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Nitrogen Use and Uptake Efficiency and Crop Performance of Baby Spinach (*Spinacia oleracea* L.) and Lamb's Lettuce (*Valerianella locusta* L.) Grown under Variable Sub-Optimal N Regimes Combined with Plant-Based Biostimulant Application



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Abstract: An optimized nitrogen (N) fertilization may have a positive effect on leafy vegetables by increasing growth, yield and nutrient content of plants. Nevertheless, crop performance must be coupled with an increase in Nitrogen Use Efficiency (NUE) in order to limit external N inputs and to avoid N surpluses associated with environmental and health problems. The aim of the current study was to assess the effects of a legume-derived plant hydrolysates (LDPH; Trainer[®]) and N fertilization levels (0, 2.25 and 4.5 g N m⁻² for spinach and 0, 2.5 and 5.0 g N m⁻² for lamb's lettuce; N0%, N50%, N100%, respectively) on agronomical, biochemical, qualitative responses and NUE of these two important greenhouse leafy vegetables. Spinach and lamb's lettuce were sprayed four times during the growing period (at a concentration of 4 mL L^{-1} of LDPH). In baby spinach, the LDPH application elicited a significant increase at the three levels of N fertilization: +16.8%, +14.2%, and 39.4% at 0, 2.25 and 4.5 g N m⁻², respectively. Interestingly, in lamb's lettuce, the N50% plants treated with LDPH reached similar values of marketable yield in comparison to treated and non-treated plants under N100% conditions. The presumed mechanism involved in the enhancement of yield response in the two leafy greens could be associated to a better activity of the photosystem II (higher SPAD index), biochemical (higher content of chlorophyll a, b and total) and leaf nitrate status. The foliar application of LDPH produced a major fortification in lipophilic and hydrophilic antioxidant activities (+11.6 and 6.3% for spinach and lamb's lettuce, respectively). The biostimulant application also improved N-use efficiency and N-uptake efficiency compared to untreated plants: +17.8% and +18.8%, and +50% and +73.3%, for spinach and lamb's lettuce, respectively.

Keywords: N fertilization; nitrogen use efficiency; antioxidant activity; leaf quality; protein hydrolysate; *Spinacia oleracea* L.; sustainable agriculture; *Valerianella locusta* L.

1. Introduction

Chemical fertilizers, especially nitrogen (N), are basically the main input for boosting yield and concomitantly one of the most expensive inputs in terms of economics and environment. Many crops require high amounts of this element to maximize yield [1], but N fertilization requires a particular care



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because it is involved in many environmental and health risks [2]. The main environmental impacts of N can be summarized in the contamination of surface and groundwater resources and greenhouse gases emissions [3,4]. The effects on human health strongly depend on the accumulation of nitrate in edible plant tissue; when nitrate is reduced to nitrite in human body it can cause methemoglobinemia, which is dangerous to children [5–7]. Moreover, nitrite can also react with several chemical compounds (amines and amides), producing N-nitrous compounds, known as probably carcinogenic to humans [7–9].

On the other hand, it is certainly necessary to adapt the correct management of N fertilization through a balanced application of the elements in order to reach the right dose, nevertheless by choosing the convenient chemical form and application time. Moreover, another possible perspective is to raise the nitrogen use efficiency (NUE) that is linked to the capacity of plants to uptake nutrients, nevertheless to their systems of transport, storage and mobilization and to the N loss into the environment [10]. NUE is expressed as the harvestable yield per the amount of available N in the soil or per N supply [11–13].

In recent years, the approach to improve NUE, passed through biotechnology and plant breeding strategies, but currently it is necessary to evaluate alternative means, which are environmentally friendly, such as the use of plant biostimulants. These products can be used to complement fertilizers in order to reduce the inputs and increase the NUE [14]. They act in several ways: on plant growth, physiology, carbon and nitrogen metabolism, productivity, product quality and tolerance to abiotic stress [14]. Moreover, some studies found that plant biostimulants, particularly commercial legume-derived proteins, have a great potential to reduce nitrate accumulation in the leaves of some green leafy vegetables [15]. It is of a major result because these crops have the genetic predisposition to greatly accumulate nitrate in their leaves [16]. It is known that the different crops ability of nitrate accumulation can depend on different localization and activity of nitrate reductase (NR) [17,18], but also on unbalanced relationship between nitrate uptake and NR activity, as well as the different capacity of uptake, translocation and accumulation of plants [16]. Moreover, this behavior is worsened by specific environmental conditions, where nitrate accumulation increases at low solar radiation [19–21].

In addition, the cultivation in protected environment causes a similar effect, because the plastic film cover reduces the solar radiation transmission. Likewise, the photoperiod and growing period affect nitrate accumulation; in fact, both conditions are matched to conditions of low solar radiation.

Green leafy vegetables play a key part in the economical market of many countries, both in the Mediterranean area and Nord-Europe, because they are widely used in ready-to-eat salads. In addition to typical leafy greens such as lettuce and rocket, also spinach and lamb's lettuce are largely spreading. Italy is a leading country in the European production of green leafy vegetables destined for the ready-to-eat market, with more than 150 kilotons harvested per year in protected conditions [22,23]. Among these crops, spinach is the less-efficient in terms of N uptake and use [24], requiring high rates of fertilization to grow well and reach higher leaf quality (dark green leaves) [25].Instead, lamb's lettuce is still under-studied, and its behavior regarding NUE under different N regimes is unknown.

Previous studies regarding vegetable crops including leafy greens have documented that the application of plant biostimulants triggers several molecular and physiological processes, accompanied by improvement in growth, yield, quality, NUE and tolerance to abiotic stress [22,26–37]. The capacity of biostimulants to improve NUE is the utmost reason for which they are spreading in the market, considering their economic and environmental motives [38]. However, relatively few researches regarding biostimulants effects on plants grown under sub-optimal N conditions are available [33,35,39–41], especially about green leafy vegetables. The reduction of N inputs in leafy vegetables is very important, both for containing the phenomenon of nitrate accumulation in leaves and for reducing the economic and environmental impacts of fertilization. Di Mola et al. [42] reported that the foliar application of different biostimulants (in particular seaweed extract and legume-derived hydrolysate protein) on greenhouse baby lettuce boosted plant growth, mainly in sub-optimal N fertilization. Furthermore, in baby rocket cultivated under greenhouse conditions, Di Mola et al. [43]

found that the application of plant-based biostimulants boosted the marketable yield at low N levels compared to the control.

The aim of this study was to assess the effect of foliar application of legume-derived protein hydrolysates on N demand and uptake efficiency of two important leafy greens. Therefore, two experiments were carried out for evaluating the possible beneficial effects of a plant-derived protein hydrolysates applied on greenhouse spinach and lamb's lettuce grown under variables N conditions, in order to depict its influence on NUE, yield and leaf quality.

2. Materials and Methods

2.1. Experimental Setting, Leafy Vegetables Tested and Cultural Practices

Two consecutive experiments were carried out in a plastic tunnel during winter 2018/2019 and spring 2019 seasons at the experimental site "Gussone Park" of the Department of Agricultural Sciences (40°48.870′ N; 14°20.821′ E; 70 m a.s.l.), University of Naples Federico II, Italy. The two tested crops were cultivated in large pots (diameter 0.70 m and height 0.60 m) filled with sandy soil, with the following physical and chemical proprieties: pH 7.4, 2.5% organic matter, 0.9 g kg⁻¹ total N (Kjeldhal method), 252.6 mg kg⁻¹ P₂O₅ and 490.9 mg kg⁻¹ of K₂O.

For the first experiment (Winter 2018/19), baby spinach (*Spinacia oleracea* L. cv. Platypus RZ F1, Rijk Zwaan, Bologna, Italy), a widely spread cultivar in Italy with dark green leaves, was sown on January 17th (1000 seeds per square meter) and harvested on March 12th. While for the second experiment (Spring 2019), lamb's lettuce (*Valerianella locusta* L. cv. Princess HM CLAUSE, Torino, Italy) was sown on March 26th (1200 seeds per square meter)—this cultivar is characterized by deep green leaves and a high adaptability to different growing seasons—and harvested in five different dates from May 10th till the 25th, upon reaching the marketable size according the different treatments. The germination time was 8 and 10 days after sowing and the plant densities after germination were 900 and 1100, for spinach and lamb's lettuce, respectively. For both crops, there were no differences between the treatments in terms of plant density.

Considering the chemical composition of soil, no phosphorus or potassium was given to either crop; while N was added as ammonium nitrate (34%) in a single application 27 and 20 days after the sowing, for spinach and lamb's lettuce, respectively. Water was not a limiting factor; the crop evapotranspiration was calculated with the Hargreaves method and the deficit was fully restored by sprinkler irrigation.

2.2. Experimental Design, Nitrogen Fertilization and Biostimulant Application

A factorial combination of three nitrogen fertilization levels and two biostimulant applications (treated and non-treated control) distributed in a randomized complete-block design were adopted for both experiments. Each treatment was replicated three times accounting a total of 18 pots (3 N levels \times 2 biostimulant applications \times 3 replicates).

The optimal nitrogen dose was calculated based on the balance method that considers all inputs and outputs. For the first experiment (spinach) N levels were: optimal dose (N100%) -4.5 g m⁻², sub-optimal dose (N50%) -2.25 g m⁻² and no fertilization (N0%). While for the second experiment (lamb's lettuce) N levels were: optimal dose (N100%) -5.0 g m⁻², sub-optimal dose (N50%) -2.5 g m⁻² and no fertilization (N0%).

The plant-based biostimulant used for both green leafy vegetables was a legume-derived protein hydrolysates, promoted as Trainer [®] by Italpollina S.p.A. The legume-derived PH biostimulant obtained through enzymatic hydrolysis contains 75% of free amino acids and peptides, 22% of carbohydrates and 3% of mineral nutrients. The detailed aminogram of the product along with the phenolics, flavonoids, and elemental composition were reported in detail by Rouphael et al. [22]. For both crops, the treated plants were sprayed four times at 21, 27, 33 and 39 days after sowing, at a concentration of 4 mL L⁻¹. Untreated control spinach and lamb's lettuce plants were only sprayed with water. Each pot was

sprayed with a solution volume of 38.5 mL (=1000 L ha⁻¹) corresponding to a biostimulant application rate of 0.000154 mL per pot (=4 L of biostimulant per ha).

2.3. Marketable Yield and Sampling

In both experiments, the whole area of all the pots at harvest was cut and leaves were weighed in order to measure the marketable fresh yield. In addition, a representative sub-sample of each replicate was dried in a forced air oven at 70 °C and then weighed in order to determine dry weight and then to calculate leaf dry matter content and subsequently used for N content determination (total N and nitrate) by chemical analysis. For qualitative analysis, fresh samples were also collected from each replicate and conserved at -80 °C.

2.4. Nitrogen Determination, N-use Efficiency and Uptake Efficiency

The Kjeldahl method [44] was used to determine the concentration of N in dried leaves samples that were mineralized with sulfuric acid, while nitrate content was determined using the Foss FIAstar 5000 continuous flow Analyzer (FOSS analytical Denmark).

Nitrogen use efficiency (NUE) was calculated by dividing yield by N application dose plus the available N in the soil and expressed as ton per kg. In addition, N uptake efficiency was determined as the ratio between N content in the leaves and N application dose and it was expressed as kg kg⁻¹.

2.5. Leaf Quality: Antioxidant Activity and Compounds, Chlorophyll Content and SPAD Index

Lipophilic (LAA) and hydrophilic (HAA) antioxidant activities were determined using the protocols of Re et al. [45] and Fogliano et al. [46], respectively. The two extract fractions, lipophilic and hydrophilic, were measured by the means of a Hach DR 2000 spectrophotometer at 734 and 505 nm, respectively.

The Kampfenkel et al. [47] method was used to determine ascorbic acid spectrophotometrically. A wavelength of 525 nm was set in order to measure the absorbance of the extract. Total phenols were also assessed spectrophotometrically, and the absorbance solution was detected at 765 nm, based on the Singleton et al. method [48].

Leaves chlorophyll content was measured spectrophotometrically: the first step was the extraction of fresh material by ammoniacal acetone as described by Wellburn [49], then the absorbance of solutions was measured at 662 and 647 for chlorophyll a and b, respectively.

The soil plant analysis development (SPAD) index was measured at harvest, on 15 leaves by replicate, using a portable SPAD-502 chlorophyll meter.

2.6. Statistical Processing

In both experiments, a two-way ANOVA was conducted using the SPSS 21 software package. Duncan's Multiple Range Test (DMRT; significance level 0.05) was adopted for mean comparisons on each of the independent measured variables.

3. Results

3.1. Marketable Yield and SPAD Index

The effects of both tested factors (N fertilization rates and biostimulant application) on marketable fresh yield and SPAD index were reported in Figure 1A,B and Figure 2A,B, where the relevant F and P values and the degrees of freedom are reported in Table 1.

	Spinach		Lamb's Lettuce	
	Yield	SPAD Index	Yield	SPAD Index
Nitrogen × Biostimulant				
f value	25.198	9.580	6.195	7.554
Degrees of freedom	17	17	17	17
<i>p</i> value	0.001	0.01	0.05	0.01

Table 1. Analysis of variance of marketable fresh yield and SPAD index of spinach and lamb's lettuce (Figure 1A,B and Figure 2A,B).

In particular, the marketable yield of baby spinach was positively influenced by N fertilization, but it was further boosted by biostimulant application (Figure 1A). The LDPH application elicited a significant increase at all the levels of N: +16.8%, +14.2%, and 39.4% at 0, 2.25 and 4.5 g N per square meter, respectively.

As with baby spinach, the marketable yield of lamb's lettuce increased with higher N dose and it was positively affected by biostimulant foliar application (Figure 1B). However, no significant difference was recorded between LDPH-treated and non-treated control plants at the higher N fertilization level (N100%). Interestingly, the N50% plants treated with LDPH reached significantly similar values of marketable yield in comparison to treated and non-treated plants under N100% conditions.



Figure 1. Marketable yield of baby spinach (**A**) and lamb's lettuce plants (**B**) as affected by nitrogen (N) fertilization levels (0, 2.25 and 4.5 g N m⁻² and 0, 2.5 and 5.0 g N m⁻²; N0%, N50%, N100%, respectively) and biostimulant application (non-treated control and LDPH: Legume-derived protein hydrolysates). Different letters indicate significant differences according to the DMR test (p < 0.05). Vertical bars indicate ± standard error of means.

The SPAD index statistically increased with the higher availability of N and also with the foliar application of LDPH in both baby spinach (Figure 2A) and lamb's lettuce (Figure 2B). The average increase of the SPAD index of fertilized and sprayed spinach plants was 8.6% compared to fertilized unsprayed plants. At 0 g N per square meter, the SPAD index of spinach plants treated with LDPH was +7% compared to untreated N0% plants. Finally, for lamb's lettuce the SPAD index increases due to biostimulant application were less marked: +5.2% and +2.9% for fertilized (N50% and N100% plants) and non-fertilized plants (N0%).

SPAD index

0



0

N50%

N0%

N100%



3.2. N-Use and Uptake Efficiency

N0%

N50%

N100%

The results regarding the two efficiency parameters: N use efficiency and N uptake efficiency in baby spinach and lamb's lettuce are presented in Table 2. In both leafy vegetables, significant effects were noted on N use efficiency with both N and biostimulant treatments, but not the $N \times B$ interaction, whereas N uptake efficiency was only affected by foliar biostimulant application (Table 2).

Table 2. Nitrogen use and uptake efficiency of baby spinach and lamb's lettuce plants as affected by
nitrogen (N) fertilization levels (0, 2.25 and 4.5 g N m $^{-2}$ and 0, 2.5 and 5.0 g N m $^{-2}$; N0%, N50%, N100%,
respectively) and biostimulant applications (control and LDPH: Legume-derived protein hydrolysates).

	Spinach		Lamb's Lettuce	
Treatments	N-Use Efficiency	N-Uptake Efficiency	N-Use Efficiency	N-Uptake Efficiency
	(t kg ⁻¹)	(kg kg ^{-1})	(t kg ⁻¹)	(kg kg ⁻¹)
Fertilization				
N0%	0.35 a	0.14	0.49 a	0.20
	(0.29 - 0.40)	(0.29 - 0.45)	(0.48 - 0.56)	(0.15 - 0.26)
N50%	0.31 ab	0.17	0.33 b	0.21
	(0.25 - 0.36)	(0.25 - 0.36)	(0.25 - 0.34)	(0.12 - 0.22)
N100%	0.25 b	0.14	0.25 c	0.20
	(0.19–0.30)	(0.19–0.30)	(0.17–0.26)	(0.12–0.23)
Biostimulant				
Control	0.28 b	0.12 b	0.32 b	0.15 b
	(0.23 - 0.31)	(0.09 - 0.14)	(0.28 - 0.35)	(0.07 - 0.16)
LDPH	0.33 a	0.18 a	0.38 a	0.26 a
	(0.29–0.37)	(0.15–0.21)	(0.34–0.41)	(0.21–0.30)
Significance				
Fertilization (F)	*	NS	**	NS
Biostimulant (B)	*	**	*	**
$F \times B$	NS	NS	NS	NS

NS, *, ** Non-significant or significant at $p \le 0.05$ and 0.01. Different letters within each column indicate significant differences according to Duncan's test ($p \le 0.05$). The numbers in parenthesis are the data of 90% confidence interval.

When averaged over the N treatments, the baby spinach plants sprayed with the plant-based biostimulant showed a 17.8% and 50.0% increase compared to untreated plants, for N-use efficiency

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and N-uptake efficiency, respectively (Table 2). Moreover, irrespective of biostimulant application, the N0% and N50% plants had the highest values of NUE (Table 2). The trends of the two efficiency parameters in lamb's lettuce were similar to those of spinach but were always higher. In particular, the NUEs of unfertilized plants were significantly higher than in N50% and N100% plants around +69% and +59.5%, respectively (Table 2). When averaged over the N treatments, the foliar application of LDPH improved N-use efficiency and N-uptake efficiency compared to untreated plants, by 18.8% and 73.3% respectively (Table 2).

3.3. Total Chlorophyll, Chlorophyll a and b and Nitrate content

In spinach, the N fertilization levels statistically affected the content of total chlorophyll, chlorophyll a and b, as well as nitrate content in leaves. This latter was the only parameter also affected by the biostimulant application (Table 3). Particularly, chlorophyll (a, b, and total) content increased when N dose was raised. The two treatments N50% and N100% were not significantly different, but N100% was significantly higher than N0% (+10%, +37.3%, and +20.3% respectively).

Table 3. Chlorophyll a and b, total chlorophyll and nitrate content of baby spinach plants as affected by nitrogen (N) fertilization levels (0, 2.25 and 4.5 g N m⁻²; N0%, N50%, N100%, respectively) and biostimulant application (control and LDPH: Legume-derived protein hydrolysates).

Treatments	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Nitrate
	$(mg g^{-1} fw)$	(mg g^{-1} fw)	(mg g^{-1} fw)	(mg kg $^{-1}$ fw)
Fertilization				
N0%	0.905 b	0.547 b	1.452 b	84.9 c
	(0.846 - 0.965)	(0.418 - 0.675)	(1.268 - 1.639)	(-209.1 - 379.0)
N50%	0.976 ab	0.716 ab	1.692 ab	2932.8 b
	(0.917 - 1.035)	(0.587 - 0.844)	(1.508 - 1.876)	(20638.7–3226.8)
N100%	1.015 a	0.786 a	1.801 a	3867.5 a
	(0.955 - 1.074)	(0.657–0.914)	(1.616–1.984)	(3573.4–4161.6)
Biostimulant				
Control	0.957	0.681	1.637	476.5 b
	(0.908 - 1.005)	(0.576-0.786)	(1.487 - 1.787)	(236.3–716.6)
LDPH	0.974	0.685	1.659	4113.7 a
	(0.925–1.022)	(0.58–0.790)	(1.509 - 1.809)	(3873.6–4353.8)
Significance				
Fertilization (F)	*	*	*	**
Biostimulants (B)	NS	NS	NS	**
F×B	NS	NS	NS	NS

NS, *, ** Non-significant or significant at $p \le 0.05$ and 0.01. Different letters within each column indicate significant differences according to Duncan's test ($p \le 0.05$). The numbers in parenthesis are the data of 90% confidence interval.

As expected, our results demonstrated that increasing N fertilization from 0 to 5.0 g m⁻² elicited a significant linear increase in nitrate content compared to non-fertilized plants. Particularly, at N100%, the nitrate content in LDPH-treated plants exceeded the limits imposed by the European Regulation No. 1258/2011 for the commercialization of fresh spinach (3500 mg kg⁻¹ on fresh weight basis) as determined by the cultivation practices, growing conditions and latitude (Table 3).

In lamb's lettuce, all parameters were affected by both factors, but not by their interaction (Table 4). The chlorophyll a, b and total content increased with increasing N level; N100% had the highest values and was statistically different from the other two treatments: +19%, +24.7% and +21% over the mean value of N0% and N100%, for chlorophyll a, b and total chlorophyll, respectively. Moreover, the increases due to biostimulant applications were 26.6%, 44.0% and 32.3% for chlorophyll a, b and total chlorophyll, respectively.

Treatments	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Nitrate
	(mg g^{-1} fw)	(mg g^{-1} fw)	(mg g^{-1} fw)	(mg kg $^{-1}$ fw)
Fertilization				
N0%	0.673 b	0.347 c	1.020 c	102.8 b
	(0.621 - 0.726)	(0.312 - 0.372)	(0.954 - 1.067)	(-925.1 - 1130.7)
N50%	0.722 b	0.397 b	1.120 b	3191.8 a
	(0.672-0.777)	(0.373 - 0.429)	(1.075 - 1.189)	(2163.8-4219.7)
N100%	0.831 a	0.464 a	1.295 a	3210.0 a
	(0.780–0.885)	(0.430–0.498)	(1.228–1.362)	(2182.0–4237.9)
Biostimulant				
Control	0.655 b	0.330 b	0.985 b	562.4 b
	(0.613-0.698)	(0.302 - 0.359)	(0.931 - 1.040)	(-315.4-1363.1)
LDPH	0.829 a	0.475 a	1.304 a	3774.0 a
	(0.788 - 0.874)	(0446–0.504)	(1.251–1.361)	(2973.2–4651.8)
Significance				
Fertilization (F)	**	**	**	**
Biostimulants (B)	**	**	**	**
$F \times B$	NS	NS	NS	NS

Table 4. Chlorophyll a and b, total chlorophyll and nitrate content of lamb's lettuce plants as affected by nitrogen (N) fertilization levels (0, 2.5 and 5.0 g N m⁻²; N0%, N50%, N100%, respectively) and biostimulant applications (control and LDPH: Legume-derived protein hydrolysates).

NS, ** Non-significant or significant at $p \le 0.05$ and 0.01. Different letters within each column indicate significant differences according to Duncan's test ($p \le 0.05$). The numbers in parenthesis are the data of 90% confidence interval.

Furthermore, in lamb's lettuce nitrate content in leaves increased when nitrogen dose was raised, but without significant differences between N50% and N100%, and it was higher in the plants sprayed with biostimulants compared to untreated plants. For this crop, the European Community has not fixed any threshold, but if we consider the limit imposed for fresh spinach, only the value of biostimulant-sprayed plants overcame it.

3.4. Leaf Quality: Antioxidant Activity and Compounds

In spinach, LAA and the content of total phenols and ascorbic acid (AsA) were significantly affected by N fertilization, while the biostimulant application influenced only LAA. HAA was neither affected by N fertilization treatments nor by biostimulant application (Table 5). Irrespective of biostimulant application, LAA, total phenols, and AsA were significantly higher in N0% plants in comparison to N100% plants, around 3.9%, 29.8%, and 41.8% respectively. Interestingly, when averaged over N treatments, the foliar application of LDPH boosted LAA compared to untreated plants by 11.6% (Table 5).

In lamb's lettuce, all the measured leaf quality parameters (LAA, HAA, total phenols, and AsA) were significantly affected by N fertilization levels, while only HAA was affected by the biostimulant application (Table 6). Regarding LAA, total phenols, and AsA, the trends were similar to those observed for spinach; where the values of N0% plants were higher (+8.3%, +23.3%, and +26.9%, respectively) compared to N100% plants. Instead, HAA had an opposite trend: it was higher in fertilized plants (+18.5% compared to unfertilized plants) and it was also higher in the plants sprayed with biostimulant (+6.3%).

	LAA	HAA	Total Phenols	AsA
Treatments	(mM Trolox eq. 100g ⁻¹ dw)	(mM AA eq. 100g ⁻¹ dw)	(mg Gallic Acid eq. g ⁻¹ dw)	(mg g ⁻¹ fw)
Fertilization				
N0%	22.65 a	8.08	3.22 a	33.49 a
	(22.16-23.13)	(7.38-8.77)	(2.97-3.46)	(31.46-35.51)
N50%	22.02 ab	8.11	2.88 ab	27.45 b
	(21.54 - 22.50)	(7.41 - 8.80)	(2.63 - 3.12)	(25.42-29.46)
N100%	21.80 b	8.15	2.48 b	23.62 c
	(21.32–22.28)	(7.44–8.84)	(2.23–2.72)	(21.56–25.60)
Biostimulant				
Control	20.95 b	8.05	2.36	28.55
	(20.55-21.34)	(7.48-8.62)	(2.69-3.09)	(16.86-40.23)
LDPH	23.37 a	8.17	2.37	27.82
	(22.97–23.76)	(7.60–8.74)	(2.62–3.03)	(16.13–39.51)
Significance				
Fertilization (F)	*	NS	**	**
Biostimulants (B)	**	NS	NS	NS
$F \times B$	NS	NS	NS	NS

Table 5. Lipophilic (LAA) and hydrophilic (HAA) antioxidant activity, total phenols and ascorbic acid (AsA) of baby spinach plants as affected by nitrogen (N) fertilization levels (0, 2.25 and 4.5 g N m^{-2} ; N0%, N50%, N100%, respectively) and biostimulant applications (untreated control and LDPH: Legume-derived protein hydrolysates).

NS, *, ** Non-significant or significant at $p \le 0.05$ and 0.01. Different letters within each column indicate significant differences according to Duncan's test ($p \le 0.05$). The numbers in parenthesis are the data of 90% confidence interval.

Table 6. Lipophilic (LAA) and hydrophilic (HAA) antioxidant activity, total phenols and ascorbic acid (AsA) of lamb's lettuce plants as affected by nitrogen (N) fertilization levels (0, 2.5 and 5.0 g N m⁻²; N0%, N50%, N100%, respectively) and biostimulant applications (control and LDPH: Legume-derived protein hydrolysates).

	LAA	HAA	Total phenols	AsA
Treatments	(mM Trolox eq. 100g ⁻¹ dw)	(mM AA eq. 100g ⁻¹ dw)	(mg Gallic Acid eq. g ⁻¹ dw)	$(mg g^{-1} fw)$
Fertilization				
N0%	30.08 a (28.91–31.25)	6.26 b (5.920–6.60)	10.16 a (9.520–10.80)	63.04 a (56.543–69.54)
N50%	28.49 ab	7.18 a	8.55 b (7.913_9.19)	53.41 b
N100%	27.77 b (26.60–28.94)	(0.340–7.32) 7.65 a (7.311–7.99)	7.62 c (6.974–8.25)	49.68 b (43.177–56.17)
Biostimulant				
Control	29.40 (28.44–30.36)	6.82 b (6.537–7.09)	8.96 (8.439–9.48)	56.85 (51.537–62.15)
LDPH	28.16 (27.20–29.11)	7.25 a (6.968–7.52)	8.59 (8.067–9.11)	53.91 (48.603–59.21)
Significance				
Fertilization (F)	*	**	**	*
Biostimulants (B) $F \times B$	NS NS	* NS	NS NS	NS NS

NS, *, ** Non-significant or significant at $p \le 0.05$ and 0.01. Different letters within each column indicate significant differences according to Duncan's test ($p \le 0.05$). The numbers in parenthesis are the data of 90% confidence interval.

4. Discussion

In order to increase the supply of food produced on the available arable land—since the global population will reach 10 billion by 2050—growers must boost the yield of their produce, through the massive use of technical means, in particular N fertilization. Nowadays, it is impossible to adopt an agriculture that is not sustainable and environmentally friendly. Therefore, the objective of boosting crop productivity must occur through the reduction of N fertilizers, but also through the improvement

of nitrogen use efficiency (NUE), that assures reasonable yield and a profit margin for farmers [50].

Several researches have highlighted that plant-based biostimulants have a triggering effect on growth and yield, but they are also capable of improving the NUE in consideration of both economic and environmental motives [38,51]. The plant-based biostimulant used in this test was Trainer[®], a legume-derived protein hydrolysate (containing free amino acids and signaling molecules such as small soluble peptides), for which previous researches have already demonstrated its ability to boost crops' resources use efficiency (RUE) [15,52]—especially N uptake and assimilation [39]—as well as productivity [6,32] and quality [53,54]. Our results highlighted the ability of LDPH to enhance yield of both baby spinach and lamb's lettuce (+24.6% and +13.5% for plant sprayed with Trainer® compared to control plants, respectively), which is in line with Carillo et al.'s [35] findings on spinach, and Di Mola et al. [42,43] on other two important leafy greens (lettuce and baby leaf rocket) cultivated under variable N regimes. The positive effects of the foliar application of LDPH, irrespective of the N fertilization treatments, were more pronounced in spinach than in lamb's lettuce, demonstrating a species-specific response [15,55], especially that the same commercial plant-based biostimulant was used. The different responses between the two leafy vegetables species could be attributed to the different leaf permeability and cuticle morphology as well as the stomatal aperture and thus the efficacy of the plant biostimulant [38]. Therefore, our results highlight, that further study is warranted to assess the physiological and molecular mechanisms behind the biostimulant action and to investigate the specificity of species dependent responses in impacting leaf characteristics and consequently interacting with the different bioactive compounds of plant biostimulants. Interestingly, in our study the marketable fresh yield of LDPH-treated spinach and lamb's lettuce grown under N50% was similar to those grown under N100% (especially the non-treated plants). A number of biochemical and physiological aspects may have contributed to this result, including (i) a higher chlorophyll content (a, b and total) and SPAD index in biostimulant-treated than in non-treated plants, and (ii) improved leaf status in terms of nitrate content, triggering a more efficient translocation of assimilates to potential photosynthetic sinks, thus boosting plant growth and yield [35,42,43]. Moreover, several authors attributed the stimulation action and the increased N assimilation in response to LDPH application to multiple mechanisms of action involving (i) the hormones-like activities (i.e., auxin and giberrellins-like activities), (ii) the increase in the activity of the key enzymes glutamine synthetase and nitrate reductase, and (iii) the upregulation of specific genes responsible in N assimilation and pigment synthesis [27,33,56–58].

Similar to the effect N fertilization on agronomic performance, our findings highlighted the higher NUE of baby spinach and lamb's lettuce, even without N fertilization. The current results are in agreement with the findings of several researches such as Abdelraouf [59], Canali et al., 2011 [60], and Zhang et al. [61], which in spinach observed a linear decrease in NUE when N dose increased. Moreover, our findings about N uptake are in line to the results of Canali et al. [60], which observed that this parameter was not affected by variable nitrogen regimes.

Interestingly, our findings also indicated that foliar application of LDPH can be considered an efficient tool to reduce N additional inputs to the cropping system, hence cutting down the production costs for farmers and N surpluses into the environment [62]. Mainly because the LDPH-treated baby spinach and lamb's lettuce plants exhibited both higher NUE and higher N-uptake efficiency, irrespective of the N fertilization levels. The positive effect of foliar application of LDPH on the two N efficiency parameters can be attributed to the improvement of root architecture (i.e., more vigorous root apparatus) which is related to an overall increase in nutrient accessibility caused by

its power to boost the capacity of absorption, translocation and assimilation of macro and micro minerals, especially when N is limiting plant growth [27,56,63]. This phenomenon associated to the PH-induced remolding of root advocating N uptake and translocation was described by Colla et al. [64], as "nutrient acquisition response". The stimulation of root system architecture—in particular the increase in root hair density and length—was observed previously by several authors on a wide range of agronomic and horticultural species such as corn, sunflower, tomato, eggplant, lettuce and *Brassica* genus [27,57,64,65].

Although the application of fertilizers (nitrogen, phosphorus and potassium) generally increases the crop yield; alternatively, the excessive application of synthetic fertilizers—especially N—can result in undesirable nutritional quality changes such as a decrease in some bioactive compounds (phenols and vitamin C) and soluble sugars [66]. This was the case in the current study, whereby baby spinach and lamb's lettuce cultivated under N100% negatively modulated the synthesis and accumulation of antioxidant molecules such as total phenols and ascorbic acid along with low antioxidant activity. Similar trends were reported recently by Di Mola et al. [42,43] on baby lettuce and rocket grown under optimal and supra-optimal N regimes.

Concerning the effect of LDPH application on the quality of the two tested leafy greens, some findings demonstrated that the application of protein hydrolysates-based biostimulant was able to modify plant primary and secondary metabolism [15,55], leading to the synthesis and accumulation of phytochemicals with health-promoting properties. This was the case in the current greenhouse experiment, since baby spinach and lamb's lettuce plants treated with the commercial protein hydrolysates Trainer[®] positively modulated both the lypophilic and hydrophilic antioxidant capacity, which are considered important traits in evaluating the quality of food including leafy vegetables [23]. However, the foliar LDPH application did not affect the concentration of total phenols and ascorbic acid in both leafy vegetables. A variable effect of three commercial plant biostimulants containing mainly free amino acids (Aminovert, Megafol and Veramin) was also observed on the chemical composition, phenolic profile and bioactive properties of two greenhouse spinach cultivars [67]. Therefore, future research should focus on designing the ideotype plant biostimulants and identifying the best species × biostimulant × fertilization (N) combination(s) for the production of healthy and nutrient-dense leafy vegetables.

5. Conclusions

Sustainable agriculture is the greatest challenge of our century, and plant-based biostimulants represent an efficient and concrete possibility to reach this objective by maintaining high production and improving the NUE of leafy greens with several economic, nutritional and environmental benefits. The positive effects of the LDPH biostimulant were manifested in terms of marketable fresh yield in baby spinach, irrespective of N fertilization treatments and at low N rates (N0% and N50%) in lamb's lettuce. Such benefits were likely derived from the signaling molecules (such as small peptides) as a result of augmented leaf nitrate content, SPAD index and pigments synthesis. These stimulation actions of the LDPH application were more pronounced under sub-optimal (N0% and N50%) than under optimal (N100%) N regimes. Interestingly, foliar LDPH application in both tested leafy vegetables boosted NUE and N uptake efficiency, which is fundamental for both economic and environmental reasons. Our results also demonstrated that the foliar application of LDPH can promote the antioxidant capacity which is important for the human diet and may constitute an added value for both growers and consumers. Overall, our findings suggest that the application of protein hydrolysates can be a sustainable practice in intensive greenhouse cropping systems to enhance crop productivity and NUE under both optimal and sub-optimal (low-input conditions) N regimes.

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C.E.-N. and G.C. visualization, L.O., I.D.M. and C.E.-N. supervision, Y.R., G.C. and M.M. project administration, M.M. funding acquisition, M.M. All authors have read and agreed to the published version of the manuscript.

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