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Evaluation of the Effectiveness of a Humic Substances-Based Product for Lettuce Growth and Nitrogen Use Efficiency under Low Nitrogen Conditions

Santiago Atero-Calvo [1], Francesco Magro [2], Giacomo Masetti [2], Eloy Navarro-León [1,*], Juan Jose Rios [1] and Juan Manuel Ruiz [1]

[1] Department of Plant Physiology, Faculty of Sciences, University of Granada, 18071 Granada, Spain; satero@ugr.es (S.A.-C.); jjr@ugr.es (J.J.R.); jmrs@ugr.es (J.M.R.)
[2] Sofbey S.A., Via San Martino 16/A, CH-6850 Mendrisio, Switzerland; fmagro@sipcam.com (F.M.); gmasetti@sofbey.com (G.M.)
* Correspondence: enleon@ugr.es

Abstract: Increasing crop yield with low-N supplies has become one of the main aims of current agriculture to reduce the excessive use of chemical fertilizers. A sustainable strategy to improve crop productivity, N assimilation, and N Use Efficiency (NUE) under limit-N growth conditions is the application of biostimulants, such as humic substances (HS). Here, we evaluated the effectiveness of an HS-based biostimulant, BLACKJAK®, in improving lettuce growth and NUE under N-deficit conditions. Thus, BLACKJAK® was applied radicularly (R) and foliarly (F) at the following doses: R-HS 0.40 mL/L, R-HS 0.60 mL/L, F-HS 7.50 mL/L, and F-HS 10.00 mL/L. Three N levels were applied: optimal (7 mM) and N-deficit (3 mM and 1 mM). The results showed that shoot dry weight (DW) was reduced at 3 mM N (−32%) and 1 mM N (−42%). However, R and F BLACKJAK® enhanced plant growth at all three N levels, especially with F-HS at 10.00 mL/L, which showed an increase of 43% in shoot DW at 3 and 1 mM N, compared to plants not treated with HS. BLAKCJAK® also improved photosynthesis, NO_3^- and organic N accumulation, the activity of N assimilation enzymes, and the concentration of amino acids and proteins, regardless of the N level. In addition, HS enhanced NUE parameters under all N conditions, except for R-HS 0.60 mL/L at 1 mM N. Hence, our study suggests that the HS-based product BLACKJAK® could be a good candidate for reducing chemical fertilizer use and improving lettuce growth and NUE under low N conditions, although further research is required.

Keywords: biostimulant; humic substances; lettuce; nitrogen deficiency; nitrogen use efficiency; photosynthesis

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1. Introduction

Nitrogen (N) is an essential nutrient, which is required by plants in high amounts. It is a key constituent of essential biomolecules, including amino acids, proteins, nucleic acids, and membranes, constituting about 2% of dry tissue [1,2]. Consequently, an increase in N application results in higher plant growth and productivity. For this reason, along with the increase in food demand due to global population growth, N fertilization has been the main strategy employed by farmers to increase crop yield since the "Green Revolution" [3–5]. However, the excessive application of chemical fertilizers leads to different ecological issues because most of the applied N is not taken up by crops. Instead, it reaches groundwater and causes eutrophication. Additionally, N-derived greenhouse gases, such as nitric oxide, are emitted into the atmosphere [6,7]. Hence, the overuse or improper use of conventional N fertilizers has a negative impact on the environment.

Plants absorb N mainly as nitrate (NO_3^-), which is reduced to ammonium (NH_4^+) and assimilated and incorporated into organic forms (i.e., amino acids) by the activity

of key enzymes such as nitrate reductase (NR) and glutamine synthetase (GS) [1,8]. As most of the applied N is not employed by plants for biomass production, one of the main aims of current agronomic research is the increase in N use efficiency (NUE), defined as the increase in biomass per unit of N input. NUE depends on different aspects related to N metabolism, such as its uptake, transport, and assimilation [6,9,10]. Enhanced NUE allows the maintenance of crop production with lower N inputs [11], reducing the excessive use of synthetic fertilizers and their environmental impacts. In this regard, different agronomic approaches, such as plant breeding programs and biostimulant-based products, are developed to improve NUE in a sustainable way.

The Fertilising Products Regulation (EU 2019/1009) of the European Parliament and of the Council defined plant biostimulants as: "A plant biostimulant shall be an EU fertilising product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (a) nutrient use efficiency, (b) tolerance to abiotic stress, (c) quality traits, and (d) availability of confined nutrients in the soil or rhizosphere" [12]. Thus, beneficial microorganisms such as arbuscular mycorrhizal fungi and plant growth-promoting rhizobacteria, protein hydrolysates, amino acids, algae and plant extracts, silicon, and humic substances, are considered as plant biostimulants that can improve NUE and plant growth under abiotic stress conditions such as N deficiency [2,13–15].

The biodegradation of soil organic matter through the action of microorganisms, also called "humification", leads to the generation of humic substances (HS). HS include humic acids (HA), which are soluble in alkaline pH and precipitate at acidic pH (~1–2), and fulvic acids (FA), which are soluble in alkaline and acidic solutions [16,17]. Chemically, the HS structure is constituted by different functional groups containing oxygen (O), such as carboxyl (C(=O)OH), carbonyl (−C=O), and hydroxyl (-OH), in addition to N in amines and amides and sulfur (S) in sulfhydryl groups [18,19]. HS can be extracted from different sources, such as leonardite, coal, compost, or peat, and then purified and marketed mainly as liquid HS-based products, which can be applied through irrigation (via radicular) or by foliar sprays [18–20]. According to various studies, the enhancement of soil–plant systems by HS is related to both indirect and direct effects. The indirect effects include improvements in the soil's physicochemical properties, such as porosity, water-holding capacity, cation exchange capacity, and carbon sequestration. These changes positively affect plant growth indirectly [16,17]. Additionally, HS can form complexes with mineral nutrients, significantly improving their solubility and absorption by plants. Consequently, HS applications can enhance nutrient use efficiency. Therefore, HS are shown as a potential tool to improve NUE and reduce the overuse of chemical fertilizers [21,22].

In the present study, a leonardite HS-based product named BLACKJAK® was employed as a biostimulant. Its efficiency in increasing nutrient use efficiency in lettuce plants under optimal growth conditions was previously demonstrated by our research group [22]. However, its effectiveness under low N conditions has not been tested. In this way, the hypothesis of the present experiment was that application of the HS-based product BLACKJAK® enhances plant growth, N assimilation, and use efficiency under low N conditions. Hence, the specific objectives of this study were to determine whether the application of BLACKJAK® compensates for the reduction in N supply and which application mode (root or foliar) and dose would be most appropriate.

2. Materials and Methods

2.1. Growth Conditions and Vegetal Material

The present study was carried out in an environmentally controlled growth chamber in the Department of Plant Physiology, Faculty of Sciences, University of Granada (Granada, Spain). The environmental conditions of this chamber were adjusted for the optimal growth of plant material: temperatures of 25/15 °C (day/night), 65–70% relative humidity, and a 16/8 h photoperiod. Seedlings of *Lactuca sativa* L. var. Capitata were used as plant

material for this experiment. These seedlings were germinated and grown in a greenhouse in Southern Spain (Saliplant S.L., Granada, Spain) for 45 days. Afterwards, they were transferred to the growth chamber. During the experiment, all plants were watered with a nutritive solution, with some modification for the lettuce culture: 4 mM KCl, 4 mM $CaCl_2$, 1 mM KH_2PO_4, 2 mM $MgSO_4$, 1 mM NaH_2PO_4, 1 µM $ZnSO_4$, 2 µM $MnCl_2$, 0.25 µM $CuSO_4$, 0.1 µM Na_2MoO_4, 50 µM H_3BO_3, and 125 µM Fe-EDDHA. Every three days, plants received the nutritive solution, whose pH was adjusted to 5.5–6.

2.2. Nitrogen Treatments, Biostimulant Application, and Experimental Design

Two factors were considered for this experiment: N fertilization level and biostimulant application. Three N levels were achieved by adding $NaNO_3$ (Panreac Química S.L.U., Barcelona, Spain) to the nutritive solution at different doses: 7 mM N (optimal N), 3 mM N, and 1 mM N. For each N dose, a control treatment without HS applications was performed. These N doses were selected following previous studies by our research group in lettuce [2]. HS substances were applied using BLACKJAK® (Sofbey S.A., Mendrisio, Switzerland, https://www.sofbey.com/product/blackjak/ (accessed on 26 June 2024) at four different doses: radicular (R) (0.40 and 0.60 mL/L) and foliar (F) (7.50 and 10.00 mL/L). This biostimulant consisted of a leonardite-suspension concentrate-based product whose chemical characterization has been previously described [23]. The doses were selected in a previous screening, where lettuce biomass production and nutritional quality were evaluated [21]. The biostimulant was applied three times, with a periodicity of ten days. Radicular applications were performed using BLACKJAK® diluted in the nutritive solution, which decreased its pH to 4.5. Foliar applications were conducted using a BLACKJAK® dilution in distilled water, which was directly sprayed onto the leaves 2 h after switching on the growth chamber. The experimental design consisted of a randomized complete block with 15 treatments, with eight plants per treatment.

2.3. Plant Sampling and Growth Measurements

Ten days after the last HS application, lettuce plants were sampled. Shoots from all plants per treatment were washed with distilled water, surface dried with filter paper, and weighed to obtain the fresh weight (FW) of the shoots. Shoots from half of the plants in each treatment (4 plants) were stored together at −80 °C in liquid nitrogen for biochemical analysis. Shoots from the other half were lyophilized and weighed to determine shoot dry weight (DW) and the N forms.

Before the treatments started, plants of similar size were sampled, dried, and weighed (initial DW, DWi). The shoot relative growth rate (RGR) was determined using the equation $(\ln DWf - \ln DWi)/(Tf - Ti)$, where DWf is the final DW and Tf and Ti are the sampling (30 days) and initial (0 days) times, respectively.

2.4. Chlorophyll (Chl) and Carotenoid Concentration

Lettuce leaves (0.1 g) were ground with methanol (1 mL) and then centrifuged (5000× *g*, 5 min). The absorbance was recorded at 666 nm, 653 nm, and 470 nm. According to the equations described in [24], Chls (*a* and *b*) and carotenoids were calculated. The total Chls were estimated as the sum of Chl *a* and *b*.

2.5. Analysis of Leaf Gas Exchange Parameters

Determination of the leaf gas exchange parameters was carried out using an infra-red gas analyzer LICOR 6800 (IRGA: LICOR Inc., Lincoln, NE, USA). Before measurements, system warmup tests were performed according to the manufacturer's recommendations. The different environmental conditions of the leaf chamber were adapted to 400 µmol mol^{-1} of CO_2, 25 °C leaf temperature, 70% relative humidity, 350 µmol m^{-2} s^{-2} of photosynthetically active radiation, and 10,000 rpm chamber fan mixing speed. The measurements were taken in intermediate leaves from six plants per treatment between 10.00 a.m. and 02.00 p.m. In this way, net photosynthetic rate (*A*), transpiration rate (*E*), intercellular CO_2

(*Ci*), and stomatal conductance (*gs*) were estimated. The ratio between *A* and *E* was used to determine the water use efficiency (WUE).

2.6. Estimation of Chlorophyll a Fluorescence

Intermediate leaves from six plants per treatment were adapted to dark conditions for 30 min, with the assistance of a special clip. Afterward, fluorescence was induced at 650 nm of red light using the Chl Fluorimeter Handy PEA (Hansatech Ltd., King's Lynn, Norfolk, UK). The RC/ABS (proportion of active reaction centers) and PI_{ABS} (performance index) were registered in fluorimeter software version 1.13.

2.7. Nitrate (NO_3^-), Organic Nitrogen, and Total Nitrogen Concentration

An aqueous extraction for 24 h from 0.1 g of dry leaves was employed to determine NO_3^- concentration through the reaction of NO_3^- with salicylic acid against a curve of potassium nitrate [25]. For organic nitrogen determination, 0.1 g of dry leaves were mineralized with pure sulfuric acid and hydrogen peroxide (33%). After dilution with distilled water, organic N was determined by mixing an aliquot of digested material with a medium containing sodium hydroxide, sodium dichloroisocyanurate, sodium salycilate, and sodium nitroprusside [26]. Total nitrogen was calculated as the sum between NO_3^- and organic N.

2.8. Enzyme Extraction and Activity Determination

A total of 0.1 g of frozen leaves was homogenized in KH_2PO_4 (50 mM, pH 7.5) buffer, containing 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), and 1% (*w/v*) polyvinylpolypyrrolidone (PVPP). After centrifugation (20,650× *g*, 20 min), supernatants were used to determine NR and glutamate dehydrogenase (GDH) activity. Similarly, the same amount of plant material was homogenized in maleic acid buffer (100 mM, pH 6.8 adjusted with KOH), containing 100 mM sucrose, 2% (*v/v*) β-mercaptoethanol, and 20% (*v*/*v*) ethyleneglycol, for the determination of GS activity [27].

The reaction of the nitrite (NO_2^-) formed with 1% sulphanilamide dissolved in HCl 1.5 M and N-(1-Naphtyl)ethylenediamine dihydrochloride 0.02% (*v/v*) dissolved in HCl 0.2 M was recorded at 540 nm for NR activity estimation, after 30 min of incubation at 30 °C [28]. GDH activity was measured by NADPH oxidation for 1.5 min at 340 nm [29]. The formation of γ-glutamylhydroxamate after incubation at 30 °C for 30 min was colorimetrically measured at 540 for GS activity determination [28]. The protein content of the extracts was estimated according to the method of Bradford [30], using a standard curve of bovine serum albumin (BSA).

2.9. Concentration of Soluble Amino Acids and Proteins

Soluble amino acids (AAs) and proteins were determined in supernatants obtained from 0.1 g of frozen leaves ground in KH_2PO_4 buffer (50 mM, pH 7) and centrifuged at 12,360× *g* for 15 min. The soluble amino acid concentration was estimated after the reaction of 100 µL of supernatant with ninhydrine at 100 °C for 20 min. Afterwards, 8 mL of propan-2-ol were added, incubated at room temperature for 30 min, and the resultant mixture was measured at 570 nm [31]. The soluble proteins were determined following the Bradford method at 595 nm, using a curve of BSA [30].

2.10. Nitrogen Use Efficiency (NUE) Parameters

Different agronomic parameters related to NUE were calculated according to Iqbal et al. [32] and Navarro-León et al. [2]. For this purpose, total nitrogen accumulation (TNA) was estimated as the product of total N and shoot DW. The N uptake efficiency (NUpE) was estimated as shoot N divided by applied N. The N utilization efficiency (NUtE) was calculated as shoot DW divided by shoot N. Thus, NUE was estimated as the product of NUpE and NUtE. Furthermore, the apparent N fertilizer recovery (ANR) was calculated as $[(TNA_B - TNA_C)/ND] \times 100$ (%), where TNA_B and TNA_C are the total N accumulation of

plants supplied and not supplied with biostimulants, respectively, and ND is the amount of N applied.

2.11. Statistical Procedures

All data were treated through a simple analysis of variance (ANOVA), with a 95% confidence interval. The means for each parameter were compared using Fisher's least significant difference test (LSD). The significance values were expressed as: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), NS (Not significant).

3. Results

3.1. Lettuce Growth

The application of the HS-based biostimulant increased plant growth under optimal growth conditions (7 mM N) (Figures 1 and 2A–C). In this way, all doses enhanced shoot FW (25%, 27%, 20%, and 24% for R-HS 0.40 mL/L and 0.60 and F-HS 7.50 and 10.00 mL/L, respectively) (Figure 2A). However, shoot DW was increased only by R-HS 0.60 mL/L (29%) and F-HS 7.50 mL/L (23%), whereas shoot RGR was improved by all doses except R-HS 0.40 mL/L, with respect to non-HS treated plants (Figure 2B,C).

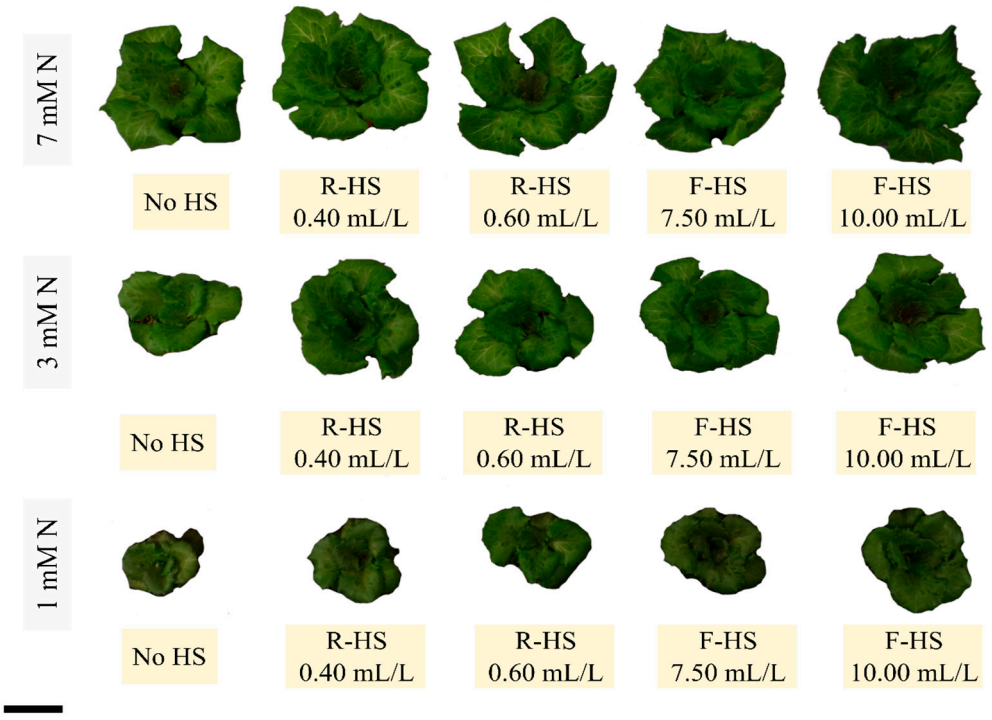

Figure 1. Effect of different N supplies and HS application on lettuce plant growth. Plants were subjected to optimal N conditions (7 mM) and N deficiency (3 mM and 1 mM) and were treated with radicular (R-HS) and foliar (F-HS) humic substances (HS). Scale bar represents 5 cm.

The reduction in N fertilization to 3 mM N and 1 mM N resulted in lower plant growth, although HS applications increased lettuce growth (Figures 1 and 2A–C). Thus, under 3 mM N conditions, all BLACKJAK® doses increased shoot FW (36%, 42%, 24%, and 30%) (Figure 2A), shoot DW (37%, 29%, 30%, and 43%) (Figure 2B), and shoot RGR (19%, 17%, 17%, and 23%) (Figure 2C), for R-HS 0.40 and 0.60 mL/L and F-HS 7.50 and 10.00 mL/L, respectively, compared to plants without HS applications. F-HS 10.00 mL/L was the dose that increased shoot DW and shoot RGR the most, although no significant differences were observed between doses (Figures 1 and 2A–C). Under 1 mM N conditions, shoot FW was also increased by all HS doses (63%, 42%, 71%, and 72% for R-HS 0.40 and 0.60 mL/L and F-HS 7.50 and 10.00 mL/L, respectively) (Figure 2A). Shoot DW was

enhanced by 30%, 26%, and 43%, while the shoot RGR was improved by 14%, 13%, and 20%, at R-HS 0.40 mL/L, F-HS 7.50 mL/L, and 10.00 mL/L (Figure 2B,C). In this way, and as commented on for 3 mM, F-HS at 10.00 mL/L showed the highest improvement in lettuce growth (Figures 1 and 2A–C).

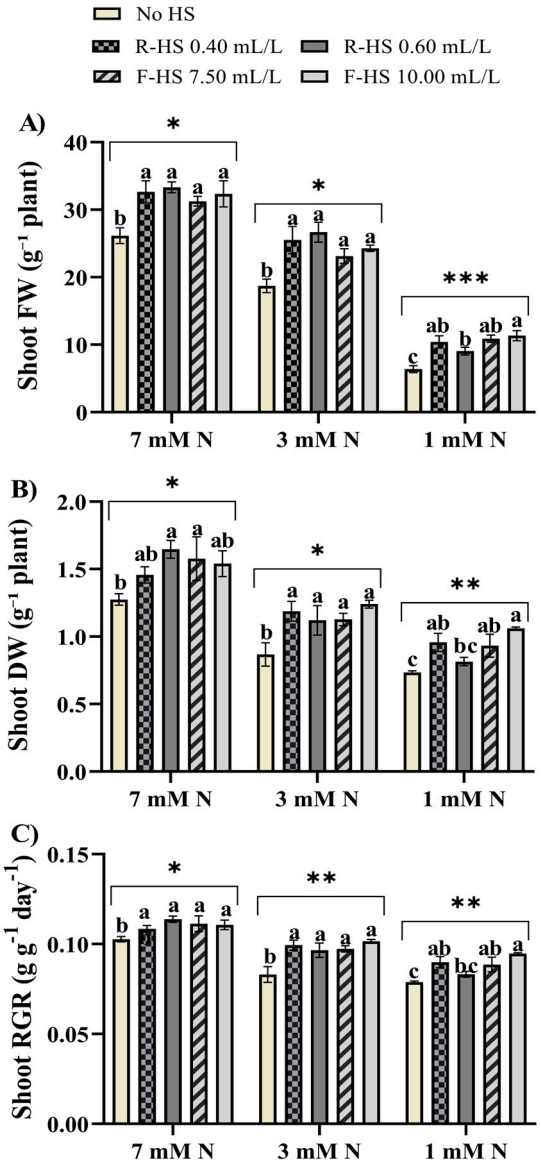

Figure 2. Effect of different N supplies and HS application on shoot FW (**A**), shoot DW (**B**), and shoot relative growth rate (RGR) (**C**). Lettuce plants were subjected to optimal N conditions (7 mM) and N deficiency (3 mM and 1 mM) and were treated with radicular (R-HS) and foliar (F-HS) humic substances (HS). Values are expressed as means ± standard error (n = 8). Columns marked with the same letters were not significantly different based on Fisher's least significance test (LSD; p = 0.05). The levels of significance are expressed as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3.2. Photosynthetic Pigments, Leaf Gas Exchange, and Chl a Fluorescence

Regardless of the N dose applied, lettuce plants subjected to HS showed an increase in Chl *a*, Chl *b*, total Chls, and carotenoid concentrations with respect to the control (non-treated) plants, except for Chl *b* with an F-HS dose of 7 mM N (Table 1).

Table 1. Effect of different N supplies and HS application on photosynthetic pigment concentration.

		Chl *a* (mg g^{-1} FW)	Chl *b* (mg g^{-1} FW)	Total Chls (mg g^{-1} FW)	Carotenoids (µg g^{-1} FW)
7 mM N	No HS	0.172 ± 0.002 d	0.111 ± 0.001c	0.283 ± 0.004 d	20.24 ± 0.29 d
	R-HS 0.40 mL/L	0.216 ± 0.006 b	0.123 ± 0.002 b	0.334 ± 0.008 b	24.44 ± 0.47 b
	R-HS 0.60 mL/L	0.248 ± 0.006 a	0.131 ± 0.002 a	0.379 ± 0.008 a	27.08 ± 0.51 a
	F-HS 7.50 mL/L	0.202 ± 0.004 c	0.115 ± 0.001 c	0.318 ± 0.004 c	23.02 ± 0.35 c
	F-HS 10.00 mL/L	0.200 ± 0.001 c	0.113 ± 0.001 c	0.313 ± 0.001 c	22.58 ± 0.21 c
	p-value	***	***	***	***
	$LSD_{0.05}$	0.012	0.004	0.015	1.09
3 mM N	No HS	0.180 ± 0.002 d	0.113 ± 0.001 c	0.291 ± 0.002 d	21.59 ± 0.30 c
	R-HS 0.40 mL/L	0.222 ± 0.004 ab	0.139 ± 0.002 a	0.360 ± 0.006 a	25.00 ± 0.40 a
	R-HS 0.60 mL/L	0.208 ±0.002 c	0.126 ± 0.001 b	0.334 ± 0.003 c	23.77 ± 0.16 b
	F-HS 7.50 mL/L	0.215 ± 0.001 bc	0.129 ± 0.001 b	0.344 ± 0.001 c	24.04 ± 0.10 b
	F-HS 10.00 mL/L	0.229 ± 0.005 a	0.135 ± 0.003 a	0.364 ± 0.007 b	25.47 ± 0.45 a
	p-value	***	***	***	***
	$LSD_{0.05}$	0.009	0.005	0.013	0.89
1 mM N	No HS	0.159 ± 0.002 c	0.105 ± 0.002 d	0.261 ± 0.003 d	19.07 ± 0.21 d
	R-HS 0.40 mL/L	0.219 ± 0.012 ab	0.128 ± 0.006 bc	0.324 ± 0.009 c	22.49 ± 0.25 c
	R-HS 0.60 mL/L	0.202 ± 0.004 b	0.126 ± 0.002 c	0.329 ± 0.006 c	23.15 ± 0.36 c
	F-HS 7.50 mL/L	0.212 ± 0.004 ab	0.136 ± 0.002 ab	0.348 ± 0.005 b	24.54 ± 0.34 b
	F-HS 10.00 mL/L	0.230 ± 0.005 a	0.139 ± 0.002 a	0.369 ± 0.006 a	25.57 ± 0.37 a
	p-value	***	***	***	***
	$LSD_{0.05}$	0.018	0.009	0.002	0.90

Lettuce plants were subjected to optimal N conditions (7 mM) and N deficiency (3 mM and 1 mM) and were treated with radicular (R-HS) and foliar (F-HS) humic substances (HS). Values are means (n = 9). Fisher's least significance test (LSD; p = 0.05) was used to compare between means. Different letters indicate significant differences. The levels of significance are expressed as $p < 0.001$ (***).

Similarly, under the three levels of N, the HS-based product increased *A* compared to the control (no HS) treatment. Specifically, higher *A* values were registered with radicular-HS, in particular with R-HS 0.60 mL/L. Furthermore, R-HS 0.40 mL/L enhanced *E*, whereas F-HS 7.50 mL/L decreased it, under 3 mM and 1 mM N, respectively. All HS doses increased *Ci* in plants under 7 mM N, whereas F-HS 7.50 mL/L and all HS treatments decreased *Ci* in plants treated with 3 mM and 1 mM N, respectively. In addition, HS applications improved WUE under the three N growth conditions, with R-HS 0.60 mL/L showing the highest increase at 7 mM N and 1 mM N. Regarding RC/ABS, only R-HS 0.40 mL/L significantly enhanced it under 7 mM N, whereas the same HS dose, along with F-HS 10.00 mL/L, increased RC/ABS under 3 mM N. Furthermore, all HS doses improved RC/ABS under the lowest N condition (1 mM), with F-HS at 10.00 mL/L showing the highest values. PI_{ABS} was significantly improved in plants subjected to all HS doses, but only under 1 mM N (Table 2).

Table 2. Effect of different N supplies and HS application on leaf gas exchange and Chl a fluorescence.

		A	*E*	*Ci*	WUE	RC/ABS	PI_{ABS}
7 mM N	No HS	7.03 ± 0.45 b	2.63 ± 0.01	273.5 ± 4.3 b	2.53 ± 0.10 c	0.308 ± 0.001 b	1.96 ± 0.05
	R-HS 0.40 mL/L	8.36 ± 0.14 a	2.94 ± 0.14	301.4 ± 5.4 a	2.88 ± 0.18 bc	0.334 ± 0.004 a	2.00 ± 0.04
	R-HS 0.60 mL/L	9.27 ± 0.47 a	2.79 ± 0.24	292.1 ± 0.5 a	3.38 ± 0.15 a	0.309 ± 0.003 b	1.96 ± 0.04
	F-HS 7.50 mL/L	8.53 ± 0.22 a	2.80 ± 0.20	292.0 ± 5.4 a	3.11 ± 0.17 ab	0.305 ± 0.003 b	1.91 ± 0.06
	F-HS 10.00 mL/L	8.55 ± 0.52 a	3.12 ± 0.49	297.4 ± 8.2 a	3.33 ± 0.07 a	0.314 ± 0.005 b	1.98 ± 0.05
	p-value	**	NS	*	*	***	NS
	$LSD_{0.05}$	1.14	0.78	15.6	0.40	0.010	0.14
3 mM N	No HS	5.59 ± 0.12 b	2.53 ± 0.08 b	320.3 ± 2.7 a	2.25 ± 0.05 b	0.297 ± 0.001 c	1.78 ± 0.04
	R-HS 0.40 mL/L	7.55 ± 0.50 a	3.24 ± 0.48 a	309.8 ± 9.1 ab	2.79 ± 0.24 a	0.311 ± 0.006 ab	1.86 ± 0.03
	R-HS 0.60 mL/L	7.59 ± 0.27 a	2.94 ± 0.14 ab	308.8 ± 1.1 ab	2.59 ± 0.03 ab	0.305 ± 0.003 bc	1.82 ± 0.02
	F-HS 7.50 mL/L	7.45 ± 0.39 a	2.62 ± 0.13 ab	298.6 ± 1.2 b	2.85 ± 0.08 a	0.303 ± 0.005 bc	1.82 ± 0.05
	F-HS 10.00 mL/L	7.22 ± 0.18 a	2.86 ± 0.09 ab	310.9 ± 0.5 ab	2.53 ± 0.03 ab	0.323 ± 0.006 a	1.78 ± 0.08
	p-value	**	*	*	**	**	NS
	$LSD_{0.05}$	0.94	0.69	12.6	0.34	0.013	0.14

Table 2. *Cont.*

		A	*E*	*Ci*	WUE	RC/ABS	PI_{ABS}
1 mM N	No HS	2.69 ± 0.49 c	1.71 ± 0.30 ab	335.7 ± 1.2 a	1.55 ± 0.03 c	0.293 ± 0.002 c	1.53 ± 0.03 b
	R-HS 0.40 mL/L	5.06 ± 0.41 ab	2.07 ± 0.25 a	307.6 ± 5.2 bc	2.51 ± 0.14 b	0.312 ± 0.005 b	1.74 ± 0.04 a
	R-HS 0.60 mL/L	5.74 ± 0.28 a	1.91 ± 0.13 ab	290.6 ± 3.1 d	3.02 ± 0.09 a	0.310 ± 0.003 b	1.86 ± 0.06 a
	F-HS 7.50 mL/L	4.38 ± 0.06 b	1.43 ± 0.14 b	300.8 ± 2.5 c	2.66 ± 0.04 b	0.318 ± 0.003 ab	1.84 ± 0.05 a
	F-HS 10.00 mL/L	4.31 ± 0.01 b	1.71 ± 0.08 ab	311.1 ± 3.2 b	2.54 ± 0.12 b	0.329 ± 0.009 a	1.79 ± 0.04 a
	p-value	***	*	***	***	***	***
	$LSD_{0.05}$	0.92	0.57	9.6	0.27	0.014	0.14

Lettuce plants were subjected to optimal N conditions (7 mM) and N deficiency (3 mM and 1 mM) and were treated with radicular (R-HS) and foliar (F-HS) humic substances (HS). A (Net photosynthetic rate), E (Transpiration rate), Ci (Intercellular CO_2), WUE (Water Use Efficiency), RC/ABS (Proportion of active reaction centers), and PI_{ABS} (Performance index). A is expressed as μmol m^{-2} s^{-1}; E is expressed as mmol m^{-2} s^{-1}; Ci is expressed as μmol mol^{-1}. Values are means (n = 6). Fisher's least significance test (LSD; p = 0.05) was used to compare between means. Different letters indicate significant differences. The levels of significance are expressed as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). NS (Not significant).

3.3. Accumulation of Nitrogen Forms

The application of the HS-based product, both radicularly and foliarly, increased NO_3^-, organic, and total N concentrations at all doses and N levels, except for F-HS at 10.00 mL/L, which decreased organic N and showed no differences in total N under 3 mM N (Figure 3A–C). In addition, F-HS at 10.00 mL/L increased all N forms to the greatest extent at 7 mM N (Figure 3A–C). Under 3 mM N, the R-HS effect was more pronounced, especially for organic N (Figure 3B). Meanwhile, F-HS 7.50 mL/L showed higher NO_3^- and organic N accumulation under 1 mM N (Figure 3A,B).

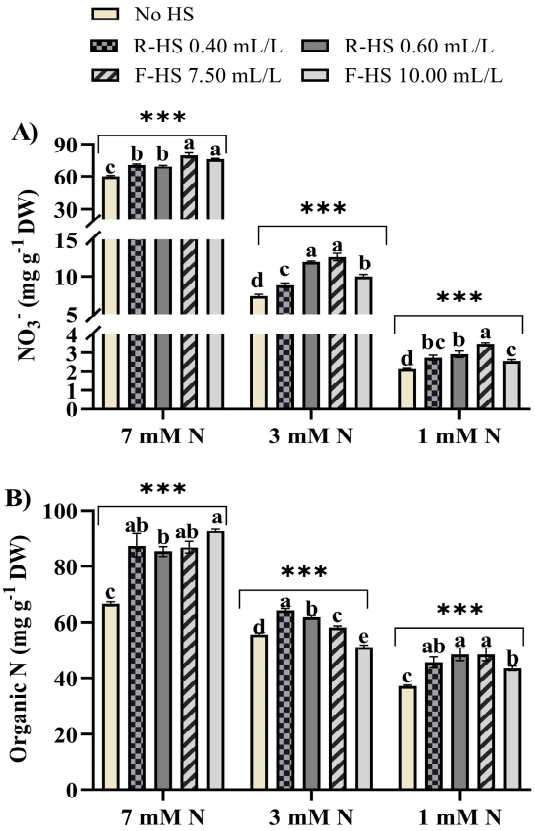

Figure 3. *Cont.*

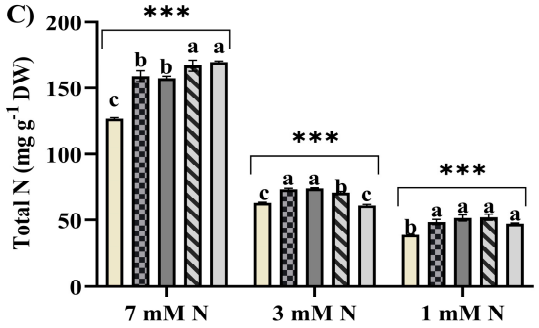

Figure 3. Effect of different N supplies and HS application on NO_3^- (**A**), organic N (**B**), and total N (**C**). Lettuce plants were subjected to optimal N conditions (7 mM) and N deficiency (3 mM and 1 mM) and were treated with radicular (R-HS) and foliar (F-HS) humic substances (HS). Values are expressed as means ± standard error (n = 9). Columns marked with the same letters were not significantly different based on Fisher's least significance test (LSD; p = 0.05). The level of significance is expressed as $p < 0.001$ (***).

3.4. Nitrogen Assimilation in Lettuce Plants

Under optimal N growth conditions, the application of the HS-based product significantly enhanced GS activity and AA concentration at both F-HS doses. In addition, radicular and foliar BLACKJAK® applications at all doses significantly enhanced NR, GS, and GDH activities, as well as soluble AAs and protein concentration under 3 mM and 1 mM N, except for GS activity at R-HS 0.60 mL/L and F-HS 10.00 mL/L under 1 mM N (Table 3).

Table 3. Effect of low N and HS application on NR, GS, and GDH activity, as well as soluble AA and soluble protein content.

		NR	GS	GDH	Soluble AAs	Soluble Proteins
7 mM N	No HS	102.8 ± 4.6	1.20 ± 0.02 bc	1.16 ± 0.20	0.43 ± 0.02 b	8.20 ± 0.53
	R-HS 0.40 mL/L	111.1 ± 10.7	1.15 ± 0.02 c	1.23 ± 0.12	0.44 ± 0.02 b	7.61 ± 0.58
	R-HS 0.60 mL/L	115.3 ± 10.0	1.19 ± 0.02 bc	1.32 ± 0.16	0.48 ± 0.03 ab	8.08 ± 0.50
	F-HS 7.50 mL/L	111.7 ± 7.6	1.21 ± 0.02 b	1.34 ± 0.20	0.44 ± 0.01 b	8.11 ± 0.14
	F-HS 10.00 mL/L	119.6 ± 5.6	1.33 ± 0.02 a	1.43 ± 0.22	0.52 ± 0.01 a	8.99 ±0.42
	p-value	NS	***	NS	*	NS
	$LSD_{0.05}$	23.0	0.06	0.53	0.07	1.42
3 mM N	No HS	83.4 ± 2.5 b	0.97 ± 0.03 b	0.51 ± 0.06 b	0.40 ± 0.02 b	5.71 ± 0.22 b
	R-HS 0.40 mL/L	120.2 ± 5.2 a	1.14 ± 0.03 a	1.63 ± 0.30 a	0.52 ± 0.01 a	6.71 ± 0.17 a
	R-HS 0.60 mL/L	127.3 ± 14.4 a	1.23 ± 0.07 a	1.52 ± 0.06 a	0.53 ± 0.02 a	7.08 ± 0.22 a
	F-HS 7.50 mL/L	121.5 ± 6.6 a	1.13 ± 0.06 a	1.37 ± 0.17 a	0.53 ± 0.04 a	6.72 ± 0.35 a
	F-HS 10.00 mL/L	126.7 ± 16.0 a	1.23 ± 0.07 a	1.21 ± 0.05 a	0.50 ± 0.01 a	6.75 ± 0.41 a
	p-value	*	**	***	***	*
	$LSD_{0.05}$	29.7	0.16	0.46	0.01	0.82
1 mM N	No HS	113.2 ± 1.8 b	4.66 ± 0.04 b	0.60 ± 0.06 b	0.31 ± 0.03 b	2.12 ± 0.16 b
	R-HS 0.40 mL/L	149.3 ± 1.8 a	5.78 ± 0.38 a	0.83 ± 0.07 a	0.44 ± 0.02 a	4.68 ± 0.15 a
	R-HS 0.60 mL/L	142.4 ± 6.3 a	5.31 ± 0.36 ab	0.92 ± 0.09 a	0.49 ± 0.04 a	4.87 ± 0.16 a
	F-HS 7.50 mL/L	152.7 ± 9.7 a	5.58 ± 0.14 a	1.01 ± 0.08 a	0.43 ± 0.03 a	4.55 ± 0.18 a
	F-HS 10.00 mL/L	147.8 ± 13.1 a	5.30 ± 0.38 ab	0.99 ± 0.07 a	0.43 ± 0.02 a	4.28 ± 0.25 a
	p-value	*	*	**	***	***
	$LSD_{0.05}$	26.4	0.85	0.22	0.08	0.63

Lettuce plants were subjected to optimal N conditions (7 mM) and N deficiency (3 mM and 1 mM) and were treated with radicular (R-HS) and foliar (F-HS) humic substances (HS). NR (Nitrate reductase) is expressed as μM NO_2^- min^{-1} mg $protein^{-1}$, GS (Glutamine synthetase) is expressed as μmol ɣ-glutamylhydroxamate^{-1} min^{-1} mg $protein^{-1}$, GDH (Glutamate dehydrogenase) is expressed as ΔAbs min^{-1} mg $protein^{-1}$, soluble AAs (amino acids) and proteins are expressed as mg g^{-1} FW. Values are means (n = 9). Fisher's least significance test (LSD; p = 0.05) was used to compare between means. Different letters indicate significant differences. The levels of significance are expressed as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). NS (Not significant).

3.5. Nitrogen Use Efficiency

Radicular and foliar applications of BLACKJAK® significantly increased TNA and NUpE at all N levels (7, 3, and 1 mM N), compared to plants not treated with HS (Figure 4, Table S1).

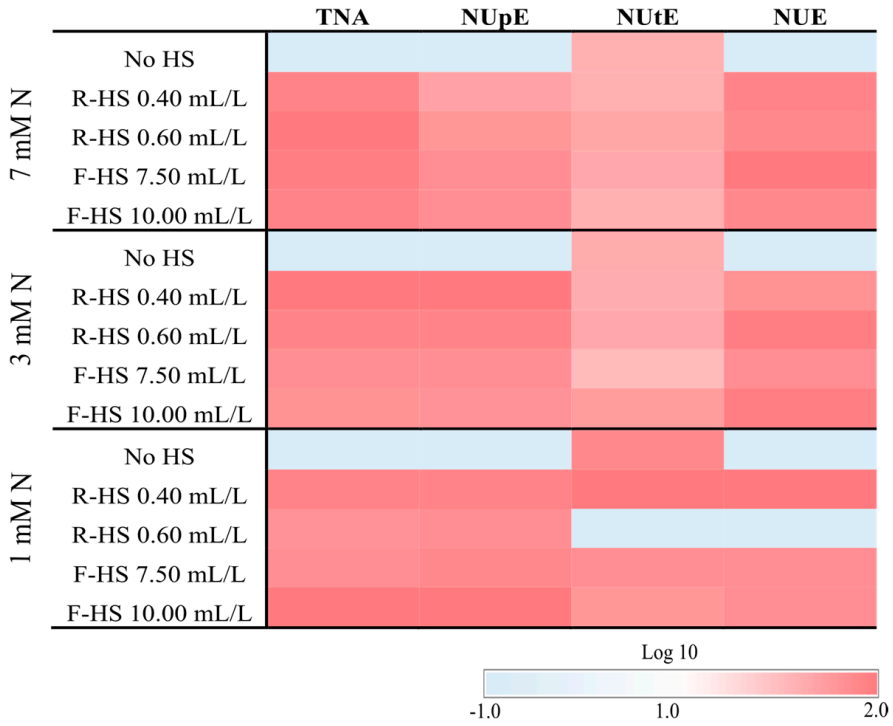

Figure 4. Heat map showing the effect of different N supplies and HS applications on Total Nitrogen Accumulation (TNA) and different nitrogen use efficiency parameters: NUpE (Nitrogen Uptake Efficiency), NUtE (Nitrogen Utilization Efficiency), and NUE (Nitrogen Use Efficiency). Lettuce plants were subjected to optimal N conditions (7 mM) and N deficiency (3 mM and 1 mM) and were treated with radicular (R-HS) and foliar (F-HS) humic substances (HS). Color scale refers to the logarithmic transformation (Log10). Higher values are shown in red, intermediate values in white, and lower values in blue.

The different treatments did not significantly affect NUtE at the three N levels. Furthermore, R-HS 0.60 mL/L reduced NUtE at 1 mM N (Figure 4). However, BLACKJAK® enhanced NUE at all N supplies, except for R-HS 0.60 mL/L, which did not show differences with respect to plants without HS (Figure 4). Thus, NUE was improved with R-HS 0.40 mL/L, R-HS 0.60 mL/L, F-HS 7.50 mL/L, and F-HS 10.00 mL/L by 32%, 42%, 46%, and 37%, respectively, at 7 mM N, and by 51%, 57%, 43%, and 57%, respectively, at 3 mM N. At 1 mM N, R-HS 0.40 mL/L, F-HS 7.50 mL/L, and 10.00 mL/L increased NUE by 78%, 61%, and 72%, respectively (Figure 4).

A comparison of the HS doses employed showed that the application of R-HS 0.40 mL/L increased the ANR percentage at 3 mM N, whereas R-HS 0.60 mL/L decreased it at 1 mM N (Figure 5).

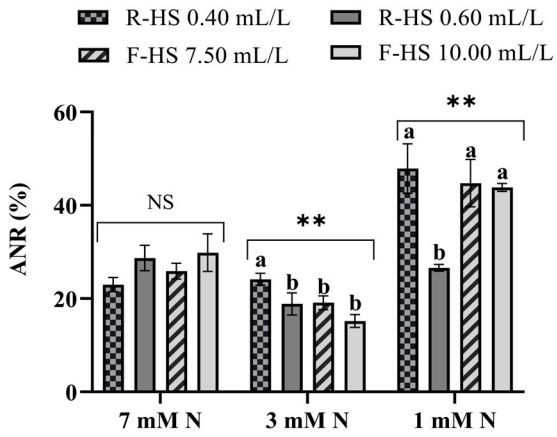

Figure 5. Effect of different N supplies and HS application on Apparent Nitrogen Recovery (ANR). Lettuce plants were subjected to optimal N conditions (7 mM) and N deficiency (3 mM and 1 mM) and were treated with radicular (R-HS) and foliar (F-HS) humic substances (HS). Values are expressed as means ± standard error (n = 9). Columns marked with the same letters were not significantly different based on Fisher's least significance test (LSD; $p = 0.05$). The level of significance is expressed as $p > 0.05$ (NS) and $p < 0.01$ (**).

4. Discussion

Among the objectives of modern agriculture, a key focus is maintaining crop production under low-N conditions to reduce the overuse of chemical fertilizers, which have serious detrimental effects on the environment [33]. To achieve this goal, the external applications of different compounds, known as biostimulants, continue to be a focus of research. HS-based products are developed by companies from different sources such as coal, lignite, or peat, with different proportions of HA and FA, as an alternative to conventional fertilizers to increase crop yield and abiotic stress tolerance [34]. In the present experiment, BLACKJAK®, a leonardite-suspension concentrate-based product, was employed. The effectiveness of this fertilizer in increasing plant growth under optimal growth conditions has been proven [22]. However, no previous studies have focused on its efficiency under low-N conditions.

Plant growth is a reliable indicator of mineral nutrient toxicity or deficiency. As N is a major constituent of plant tissues, its reduction in soil or irrigation water quickly results in decreased plant growth and productivity [35,36]. Indeed, in our study, reducing the N concentration in nutritive solution to 3 mM and 1 mM caused decreases of 28% and 76% in shoot FW and 32% and 42% in shoot DW, respectively. These results underscore the importance of proper N fertilization to ensure adequate plant growth. Similarly, Navarro-León et al. [2] observed a decrease of 15% and 30% in lettuce biomass with a reduction of 30% and 60% in N application, respectively. However, plant growth was improved by all BLACKJAK® doses and modes of application under the three N levels compared to plants not treated with HS, except for R-HS 0.60 mL/L at 1 mM N, which increased shoot DW, although it was not statistically significant. Better results for lettuce growth under limited N conditions were offered by F-HS 10.00 mL/L application. As reported previously, HS can improve the primary and secondary metabolism of plants, including photosynthesis capacity, nutrient uptake and assimilation, and phytohormonal profile [16,17]. Consequently, HS may enhance plant tolerance to abiotic stresses such as nutrient deficiency [22,34]. In this way, other studies have reported that HS applications enhance plant growth under low-N conditions, which agrees with our results. Thus, a lignite HS-based product increased tomato growth under 50% N fertilization [37]. Hence, our data indicate that, overall, BLACKJAK® could be employed to offset N reduction in lettuce plants, regardless of the mode of application (root or foliar) at most of the doses employed.

In addition, the N-based nutritional status of a plant is currently supported by photosynthetic pigment concentrations, as N is one of the main components of the structure of

Chls. In addition, some AAs, such as glutamic acid, serve as precursors in Chl biosynthesis [38,39]. For this reason, pigment concentrations decrease after N reduction [40,41]. Our data revealed that radicular and foliar HS applications enhanced Chl and carotenoid accumulation in lettuce leaves under the three N concentrations (7, 3, and 1 mM N), compared to plants without HS. The impact of HS on pigment levels could be associated with the fact that N is in the HS's molecular structure, in the form of amines and amides, possibly enhancing N assimilation for the synthesis of AAs [19,42,43]. Thus, the accumulation of photosynthetic pigments through HS application has been observed in different species such as rice [43], spinach [44], or pistachio [45].

The photosynthetic pigments participate in light absorption, transport, and, ultimately, its conversion to chemical energy through photosynthesis. Hence, there is normally a positive correlation between pigment content and photosynthesis efficiency or, in other words, between N status and photosynthesis capacity [38]. Different instruments, such as LICOR 6800, allow us to estimate the photosynthesis rate (or *A*) of plants, studying if this physiological process is affected by adverse growth conditions. Furthermore, photosynthesis improvement has been proposed as a physiological HS mechanism of action to increase plant growth. Thus, different genes involved in the Calvin cycle, including those that encode the Rubisco enzyme, have been found to be upregulated in *Zea mays* L. treated with HA, which results in a higher photosynthesis rate [46]. In the present experiment, the reduction in N irrigation caused a significant decrease in *A*, in agreement with other studies [47–49]. Nevertheless, HS application enhanced the photosynthesis rate in lettuce plants under different N conditions, which could explain the improved plant growth compared to lettuce without HS [50]. Similar results were reported by Zhang et al. [51], who observed a significant increase in *A* in maize plants grown under N-deficient conditions and treated with radicular HA.

In addition, the reduction in N fertilization may decrease WUE as previously observed [52], which is consistent with our results. WUE is defined as biomass production per unit of water consumed [53], and higher values are associated with the maintenance of cell turgor and water status, which are especially important for plant growth under restricted conditions [52]. Indeed, WUE was improved through BLACKJAK® application, which may help the lettuce to maintain leaf water status under low levels of N. Furthermore, the analysis of Chl *a* fluorescence emission provides an approximation of the photosynthetic status of a plant. RC/ABS indicates the proportion of active reaction centers, whereas PI_{ABS} refers to the effectiveness of photosystems I and II [2]. The higher values of RC/ABS and PI_{ABS} in lettuce plants grown under 1 mM N and subjected to HS, compared to plants not treated with HS, indicate a better photosynthesis performance induced by HS applications, as reported by other authors [44]. Hence, our results suggest that BLACKJAK® improves the photosynthesis capacity of lettuce under limited N conditions, and ultimately, the nutritional N status, due to the close connection between photosynthesis and N metabolism.

The effectiveness of BLACKJAK® in N uptake and assimilation was tested by measuring N accumulation, the activity of various N metabolism enzymes, and the levels of AAs and soluble proteins. The source of N applied to lettuce in our study was $NaNO_3^-$. Thus, it is relevant to estimate the capacity of plants to uptake and accumulate NO_3^-. In addition, higher accumulation of organic N forms is indicative of improved N assimilation efficiency, which ultimately has a positive impact on plant growth and productivity [32,33]. Therefore, as observed in different studies [2,6,54], plants with a decrease in vegetative growth show a reduction in NO_3^- and organic N accumulation, as well as total N, as observed in our study. Nevertheless, radicular and foliar BLACKJAK® treatments enhanced NO_3^-, organic N, and total N concentration at all three N levels, which correlated with the improved shoot biomass. In a similar way, purified HA applied to roots caused the translocation of NO_3^- from roots to shoots, increasing its concentration in cucumber leaves [55]. Vujinović et al. [56] found, in maize plants, that water-extractable HS and dissolved HS extracted from organic and conventional farming increased NO_3^- uptake. This improvement in N absorption has been postulated as one of the main HS action mechanisms to enhance

N nutritional status by reducing soil pH and regulating the activity of the root plasma membrane H^+-ATPase [57], as well as the expression of different N transporters [58,59]. Hence, BLACKJAK® increases N uptake and accumulation, probably by inducing the expression of NO_3^- transport genes. However, further research at the molecular level is needed to elucidate this hypothesis.

The assimilation of N into organic forms (i.e., AAs and proteins) is carried out by different key enzymes, such as NR, that reduce NO_3^- to NH_4^+, which is then assimilated by GS and GDH to produce glutamic acid [60,61]. Navarro-Morillo et al. [6] observed in pepper plants that enhanced activity of N metabolism enzymes led to a higher accumulation of AAs and soluble proteins, resulting in greater shoot growth under N-deficient conditions. These results agree with our study, in which radicular and foliar BLACKJAK® applications enhanced the activity of NR, GS, and GDH at most of the doses employed, resulting in higher concentrations of AAs and soluble proteins under 3 and 1 mM N. Similarly, HS derived from vermicompost and applied two times directly to lettuce leaves also increased the NO_3^- concentration, NR activity, and total protein [62]. Ertani et al. [63] found that lignosulfonate humates and leonardite HA added to the nutrient solution improved GS activity and protein content in maize plants, which was later observed in the same culture by Canelas et al. [64] using soluble HS. The expression of genes involved in N assimilation has also been identified as an HS action mechanism that improves N status and plant productivity. For instance, it has been found that different genes involved in N metabolism, such as NR and those implicated in AA metabolism, were upregulated through HS applications in *Zea mays* L. [58] or by HA in *Brassica napus* L. [59]. Overall, our data suggest that BLACKJAK® applied along with the nutrient solution or directly to lettuce leaves significantly enhances N assimilation under low-N growth conditions. Nevertheless, future studies focusing on molecular biology are necessary to reveal the mechanisms of action of BLACKJAK® in improving N metabolism.

In addition, enhancing NUE, especially under N-deficient growth conditions, has been a focus of researchers due to changing environmental conditions and the aim of reducing synthetic fertilizer use [33]. Recently, we demonstrated the potential of BLACKJAK® to improve NUE in lettuce plants under optimal growth conditions [22], which was observed again in the present experiment. Similarly, under 3 and 1 mM N irrigation, radicular and foliar BLACKJAK® also enhanced NUE compared to plants not treated with HS, which indirectly influences plant growth in a positive manner under these adverse growth conditions. However, R-HS 0.60 mL/L at 1 mM N did not improve NUE due to a reduction in NUtE. This could be related to the fact that this higher radicular dose did not enhance shoot DW at 1 mM N, despite showing higher N accumulation. In addition, ANR indicates the efficiency of N absorption by plants treated with a biostimulant compared to plants not subjected to biostimulant applications [65]. In our study, all HS enhanced the ANR percentage, especially with R-HS 0.40 mL/L, F-HS 7.50 mL/L, and F-HS 10.00 mL/L in plants grown under 1 mM N. These results could explain the capacity of BLACKJAK® to enhance NUpE, NUE, N accumulation, and lettuce plant growth under the different N levels, as previously observed in lettuce treated with AA-based biostimulants in N-deficit conditions [2]. Similarly, Leite et al. [21] observed an increase in TNA and an improvement in NUE in sugarcane plants treated with HS and HA, which were mixed together with urea and applied directly to leaves. NUE was also improved in maize plants subjected to HA applied to soil [66], whereas radicular HS improved TNA, NUpE, and NUE in tomatoes [37]. Hence, our study suggests that BLACKJAK® could be a good candidate for improving NUE under low-N conditions, decreasing the use of chemical fertilizers and N leakage into the environment, although studies under field conditions should be conducted to test our results.

5. Conclusions

Radicular and foliar applications of biostimulant-based products are becoming an environmentally friendly approach among researchers and growers to improve crop yield

and NUE. According to the results obtained in the present study, the HS-based product BLACKJAK®, applied along with the nutrient solution or foliarly, improved lettuce growth under optimal (7 mM N) and low-N (3 mM N and 1 mM N) conditions. Specifically, F-HS 10.00 mL/L was the dose that most increased lettuce growth at 3 mM N and 1 mM N. The biostimulatory effects of BLACKJAK® could be associated with its capacity to enhance photosynthesis performance and N accumulation forms such as NO_3^- and organic N, as well as N assimilation enzymes, amino acids, and protein concentrations in the three N conditions. Consequently, regardless of the N level, NUE was improved by BLACKJAK® applications (except for R-HS 0.60 mL/L at 1 mM N). Therefore, our study suggests that the leonardite HS-based product BLACKJAK® could be a good candidate for reducing the overuse of chemical fertilizers, improving lettuce growth and NUE under low-N conditions. However, future research is required to elucidate the physiological mechanisms of action of BLACKJAK® to alleviate the low-N symptoms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14091492/s1, Table S1: Effect of different N supplies and HS application on TNA, NUpE, NUtE, and NUE.

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Conflicts of Interest: F.M. and G.M. certify that they are employed in Sofbey S.A. and hereby declare that they provided the biostimulant used in this experiment, as well as participated in writing and editing this manuscript. The remaining authors declare no conflicts of interest.

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