N level supplied. The meat-PH showed the highest capacity of accumulating nitrate at the highest N dose (2677 mg kg⁻¹ fw) (Figure 4C), but according to the maximum nitrate limits for fresh spinach, set by EU Regulation N. 1258/2011 (3500 mg kg⁻¹), this product is not hazardous.

The legume-PH application only caused a reduction in N assimilation under sub-optimal N conditions (N8) (Figure 4). While, in the highest N conditions, the legume-PH-treated plants, which showed a higher amount of N and the same percentage of reduced-N and nitrate concentration in comparison with the control (Figure 4), performed a slightly improved N assimilation pattern.

In terms of N assimilation, the legume-PH application seems to perform slightly better in optimal (N14) than in sub-optimal N conditions (N8). The plant origin PHs, as well as Trainer[®], are very rich in glutamate and aspartate (Table S2), involved in the plant N metabolism, improving the activity of the main enzymes of this process (NR, NiR, GS) [15]. Therefore it is possible to suppose that the legume-PH-Trainer[®] differentially affected, in an N-dependent pattern, an up-regulation of genes involved in nitrate/ammonium/amino-acid transporters [15] or an up-regulation of genes of enzymes involved in N metabolism [55,58], along with a slight stimulating effect on the activity of the above-cited enzymes [54]. However, the positive effect of the legume-PH in improving the N assimilation process did not come out in a preponderant way, probably because the applied level N14 (196 mg L⁻¹ of N) was slightly excessive.

In regards to the NUtE, it was only affected by the N nutrition (Table 4) and, as expected, spinach plants grown in limited N availability, were the most efficient ones at using N. While, the B treatment did not affect the NUtE at any N dose (Table 4) and this lack of a (positive) effect confirmed a scarce capacity of PHs (Trainer[®], 3.3 mL L⁻¹) to affect N conversion into its organic form. In our experimentation, it is quite likely that the growing conditions and/or the biostimulant application dose/number during the crop cycle did not allow the performance in terms of NUtE of PHs/Trainer[®] to come out.

4.3. Effect of Foliar Application of Biostimulants and Nitrogen Nutrition on the Bio-Physical and Antioxidant Profile of Spinach Leaves

The main physical traits (colour indices, dry matter-DM, and specific leaf area-SLA) of spinach leaves were affected by N rates, but are not affected by B treatment, thus the PHs at any N level did not modify either the colour or thickness/consistency of the soilless-spinach product grown for 45 days (Table 2). In relation to the leaf colour, our results were in agreement with Carillo et al. [23] where Trainer[®] did not improve the colour of the winter-spring spinach leaves, at any N doses. The literature findings on the effect of PHs/legume-PH-Trainer[®] on the physical profile of spinach leaf are quite contrasting. Trainer[®] improved the greenness and consistency of the winter-greenhouse-lettuce, independently of N levels [24], while the leaves of Trainer[®]-treated spring-wild rocket grown in a lysimeter under a plastic tunnel [25] showed a lower consistency, irrespective of N rates.

When plants face stressful conditions, the reactive oxygen species (ROS) tend to increase, and the (enzymatic and non-enzymatic) antioxidant defence system could work to protect the plants against oxidative stress damage. Plants subjected to N-nutrition

deficiency accumulated phenols (as non-enzymatic antioxidants) in large amounts (Table 6; Figure 5), by an overproduction of molecules operating anti-oxidative defence mechanisms in the cells for scavenging of ROS [59].

Under N limiting conditions the meat-PH promoted phenolic biosynthesis more than legume-PH (Figure 5). This response could be attributable to the composition of the biostimulants. The higher concentration of glycine in the meat-PH (Table S2) could have elicited phenolic biosynthesis. According to a study on lettuce [60], glycine induced a higher activity of phenylalanine ammonia-lyase (PAL) enzyme, and higher production of phenylalanine; it is worth noting that PAL catalyses the first steps in the reaction sequence of the phenylpropanoid pathway and phenylalanine is a precursor of phenols [61].

The B treatment did not affect the level of carotenoids, vitamin C, or its components, ascorbic (AA) and the de-hydro-ascorbic (DHAA) acids, essential in the defence against environmentally-induced oxidative stress. Similarly, the legume-PH-Trainer[®] did not affect carotenoids in winter-spinach [23], or on vitamin C in winter-spinach and spring lamb's lettuce [24]. However, the literature findings concerning the antioxidant profile are also not unique. In fact, the legume-PH-Trainer[®] also caused an increase in carotenoids in (lysimeter) winter lettuce [27] and (lysimeter) spring wild rocket [25], regardless of the N dose.

5. Conclusions

The study, by focusing on the morphological, productive, physiological, and qualitative response of spinach, highlights that the foliar application of PHs, characterised by a different amino-acid composition, differently boosted the growth and productive response according to a specific N level in the nutrient solution.

The best performance in terms of growth and yield in plants sprayed with the legume-PH occurred at the optimal N level (N14) and with the meat-PH at the sub-optimal N rate (N8), according to the amount of N uptake and relative boosted leaf expansion in the corresponding N*B combination.

The effect of the legume-PH on modulating the architecture of roots and chlorophyll concentration is confirmed by the present work. This positive effect is only evident under optimal N conditions, inducing an improvement of N uptake, leaf expansion, and photosynthesis activity at the canopy level.

Under the present experimental conditions, the properties of the legume-PH to boost the growth and yield of spinach in deficient and sub-optimal conditions are not confirmed. The N assimilation pattern was only slightly improved by the legume-PH under optimal N conditions. The meat-PH was not efficient in improving N assimilation, reaching the highest nitrate accumulation in the highest N dose, even if the value is below the limits fixed for fresh spinach by the EU.

In general, no capacity of PHs to reduce nitrate accumulation or to improve the cation acquisition response, the CO₂ assimilation at the leaf level, the NUtE, the physical (colour, consistency), or the antioxidant profile of spinach leaves emerges.

These findings suggest that spinach response to PHs has to be defined according to different specific parameters (PH application dose/N nutrition level/growing system), which need to be further investigated.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy12010036/s1>, Table S1: Experimental Design; Table S2: Amino-acid composition of biostimulants as Protein Hydrolysates from plant (Trainer[®]) and animal (Isabion[®]) source.

Author Contributions: Conceptualisation, A.B., G.C. and A.E.; methodology, A.B., G.C.; software, A.E.; validation, A.E.; formal analysis, C.L.; investigation, A.B., C.L. and G.C.; resources, A.E.; data curation, A.B. and A.E.; writing—original draft preparation, A.B.; writing—review and editing, G.C. and A.B.; visualisation, A.B. and G.C.; supervision, G.C. and A.E.; project administration, A.E.; funding acquisition, A.E. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are available from the corresponding Authors upon request.

Acknowledgments: The Authors are grateful to Paolo La Rotonda for the valuable help in the experimental trial and in the laboratory analysis.

Conflicts of Interest: The Authors declare no conflict of interest.

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