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Innovative Cultivation Practices for Reducing Nitrate Content in Baby Leaf Lettuce Grown in a Vertical Farm

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Abstract: The aim of this research is to introduce innovative cultivation practices that result in reduced nitrate levels in baby leaf lettuce grown under vertical farming conditions while maintaining high productivity. For this reason, three experiments were conducted. The first experiment focused on the impact of two "white" light spectra with a blue:green:red:far-red ratio of 14:32:43:10 ($B_{low}R_{high}$) and 21:34:36:7 ($B_{high}R_{low}$). The second experiment assessed the effects of two nitrogen supply conditions: sufficient total nitrogen (N_{15}) and limited total nitrogen (N_5), and foliar biostimulant application. In the third experiment, the impact of replacing the nutrient solution in the N_{15} treatment with tap water for an additional 24 h (TW24) on leaf nitrate content was examined. Results from the lighting experiment revealed no significant effects on agronomical parameters or nitrate content between the two light spectra. Reducing nitrogen content in the nutrient solution reduced leaf nitrate content but negatively influenced agronomical characteristics. Biostimulant application and replacing the nutrient solution with water reduced leaf nitrate content compared to the control and positively affected growth. The most favorable outcomes were observed in plants supplied with sufficient nitrogen and foliar biostimulant but also cultivated for an additional 24 h with tap water (Sp-N_{15}-TW24).

Keywords: vertical farming; nitrates; baby lettuce; white light; biostimulant; protein hydrolase; hydroponics

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1. Introduction

Lettuce (*Lactuca sativa* L.) stands out as one of the most commonly used leafy vegetables, contributing to the improvement of human diets, whether consumed as fresh-cut leaves, whole heads, or as "baby leaf" [1,2]. The term "baby leaf" refers to leafy vegetables that are harvested beyond the seedling stage but typically before the formation of more than eight true leaves [3]. "Baby leaves" are considered key ingredients in minimally processed salad mixtures due to their rich nutrient content and antioxidant potential [4]. Since ready-to-eat baby leaf vegetables are harvested as entire leaves and not as whole heads, they require minimal processing, resulting in less bruising, minimal oxidation, and longer storage potential [5,6].

Concerns regarding the effect of high nitrate levels in consumed products and the possible relationship between dietary nitrate intake and various types of cancer have led to efforts towards reducing nitrate levels in leafy vegetables as a precautionary measure [7–9]. Lettuce, similar other "baby leaf" vegetables, may contain elevated nitrate levels in the edible leaf tissues [10,11]. The World Health Organization (WHO) has established an acceptable daily intake (ADI) for nitrate at 3.7 mg kg^{-1} body weight. Similarly, the European Commission has set specific thresholds for different leafy vegetables (Commission

Regulation (EU) No 1258/2011 [12]). The legislation refers to the following six categories: fresh spinach, preserved deep-frozen spinach, fresh lettuce (protected and open-grown), "iceberg" type lettuce, rucola, processed cereal-based foods, and baby foods for infants and young children, none of which is in the "baby leaf" category. The thresholds referring to vegetables range from 2000 to 5000 mg of nitrate per kg of fresh weight, depending on the vegetable type and cultivation season, whereas foods for infants and young children have a maximum level of 200 mg of nitrate per Kg of fresh weight. Specific population groups, such as vegetarians, may face a higher risk due to consistent exposure to elevated dietary nitrate intake [13].

To that end, several agronomical practices have been proven effective in improving the nitrate levels of baby leaf lettuce. For example, in soilless agriculture the nitrate content can be controlled through the manipulation of the nutrient solution [14–17]. By limiting the nitrate concentration of the supplied nutrient solution, a reduction in the nitrate content of plant tissues is to be expected [18–21]. In vertical farms, where the light characteristics can be controlled, the nitrate accumulation has been found to depended on the spectral composition [22], light intensity [23], and photoperiod [24]. LED technology has allowed for increased control over the wavelenght percentages. Neverthelss, this has also create a vast room for experementation and has ignited a complicated search regarding the optimal light characteristics per plant, per cultivar, per cultivation stage [25]. Moreover, the vast spectra combination examined in existing literature, such as the comparison of R:B ratios from 1 to 8 as monochromatic blue and red LEDs, or combination with green and white LEDs, or even fluorescent light [26–28], makes it challenging to determine the ideal spectrum. According to research by Viršilė et al., after investigating and contrasting the impacts of various light intensities (ranging from 100 to 500 μmol m^{-2} s^{-1}) and photoperiods (from 12 to 24 h) on the growth and nitrate assimilation in both red and green leaf lettuce (*Lactuca sativa* L.), they concluded that a light intensity of 300–400 μmol m^{-2} s^{-1} and a 16–18 h photoperiod were necessary for efficient nitrate assimilation in red and green lettuces cultivated for 25 days [29].

Nevertheless, a reduction of the total nitrogen level in the nutrient solution can negatively affect the growth rate [30–32]. A promising method that can be used to reduce the nitrate content of leafy vegetables at the time of harvest is replacing the nutrient solution with water for 24 h or more before harvest. In this scenario, the nutrient solution is completely replaced with tap water for a certain amount of time, allowing the plants to assimilate the excess nitrate before harvest, or the plant trays are moved to another ebb and flow system that is supplied with tap water rather than the nutrient solution. Studies have focused on replacing the nutrient solution from 24 to a number of days before harvest, showing promising results even after the 48 h period [17,33–35]. Another novel technology that is promising for enhancing plant performance and may also mitigate nitrate levels is the use of plant biostimulants via foliar spray or fertigation [36,37]. Protein hydrolysates are a promising sub-category of biostimulants specifically defined as a combination of free amino acids or oligo- and polypeptides resulting from chemical hydrolysis (at high temperatures of 120 °C) for animal-derived protein hydrolysates and enzymatic hydrolysis (at low temperatures of 60 °C) for plant-derived protein hydrolysates [38]. When applied via foliar spray, protein hydrolysates are absorbed by the cuticle, epidermal cells, and stomata, eventually reaching the foliar mesophyll [39]. The observed plant growth is often ascribed to hormone-like activities that are stimulated through the application of protein hydrolysates [40,41]. Moreover, plant-derived protein hydrolysates have demonstrated interesting results in comparison to animal-derived ones [42], and are capable of reducing yield loss under nitrogen stress [43].

The cultivation of several types of crops, such as microgreens, baby leaves, young plants and seedlings, leafy, root-, or fruit-bearing vegetables, ornamental plants, edible flowers, and medicinal plants [44–46] on multiple horizontal layers or vertical plant panels, where lighting is provided by LEDs and the nutrients are delivered through the supplied solution as dissolved fertilizers, is referred to as "vertical farming" and the structures as "vertical farms" (VFs) or Plant Factories with Artificial Lighting (PFALs) [44,45]. Extensive research has been conducted on blue and red LEDs, primarily due to their enhanced photon absorption by plants [47]. Recently, advancements in the LED industry have greatly reduced the production cost and increased the efficiency of "white light". As a result, white light is becoming more common than in the past in vertical farming [48–51]. The idea of growing plants without solar light is not a new one. Experiments were carried out in the past for life support systems in space [52–54]. It was in the early 2000s that Toyoki Kozai et al. [55] were developing systems and conducting in-depth research on PFALs in Japan, while Despommier [56] in the US promoted vertical farming as a concept and solution to improve food security in an ever-increasing urban world. As with every new technology, vertical farming is still facing many challenges regarding its cost-effectiveness, scalability, and environmental sustainability [57,58]. Moreover, commercial vertical farms move away from the "food security" dream since solely the photon cost (% of dry market price) has been estimated by Pattison et al. [59] to be 10,000 for rice or wheat, 103 for general vegetables, 18 for tomatoes, 5 for lettuce, and 1 for leafy microgreens. Hence, the usage of vertical farms appears to be limited for now between leafy microgreens and lettuce.

Our objective was to pinpoint the key factors crucial for both future research endeavors and the commercial production of baby leaf lettuce with low nitrate levels and high yields. We investigated white light spectra, nutrient solution characteristics, nutrient solution management practices, and foliar biostimulant application. Initially, we explored the impact of different white light spectra on the agronomical characteristics and nitrate content of baby leaf lettuce under the chosen light intensity of our experimental setup, aiming to identify the spectra that would result in increased yield and quality. Based on the results of the first study and given the abbreviated cultivation cycle of baby leaves, a second experiment was conducted to examine (a) whether reduced nitrogen application in the nutrient solution composition could result only in limiting the nitrate concentration without compromising yield and baby lettuce performance and (b) whether biostimulant application can mitigate the negative effect of nitrogen stress caused by low-N application or reduce the nitrate content of baby leaf lettuce subjected to sufficient total nitrogen in the nutrient solution. Furthermore, we explored the potential of replacing the control nutrient solution with tap water for an additional 24-h period to assess whether replacing the nutrient solution with water could be employed to reduce leaf nitrate content without impeding growth. In sum, this research represents a significant advancement toward defining and leveraging innovative solutions for baby leaf lettuce cultivation.

2. Materials and Methods

In the present study, three experiments were carried out. The first experiment explored the possible effects of different white light sources on the growth and nitrate content of baby leaf lettuce supplied with a control nutrient solution (total-N 15 mmol L^{-1}). In the second experiment, plants grew under one light source, supplied with either a nutrient solution with limited (total-N 5 mmol $L^{-1)}$ or control total nitrogen, and sprayed either with a biostimulant or water. At the end of the cropping season of experiment 2 half of the plants were harvested, and the rest were left for another 24 h (experiment 3). During those extra 24 h the nutrient solution was replaced with tap water (Table 1).

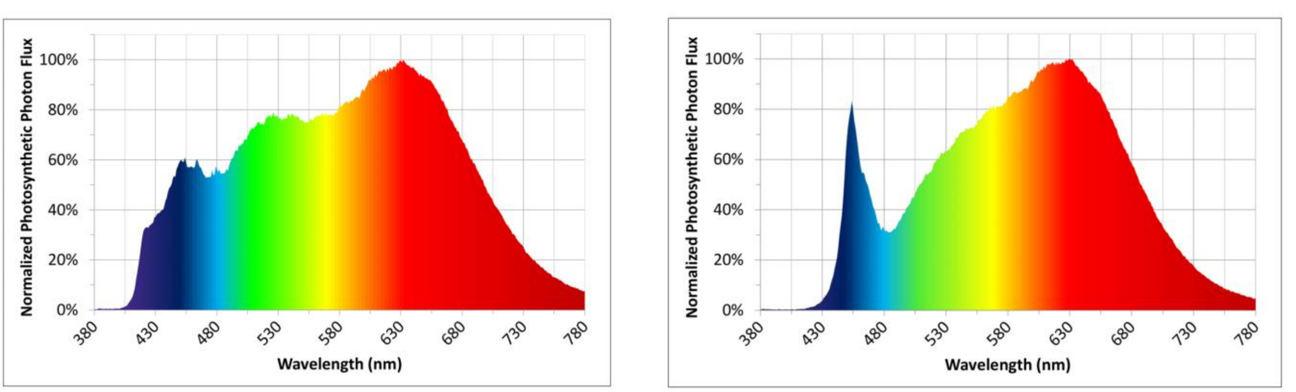
Table 1. Overview of the three experiments.

Experiment	Light Spectrum	Nutrient Solution	Biostimulant	Replacing the Nutrient Solution	Research Questions
1	$B_{low}R_{high}$ (14:43) $B_{high}R_{low}$ (21:36)	EC 2.5 dS m^{-1} Total-N 15 mmol L^{-1}	–	–	Does the slight difference in the light spectra affect the cultivation of baby leaf lettuce?
2	$B_{low}R_{high}$ (14:43)	EC 2.5 dS m^{-1} Total-N 15 mmol L^{-1} EC 1.5 dS m^{-1} Total-N 5 mmol L^{-1}	NoSpray Spray NoSpray Spray	–	Is it possible to successfully cultivate baby leaf lettuce in reduced total nitrogen conditions? If not, can biostimulant application make up for the lack of total nitrogen in the nutrient solution?
3	$B_{low}R_{high}$ (14:43)	EC 2.5 dS m^{-1} Total-N 15 mmol L^{-1}	NoSpray Spray	24 h	Can the application of biostimulants increase the positive impact of replacing the nutrient solution with water on nitrate content reduction? Can the nitrate content be further reduced by replacing the nutrient solution with water for 24 h before harvest?

B:R proportions refer to the blue and red wavelengths of the two light spectra used in the experiment. The B:R proposition is used as an indicator to differentiate the two light spectra in the text. The Normalized Photosynthetic Photon Flux and wavelength percentages are included in Figure 1 and Table 2.

Table 2. Spectral characteristics of $B_{high}R_{low}$ and $B_{low}R_{high}$ white light sources.

Color	$B_{high}R_{low}$	$B_{low}R_{high}$
Blue %	21	14
Green %	34	32
Red %	36	43
Far Red %	7	10
R:B	1.7	3.1
G:B	1.6	2.3
R:FR	5.1	4.3
Efficiency µmol J^{-1}	1.8	2.4



$B_{high}R_{low}$ $B_{low}R_{high}$

Figure 1. Normalized Photosynthetic Photon Flux for spectra $B_{high}R_{low}$ and $B_{low}R_{high}$ provided by Colasse.

2.1. Plant Material and Cultivation System

For the sowing, untreated "pills" of butterhead lettuce (var. Cecilia RZ and Rijk Zwaan) were used at a sowing density of 1500 plants m^{-2}. Sowing took place on rockwool plugs (25 × 25 × 40 mm) that were connected, creating a sheet with 200 sowing positions (AO Plug, Grodan, Roermond, the Netherlands). The rockwool sheets were cut in half so that 8 pieces of rockwool were placed in one layer, each of which accommodated 100 seeds and represented one replicate (Figure S1). For all cultivation experiments, the rockwool sheets were placed in a Vegeled trolley (Vegeled™ by Collasse SA, Seraing, Belgium). The Vegeled trolley was custom designed so that different lighting profiles could be easily removed and re-installed according to the experiment's needs, and each layer was connected to a dimmer in order to control and adjust the light intensity independently. The footprint of each layer was 135 × 56.5 cm. The irrigation method used was "ebb and flow" with a recirculating nutrient solution. The nutrient solution tank was common for all layers. The irrigation frequency was set to 5 min per hour, which was the amount needed for the nutrient solution level to reach 20 mm, which was the middle of the rockwool plugs, and that level was maintained for around 2.5–3 min before being drained back to the tank. The climate conditions at the cultivation layers were measured using a Sigrow Pro sensor (Sigrow B.V., Wageningen Campus, Wageningen, The Netherlands). The average values during the day and night were 28 °C and 25 °C, 60% and 50%, and 400 ppm for temperature, relative humidity, and carbon dioxide concentration, respectively. The photoperiod was set to 12 h, and the light intensity measured at the canopy level, 30 cm away from the LED fixtures, was 450 μmol m^{-2} s^{-1}.

2.2. Different "White" Light Sources

In the first experiment, the possible effects of two different "white" light fixtures with different efficiency and market prices with slight but existing spectral differences were explored. In one layer, the control lighting profile was the "Neutral" spectrum (blue:green:red:far-red 14:32:43:10, ($B_{low}R_{high}$), whereas on the other layer, the "SunLike™" ($B_{high}R_{low}$) [60] was installed (blue:green:red:far-red composition of 21:34:36:7) (Figure 1, Table 2). Both lighting systems were Vegeled™ Eos series.

2.3. Nutrient Solutions and Biostimulant Application

Soilless cultivation systems offer effective means to manage nitrates by adjusting nutrient solution salinity and nitrogen availability. The nutrient solutions were designed using "NUTRISENSE DSS" software (www.nutrisense.online, accessed on 1 January 2024 [61]). The control solution had an EC of 2.5 dS m^{-1}, and contained nitrogen at a level of 15 mmol L^{-1} (N_{15}); the limited fertilization solution had an EC level of 1.5 dS m^{-1}; a total nitrogen treatment had 1/3 that of the control's, hence 5 mmol L^{-1} (N_5); and finally, on the third experiment, the N_{15} nutrient solution was replaced with tap water for 24 h prior to harvest (TW) (Table 3). The required amounts of fertilizer for the preparation of the two nutrient solutions are included in Table S1.

Table 3. Chemical composition of the control nutrient solution (N_{15}), limited nitrogen solution (N_5) and tap water (TW), according to chemical analysis and NUTRISENSE DSS software.

Parameter	Units	N_{15}	N_5	TW
EC	dS m^{-1}	2.5	1.5	0.32
pH		5.6	5.6	7.3
NO_3^-	mmol L^{-1}	14.32	4.89	0
NH_4^+	mmol L^{-1}	1.04	0.53	0
K^+	mmol L^{-1}	6.48	3.79	0
Ca^{2+}	mmol L^{-1}	7.24	4.22	0.9
Mg^{2+}	mmol L^{-1}	1.57	0.92	0.3
SO_4^{2-}	mmol L^{-1}	2.78	2.26	0.2
$H_2PO_4^-$	mmol L^{-1}	1.2	1.2	0
Fe	μmol L^{-1}	40	40	0
Mn^{2+}	μmol L^{-1}	5	5	0
Zn^{2+}	μmol L^{-1}	5	5	2.15
Cu^{2+}	μmol L^{-1}	0.8	0.8	0
B	μmol L^{-1}	40	40	0
Mo	μmol L^{-1}	0.5	0.5	0
Cl^-	mmol L^{-1}	4	4	0.4
Na^+	mmol L^{-1}	0.6	0.6	0.6

In the sense that the nutrient solutions' EC and pH values would have to be re-adjusted when diverged from the desired range or when the water level was reduced to a significant amount, the water level of the tank, EC, and pH values were measured every two days with Bluelab pens (Bluelab, Tauranga, New Zealand) throughout each of the experiments.

For each nutrient solution treatment, two layers were used, each time supplied with LED profiles from the Vegeled™ Eos series with the $B_{low}R_{high}$ light spectrum: blue:green:red:far-red 14:32:43:10. This light fixture was chosen based on its market price and higher efficiency compared to the $B_{high}R_{low}$ at the time of the experiment. Before the biostimulant application, the rest of the blocks were covered in order to avoid droplets reaching the plants from the "NoSpray" treatment. After biostimulant application, the plants in the "Spray" treatment were covered, and the rest were sprayed with tap water. Foliar protein hydrolysate biostimulant application started at the cotyledon stage and was repeated every 3 to 4 days. For the N_{15} treatment, which took 13 days from sowing to harvest, 3 applications were completed, whereas for the N_5 treatment, which needed 23 days to reach the 6–8 leaf stage, 5 applications were administered. Each foliar spray was applied by either spraying 0.5 L m^{-2} of solution for the Spray treatments or only water for the NoSpray treatments. The biostimulant used in this experiment was Tyson®, a hydrolyzed plant protein, by Mugaver fertilizers (Mugavero fertilizers, Italy). This biostimulant was chosen due to its promising effects demonstrated in experiments by Consentino et al. [42,43] and Sabatino et al. [62,63]. During the foliar spray, 4 rockwool blocks, with 100 plants each, were selected randomly and sprayed. The biostimulant application was administered via foliar spray at a dosage of 3 mL L^{-1} according to the supplier's suggestion. The composition is presented in Table 4.

Table 4. Composition of Tyson® protein hydrolysate biostimulant.

Tyson®	
Total nitrogen	5.0%
Organic nitrogen	4.5%
Organic carbon	25.0%
Free amino acids	13.4%

2.4. Replacing the Nutrient Solution with Tap Water 24 h Prior to Harvest

For the determination of the effectiveness of replacing the nutrient solution with tap water 24 h prior to harvest on the reduction of the nitrate content of baby leaf lettuce, plants were cultivated with the control nutrient solution (N_{15}) for 13 days. At the end of the 13th day, the nutrient solution was replaced by tap water (TW). After 1 h it was visible that there was some nutrient solution residue that had leached from the rockwool to the TW. Hence, the solution was replaced with fresh TW until the EC and pH values were as expected. The plants that were both sprayed with tap water or protein hydrolysate biostimulant during the 13-day period were harvested at the end of the light cycle of the 14th day. The chemical analysis of the tap water has been included in Table 3.

2.5. Measurements

The measurements conducted were the same for all three experiments. Harvest was conducted in regards to the definition of baby leaves when plants reached the stage of 6–8 true leaves. At harvest, 6 plants per replicate were collected to measure the individual leaf number, area, and fresh weight using the LI-3100C (LI-COR, Inc. Lincoln, NE, USA) and a Mettler PE-3600 scale (Mettler Toledo LLC, Columbus, OH, USA). In addition, 50 plants per replicate were harvested, and their total fresh weight was measured to more accurately define the yield (g m^{-2}). Subsequently, the fresh leaves of individual plants in each replicate were placed in a drying oven (STF-N 400, FALC Instruments S.L.R, Treviglio, Italia) at a temperature of 65 °C for a duration of 7 days. Following the 7-day drying period, the leaf dry weight was measured for individual plants of each replicate per treatment. The samples were then pulverized at the highest speed setting (6000–6500 rpm) of a MF 10 Microfine grinder (IKA Werke, Staufen, Germany). The resulting grated leaf tissues were carefully collected per replicate, per treatment, in sealable plastic bags to prevent moisture-induced degradation of the samples. The relative growth rate was calculated using the equation reported by De Groot et al. [64].

For the determination of the leaf nitrate content, the method of nitrification of salicylic acid was used [65]. Two technical replicates were executed by aliquoting 0.1 g of samples into 15 mL centrifuge tubes and then adding 10 mL of distilled water. The resultant mixture was subjected to a 45 °C water bath for 1 h with agitation every 15 min. The resulting solution underwent filtration through 125 mm Macherey-Nagel filter papers, and the filtrate was then transferred to sterile 15 mL tubes. Following this, 0.2 mL of the sample solution was dispensed into 25 mL centrifuge tubes, and 0.8 mL of a salicylic and sulfuric acid solution was introduced. This mixture underwent agitation for 20 min on an orbital shaker, Unitwist 300 (Biotech, Madrid, Spain). Subsequently, 19 mL of NaOH solution (2N) was added, and the samples were stirred for an additional 20 min. The same protocol was applied to the standard samples employed in the calibration curve. All samples and standards were loaded onto a microplate and subjected to photometric analysis at 410 nm utilizing the aforementioned spectrophotometer. To ascertain nitrate concentrations in fresh leaf tissues (mg kg^{-1} of leaf fresh weight), the $NO_3{}^-$ content of dry leaf tissues (mg kg^{-1} leaf dry weight) was converted by multiplication with the DW/FW ratio.

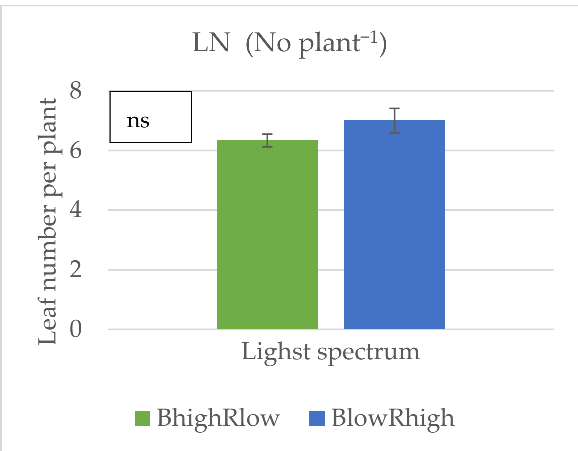
2.6. Statistical Analysis

Statistical analysis was conducted using the Statistica 10 software package for Windows (StatSoft Inc., Tulsa, OK, USA). The first and third experiments were analyzed using One-Way ANOVA and Duncan's multiple range test at a significance level of $p \leq 0.05$ for all measured variables. The lighting experiment was treated as a randomized design, even though the lighting treatments were not truly randomized. The second experiment was analyzed using Two-Way ANOVA and Duncan's multiple range test at a significance level of $p \leq 0.05$ for all measured variables. The analysis in Table 7 included data from the second experiment and the third experiment regarding the relative leaf nitrate content (compared to the control), yield, and yield/time index, which were all analyzed using One-Way ANOVA and Duncan's multiple range test at a significance of $p \leq 0.05$.

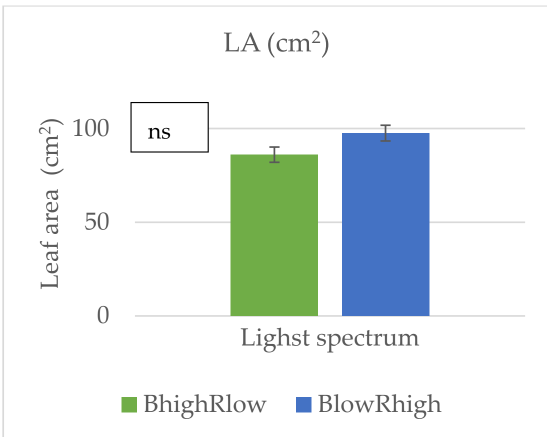
3. Results

3.1. Effect of Different "White Light" Spectra on the Yield and Quality of Baby Leaf Lettuce

The effect of two different "white light" spectra, with blue:green:red:far-red composition of 14:32:43:10 for $B_{low}R_{high}$ and 21:34:36:7 for $B_{high}R_{low}$, on the agronomical characteristics and nitrate content of baby leaf lettuce, was explored. None of the measured parameters—leaf number, leaf area, leaf fresh weight, leaf dry weight, dry matter content, or leaf nitrate content were affected by the spectral differences (Figure 2). Moreover, the leaf nitrate content remained below the safe threshold for consumption.

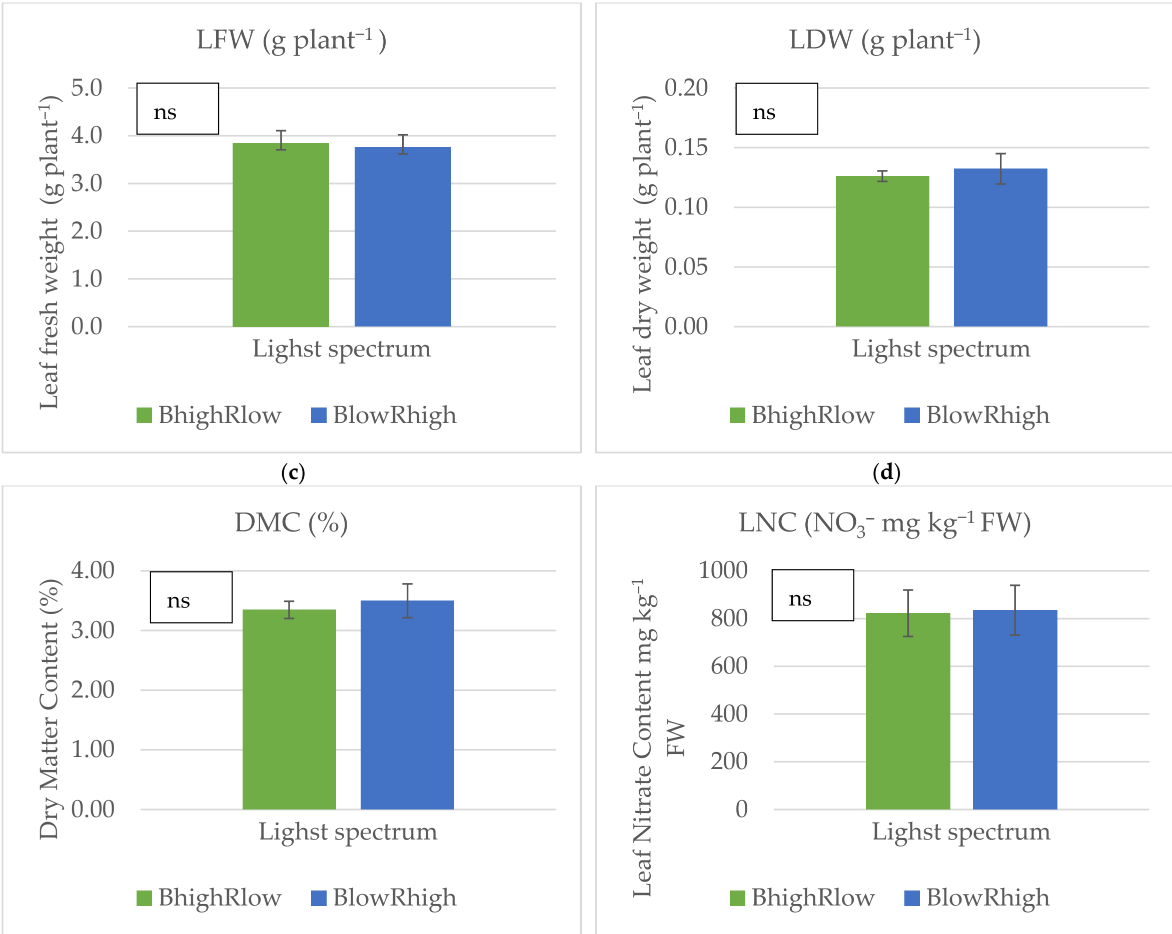


(a)



(b)

Figure 2. *Cont.*



(**c**) (**d**)

(**e**) (**f**)

Figure 2. Impact of two different kinds of "white light", a $B_{high}R_{low}$ and a $B_{low}R_{high}$, on the (**a**) leaf number (LN), (**b**) leaf area (LA), (**c**) leaf fresh weight (LFW), (**d**) dry weight (LDW), (**e**) dry matter content (DMC), (**f**) and leaf nitrate content expressed in fresh weight (LNC) of baby leaf lettuce. Values are means (n = 4 ± standard error). No statistical differences were observed according to Duncan's multiple range test at $p < 0.05$, "ns" indicates non-significant.

3.2. Effect of Different Total Nitrogen Levels and Biostimulant Application on Yield and Quality of Baby Leaf Lettuce?

The combined effect of reducing the total nitrogen level of the supplied nutrient solution and the foliar application of protein hydrolysate biostimulant on the growth and nitrate content of baby leaf lettuce was explored. Given that baby leaf lettuce is usually harvested around the 6–8 leaf stage, the plants were cultivated until that number was observed. It is important to emphasize that limiting the total nitrogen led to the elongation of the cultivation period from 13 to 22 days after sowing (DAS). Foliar application with a protein hydrolysate biostimulant appears to significantly increase the relative growth rate. Moreover, leaf number, leaf area, and relative growth rate were affected positively by the nitrogen levels of the supplied nutrient solution (Table 5). The largest leaf number, leaf area, and relative growth rate were observed in plants that were supplied with the control nutrient solution (total-N 15 mmol L^{-1}), followed by those that were supplied with limited nitrogen. In addition, interactions between the two factors (nutrient solution composition and biostimulant application) were also observed. Application with a protein hydrolysate biostimulant significantly increased the leaf area and relative growth rate, but only when the plants were supplied with sufficient nitrogen.

Table 5. Impact of foliar protein hydrolysate application (no spray, spray: NoSp, and Sp, respectively) and the nitrogen levels of the supplied nutrient solution (5 mmol L^{-1} and 15 mmol L^{-1}: N_5, and N_{15}, respectively), leaf number (LN), leaf area (LA), and relative growth rate (RGR) of baby leaf lettuce.

Treatment	LN (No plant^{-1})	LA (cm^2 plant^{-1})	RGR (g d^{-1})
		Main effects	
		Protein hydrolysate	
NoSp	6.60	69.78	0.059
Sp	6.90	77.70	0.063
		Nitrogen levels	
N_5	6.41	58.00	0.031
N_{15}	7.25	93.36	0.103
		Interaction	
NoSp-N_5	6.50	58.24 c	0.031 c
NoSp-N_{15}	6.70	87.10 b	0.096 b
Sp-N_5	6.33	57.75 c	0.031 c
Sp-N_{15}	7.75	107.62 a	0.111 a
		Statistical significance	
Protein hydrolysate (PH)	ns	ns	*
Nitrogen levels (N)	*	***	***
PH X N	ns	ns	*

Values are means (n = 4). In each column, means within the same factor followed by different letters indicate significant differences according to Duncan's multiple range test: *, ***, and "ns" indicate significance at $p < 0.05$, 0.001, and non-significant, respectively.

Foliar application with protein hydrolysate biostimulant had a slight positive effect on the leaf fresh weight and also a negative effect on the dry matter content of sprayed plants (Table 6). Moreover, the limited nitrogen treatment demonstrated lower fresh weight despite the extended cultivation period. On the other hand, the dry matter content of the limited nitrogen treatment appeared to have significantly increased compared to the control. Interactions between the two factors demonstrated that biostimulant application increased the leaf fresh weight only under sufficient nitrogen conditions. The highest values were observed in plants sprayed with protein hydrolysate biostimulant and supplied with sufficient nitrogen, followed by those sprayed with water. For plants that were supplied with limited nitrogen, protein hydrolysate biostimulant application did not counterbalance the insufficient nitrogen of the nutrient solution and did not manage to increase their fresh weight. Plants that were sprayed with protein hydrolysate biostimulant and supplied with the control nutrient solution had significantly reduced leaf nitrate content compared to plants that were sprayed with tap water while supplied with sufficient nitrogen (Table 6).

Table 6. Impact of foliar biostimulant application (no spray, spray: NoSp, and Sp, respectively) and the nitrogen levels of the supplied nutrient solution (5 mmol L^{-1} and 15 mmol L^{-1}: N_5, and N_{15}, respectively) on the leaf fresh weight (LFW), leaf dry weight (LDW), dry matter content (DMC), and leaf nitrate content expressed fresh weight (LNC) of baby leaf lettuce.

Treatment	LFW (g plant^{-1})	LDW (g plant^{-1})	DMC (%)	LNC (NO_3^- mg kg^{-1} FW)
	Main effects			
	Biostimulant			
NoSp	2.67	0.116	4.62	631
Sp	2.94	0.112	4.25	471

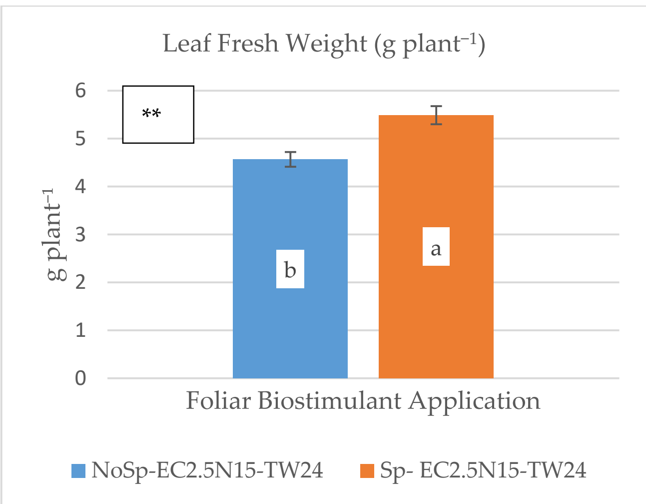
Table 6. *Cont.*

Treatment	LFW (g plant^{-1})	LDW (g plant^{-1})	DMC (%)	LNC (NO_3^- mg kg^{-1} FW)
Nitrogen levels				
N_5	2.03	0.107	5.29	404
N_{15}	3.87	0.124	3.26	698
Interactions				
NoSp- N_5	2.03 c	0.111	5.43 a	427 c
NoSp- N_{15}	3.49 b	0.124	3.58 b	836 a
Sp- N_5	2.02 c	0.104	5.16 a	391 c
Sp- N_{15}	4.32 a	0.124	2.88 c	560 b
Statistical significance				
Biostimulant (B)	*	ns	***	*
Nitrogen levels (N)	***	*	***	***
B X N	**	ns	*	**

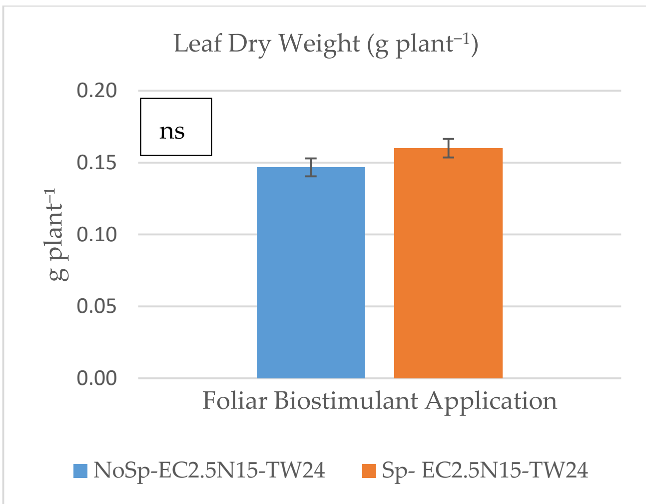
Values are means (*n* = 4). In each column, the mean within the same factor followed by different letters indicates significant differences according to Duncan's multiple range test: *, **, ***, and "ns" indicate significance at $p < 0.05$, 0.005, 0.001, and non-significant, respectively.

3.3. Does Biostimulant Aplication Affect the Leaf Nitrate Content after Replacing the Nutrient Solution with Water?

The replacement of the nutrient solution with tap water took place after the harvest of the control treatment (15 mmol L^{-1}, N_{15}). Plants that were previously sprayed with a protein hydrolysate biostimulant and plants that were sprayed solely with tap water during the cultivation period were harvested at the end of the extra 24-h cycle. During the extra 24 h in the vertical farm, supplied with tap water, leaf fresh weight and dry matter content were significantly affected by the previous biostimulant applications. The leaf fresh weight of plants that were sprayed with protein hydrolysate biostimulant maintained a significantly higher value compared to those sprayed with tap water, whereas the dry matter content remained higher in plants that were sprayed with tap water instead of biostimulant. Leaf dry weight did not differ significantly between biostimulant treatments (Figure 3a–d). The leaf nitrate content, after replacing the nutrient solution with water, of the plants previously sprayed with the biostimulant during the cultivation period demonstrated a further decrease in nitrate content compared to those that were previously sprayed with tap water.

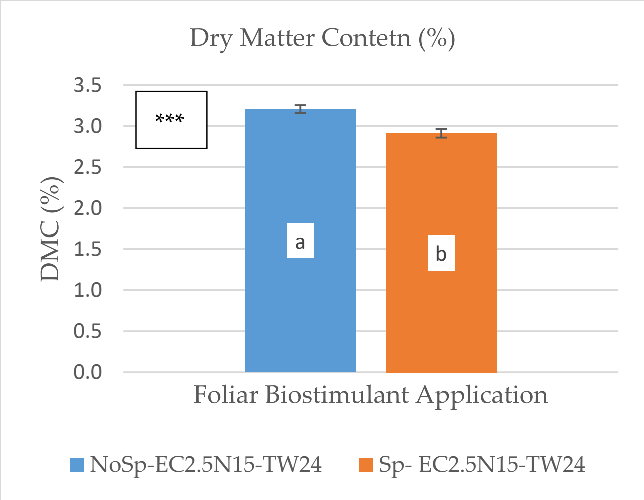


(a)

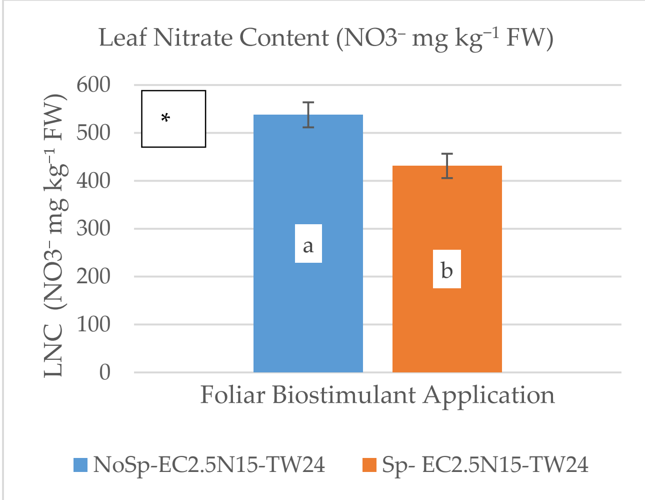


(b)

Figure 3. *Cont.*



(**c**)



(**d**)

Figure 3. Impact of replacing the nutrient solution with water for an extra 24 h (TW24) on the (**a**) leaf fresh weight (LFW), (**b**) leaf dry weight (LDW), (**c**) dry matter content (DMC), and (**d**) leaf nitrate content expressed in fresh weight (LNC) of lettuce sprayed with either the protein hydrolysate biostimulant (Sp) or tap water (NoSp). Values are means (n = 4 ± standard error). In each bar, means followed by different letters indicate significant differences according to Duncan's multiple range test: *, **, ***, and "ns" indicate significance at p < 0.05, 0.005, 0.001, and non-significant respectively.

3.4. Understanding Which Treatment Is More Effective towards Leaf Nitrate Content Decrease

To better understand the impact of each treatment on leaf nitrate content and plant yield, the control treatment, denoted as NoSp-N_{15} (non-sprayed plants cultivated with 15 mmol L^{-1} nitrogen), is compared with alternative nutrient solution management and biostimulant application treatments (Table 7). The effect of the nutrient solutions management strategies and the biostimulant application demonstrated that, compared to the control (NoSp-N_{15}), plants that were sprayed with protein hydrolysate biostimulant (Sp-N_{15}) showed a reduction in relative leaf nitrate content (67% compared to the positive control, NoSp- N_{15}). Replacing the nutrient solution with water without previously applying the biostimulant (NoSp-N_{15} -TW24) appeared to lead to a relative nitrate content of 64%, whereas plants that were previously sprayed with protein hydrolysate biostimulant had a nitrate content of 51%. Cultivating the baby leaf lettuce with limited nitrogen in the nutrient solution without foliar biostimulant application (NoSp-$N_{5)}$ and foliar biostimulant application (Sp-$N_{5)}$ resulted in 51% and 44% relative leaf nitrate content, similarly to the NoSp-N_{15} -TW24. Moreover, the yield comparison (kg m^{-2}) demonstrated statistically significant differences for the nutrient solutions, with the combination of replacing the nutrient solution with water and biostimulant substitution having the highest value. The yield of plants that were cultivated with the control nutrient solution and sprayed with protein hydrolysate biostimulant had statistically similar values to plants that were sprayed with tap water during their cultivation with sufficient nitrogen and then had the nutrient solution replaced with water and spent another 24 h. The yield of the control was significantly less compared to the aforementioned treatments but significantly greater compared to both the limited nitrogen treatments. It is noteworthy that the control group was cultivated for 13 days, the treatment of replacing the nutrient solution with water took place, extended to 14 days due to the additional day, and the limited nitrogen treatment spanned 23 days to attain the 6–8 leaf stage. In addition, an indicative index called the Yield/Time Index, expressed in kg m^{-2} $days^{-1}$, was employed to establish a comparable metric across treatments. The Yield/Time Index revealed a significant impact on nutrient solutions and biostimulant treatments, following the exact same trend as the aforementioned yield.

Table 7. Relative leaf nitrate content (LNC) comparison between the control treatment (non-sprayed plants cultivated with 15 mmol L^{-1} nitrogen; NoSp-N_{15}) and the other nutrient solution management and biostimulant application treatments, yield (kg m^{-2}) and yield/time index (kg m^{-2} $days^{-2}$) of all treatments.

Treatment	Relative LNC (%)	Yield (kg m^{-2})	Yield/Time Index (kg m^{-2} $days^{-1}$)
NoSp- N_{15}	100 a	5.23 c	0.40 c
Sp- N_{15}	67 b	6.48 b	0.50 b
NoSp-N_5	51 c	3.05 d	0.13 d
Sp-N_5	44 c	3.03 d	0.13 d
NoSp-N_{15} -TW24	64 b	6.85 b	0.49 b
Sp-N_{15} -TW24	51 c	8.23 a	0.59 a
Statistical significance			
Biostimulant X Nutrient Solutions	***	***	***

Values are means (n = 4). In each column, means within the same factor followed by different letters indicate significant differences according to Duncan's multiple range test: *** indicates significance at $p < 0.001$.

4. Discussion

4.1. Differences between "White Light" Spectra Did Not Affect the Yield and Leaf Nitrate Content of Baby Leaf Lettuce

In our experiment, two "white" light fixtures with slight spectral differences, having a blue:green:red:far-red ratio of 14:32:43:10 ($B_{low}R_{high}$) and 21:34:36:7 ($B_{high}R_{low}$), respectively, were used to cultivate baby leaf lettuce. Under Mediterranean conditions where natural light is abundant, utilizing photovoltaics during the day to directly power the LEDs could allow for the utilization of vertical farming with lower running costs. Hence, in accordance with previous research from our group on the possibility of powering the LED lighting directly through photovoltaic panels without inverters or converters [66,67], the photoperiod was set to 12 h and the light intensity was set to 450 μmol m^{-2} s^{-1} measured at the canopy level. The focus of this study was the commercial aspect of the produce, therefore the yield (g $plant^{-1}$). By the early 2000s, researchers found that the utilization of monochromatic red and blue Light Emitting Diodes (LEDs) could achieve yields comparable to those of plants cultivated under natural sunlight [68]. Apart from monochromatic LEDs of various colors of the spectrum, the white LED was also developed through phosphor technology by converting monochromatic narrowband light into broadband white light. These "white" LEDs were commercially available in 2010 [69]. This has presented numerous opportunities in the controlled environment agriculture sector and the environmental modification of plants [48,59,70]. Predominantly, research efforts have concentrated on evaluating the impact of blue, red, or their combinations due to the higher absorption of photons by plants in those wavelengths [71]. The effects of blue and red wavelengths have been found to not only affect the morphology of plants but also the nitrate content. For instance, the effect of the red light on the reduction of leaf nitrate content has been linked to increased nitrate reductase activity [72,73]. In addition, green light may offer distinct benefits to plants in some cases [74]. In another study, lettuce plants were cultivated under a combination of blue, red, and far-red basal lighting and supplemented with either UV-A, green, yellow, or orange wavelengths, resulting in a total light intensity of 300 μmol m^{-2} s^{-1} that did not demonstrate significant differences regarding the growth, though the impact on nitrite reduction was eminent. Especially between the control and the plants supplemented with green light [75]. Towards that end, the addition of green light has been found to significantly increase the activities of nitrate reductase, nitrite reductase, glutamate synthase, and glutamine synthetase, resulting in reduced nitrate content in treated plants [76]. Simultaneously, the adoption of "white light" in human lighting has reduced the initial investment costs compared to pricier, albeit typically more energy-efficient, "specialized" horticultural lamps [51,77]. Notably, variations in spectral quality, efficiency, and market price among different "white lights" should be considered before reaching a decision for commercial production [78,79]. In literature, "Neutral" white ($B_{low}R_{high}$)is the type of light between "Cool" white and "Warm" white, whose peaks lean more towards the blue and red spectrum, respectively. Hence, "Neutral" white has a higher share in the green-band [80]. On the other hand, "SunLike™" ($B_{high}R_{low}$) is a product launched in 2017, focusing on benefits especially for human-centric lighting applications [81]. To our knowledge, no public research has been published regarding its use in vertical farming and plant cultivation.

The results from our experiment indicated that agronomic characteristics, including leaf number, area, fresh weight, and dry weight, were not significantly influenced by the different "white light" spectra, nor was the leaf nitrate content. Towards that end, leaf nitrate content was at least two times smaller compared to the EU threshold for "iceberg" type lettuce (2000 mg of nitrate per kg of fresh weight), which has the lowest concentration compared to the rest of the vegetables included in Commission Regulation (EU) No 1258/2011. It has been observed that several nutrients appear in different concentrations in regards to the growth stage (microgreen, baby leaf, mature leaves) [82]. Moreover, lettuce plants harvested 2 weeks after sowing appear to have a lower nitrate content (mg kg $^{-1}$) compared to mature leaves [83]. Perhaps this is why no extra regulation exists regarding the nitrate content of baby leaves.

In our experiment, both light sources had high percentages of green photons: 36% for the $B_{high}R_{low}$ and 43% for the $B_{low}R_{high}$. Green light, despite being absorbed less efficiently compared to red and blue, can penetrate deeper into leaves and excite chlorophyll more uniformly, resulting in higher yields [84]. This could be useful in cultivation systems where plants are grown at high densities, such as in our experiment. However, a study investigating the interactive effects of light spectrum and intensity on photosynthesis found that even though at high light intensity, green light could achieve a higher quantum yield and net CO_2 assimilation rate compared to red or blue light, the maximum Rubisco carboxylation rate was not significantly affected by light spectrum [85]. We consider it important to note that, to our knowledge, the light intensity used for the cultivation of baby leaf lettuce was high (450 μmol m^{-2} s^{-1}), and accompanied by a 12-h photoperiod, this would result in a daily light integral of 19 mol·m^{-2}·d^{-1}. Further experimentation could be conducted while focusing on the reduction of the light intensity to 150 μmol m^{-2} s^{-1}, as seedling and baby leaf production is often carried out under such intensities [86], and the slight spectral differences could perhaps demonstrate a significant effect on agronomical characteristics [87,88]. Furthermore, a combination of photoperiods and light intensity resulting in a constant daily light integral could further provide significant feedback on the light needs of baby leaf lettuce. Future experiments could focus on the light intensity and spectra, including monochromatic light sources, red, blue, and their combinations. Moreover, secondary metabolites should also be analyzed, given that they can often be affected by the spectral qualities [89].

4.2. Biostimulant Application Could Not Counteract the Effects of Limited Nitrogen Supply in the Nutrient Solution but Successfully Reduced the Nitrate Content of Baby Leaf Lettuce

Baby leaves reach a growth stage similar to that of seedlings, which could mean that the nutritional needs for the cultivation of these plants would be similar to those of seedling preparation, often EC 1.2–1.8 ds m^{-2} [90,91] It has been reported that maintaining the EC values lower compared to the optimum, 1.2 ds m^{-1} instead of 2 dS m^{-1}, can lead to the same agronomical characteristics [92,93]. Another study utilizing the nutrient film technique has demonstrated high yields at an EC level of 1.4 ds m^{-2} and a plant density of 1600 plants m^{-2}, compared to EC values of 0.4, 0.8, and 1.6 ds m^{-2}, and plant densities of 400 and 100 plants m^{-2} [94]. In another experiment comparing lettuce growth cultivated on ebb and flow and floating raft systems, the results demonstrated that EC levels of 2.5 resulted in better yields than 3.5 dS m^{-1}, and that ebb and flow also resulted in reduced nitrate content on leaf tissues [95].

Given that baby-leaf lettuce is harvested at a very early stage compared to fully grown lettuce heads, we considered that preparing nutrient solutions with optimal EC levels (2.5 dS m^{-2}) might be a waste due to the limited needs of the plants that would not surpass the seedling stage and could also lead to high leaf nitrate content. In the scope of reducing fertilizer use as an agronomical practice, focusing primarily on nitrogen reduction, the total nitrogen content was reduced to 1/3 of the control. While reducing nitrogen, the total concentrations of potassium, magnesium, and calcium were followed so as to achieve the chemical balance of the nutrient solution. The resulting nutrient solution had an EC level of 1.5 dS m^{-2} and total nitrogen of 5 mmol L^{-1} (N_5). Since lettuce is a rather domesticated crop, its ability to adapt to diverse environments and nutrient limitation conditions is restricted compared to a wild edible plant, *Cichorium spinosum*, examined by our group [21], that could successfully be cultivated with reduced total nitrogen. Apart from exploring the possibility of cultivating baby leaf lettuce with lower EC levels primarily induced by the nitrogen limitation, the possibility of mitigating the nitrogen stress through the foliar application of a nitrogen-rich biostimulant was also tested.

Maintaining nitrate levels of leafy vegetables under a certain threshold, defined by the European Commission between 2000 and 5000 mg of nitrate per kg of fresh weight depending on the crop and the season [12], has been a clear goal of growers and plant scientists [96–99]. Even though the leaf nitrate content can be reduced through an increase in light intensity, photoperiod, or both, the chemical composition of the nutrient solution plays an important, if not primary, role [29,100–103]. Limited nitrogen is known to negatively affect plant growth [104,105]. On the other hand, plant biostimulants have been observed to increase plant yield even under limited nitrogen conditions and decrease leaf nitrate content [106–108]. With these two functions of plant biostimulants in mind, a protein hydrolysate biostimulant was used. Foliar application of protein hydrolysate biostimulant was administered to explore the possibility of countering the negative effects of limited nitrogen supply in the nutrient solution on the growth and the extent of reducing the leaf nitrate content of plants cultivated under sufficient nitrogen conditions for baby leaf lettuce.

The sowing density was considerably higher compared to some greenhouse experiments that had sowing densities over 600 plants m^{-2} [5,109–111] but lower compared to others that had more than 1500 plants m^{-2} [112,113]. Harvest was conducted based on the leaf number (6 to 8 true leaf stage according to the definition of baby leaves [3]) and plant size, taking into account that as soon as the plants were touching and shading each other, they should be either separated or harvested. As expected, the reduced total nitrogen content of the supplied nutrient solution (5 mmol L^{-1}, N_5) affected the growth rate in a negative way. Not only did the cultivation cycle of limited nitrogen treatment extend to 22 days instead of 13, but the leaf fresh weight, leaf area, and dry matter content were also negatively affected. Under limited nitrogen conditions, the plants required extra days to reach the commercial size and morphology of baby leaves. Hence, the decrease in leaf nitrate content that was observed in plants cultivated with 5 mmol L^{-1} total nitrogen resulted in a significant productivity reduction and should not be applied in commercial production.

A study by Di Mola et al. [107] explored the influence of plant-based biostimulants on baby leaf rockets under varied nitrogen levels and observed that biostimulants affected the yield positively. This was not observed in our experiment, perhaps due to the environmental and lighting conditions supplied by the vertical farming system in comparison to the cited experiment by Di Mola et al., which was conducted in soil. On the other hand, protein hydrolysates have been reported to play a role in regulating nitrogen uptake and reducing nitrate leaf content [114]. It is suggested that root nitrate absorption and accumulation are reduced due to the high levels of free amino acids in the applied protein hydrolysates that enrich the leaf phloem during foliar application [108]. In turn, this reduction in nitrogen concentration in plants treated with protein hydrolysates could be attributed to the utilization of their own nitrogen reserves without requiring additional nitrogen uptake [42]. It is important to note that these findings refer to soil-based experiments. To our knowledge, protein hydrolysates have not been experimented with in soilless culture, and definite little is known about the effects of foliar application of protein hydrolysate on baby leaf lettuce cultivated in vertical farms. In our experiment, the foliar application of protein hydrolysate biostimulant had a significant effect on reducing leaf nitrate content at the harvest stage.

4.3. Biostimulant Aplication Does Further Decrease Leaf Nitrate Content during the Replacement of the Nutrient Solution with Water

Effective fertigation management plays a crucial role in controlling nitrate accumulation in soilless cultures. One common practice involves lowering the electrical conductivity of the nutrient solution in the final days before harvest by either adding water or completely "replacing the nutrient solution with water" and replacing the nutrient solution with tap water [17,33]. In this section, the effect of previously sprays with protein hydrolysate on baby leaf lettuce on the effectiveness of replacing the nutrient solution with water is evaluated. As a result of replacing the nutrient solution with water, the leaf nitrate content was reduced to 537 and 431 mg kg^{-1} of fresh weight for the plants sprayed with tap water and biostimulant, respectively. These values differ significantly from one another and were significantly lower compared to the nitrate content of the control treatment (836 mg kg^{-1}, plants supplied with total nitrogen 15 mmol L^{-1} for 13 days). Hence, foliar spraying with protein hydrolysate biostimulant every 3–4 days through the 15-day cultivation period, followed by replacing the nutrient solution with water, significantly reduced the nitrate compared to plants that only received spraying with tap water.

4.4. Which Treatment Is more Effective towards Leaf Nitrate Content Decrease While Maintatining High Yields?

The goal of this study was to conclude that there is an effective and easy-to-carry out-method to reduce the leaf nitrate content of baby leaf lettuce without negatively affecting the growth or final yield. The European Commission has established defined thresholds for various leafy vegetables, as outlined in Commission Regulation (EU) No 1258/2011 [12]. These thresholds vary between 2000 and 5000 mg of nitrate per kg of fresh weight, contingent upon the type of vegetable and the season in which it is cultivated. All treatments had nitrate levels below the threshold, which can be attributed to the high light intensity used in the experiment (450 µmol m^{-2} s^{-1}). In the control treatment, the plants were cultivated with the N_{15} nutrient solution with an EC level of 2.5 dS m^{-2} containing nitrogen at a level of 15 mmol L^{-1} and were sprayed with tap water, while growing from seed to harvest under a light intensity of 450 µmol m^{-2} s^{-1} and a 12-h photoperiod. The results demonstrated a leaf nitrate content of 836 mg kg^{-1} FW, a yield of 5.23 kg m^{-2}, and a yield/time index of 0.40 kg m^{-2} days^{-1}. The foliar application of protein hydrolysate biostimulant every 3–4 days demonstrated that it could lead to a 33% decrease in leaf nitrate content in plants cultivated with the control nutrient solution (Sp-N_{15}) while increasing the yield to 6.48 kg m^{-2} and a significantly higher yield/time index of 0.50 kg m^{-2} days^{-1}. The results revealed that even though limiting fertilizer use results in EC levels close to 1.5 dS m^{-2} has been found to give better results compared to higher EC levels in some studies [90,92,94] additionally limiting the total nitrogen content to 5 mmol L^{-1} throughout the cultivation period, which in this case lasted 23 days, led to a significant compromise in both the growth and final yield of the crop (3.03 and 3.05 kg m^{-2} and 0.13 and 0.13 kg m^{-2} days^{-2} for plants that received protein hydrolysate biostimulant spray or tap water, respectively), counterbalancing the benefits of achieving lower leaf nitrate content (56% and 49% for plants that received protein hydrolysate biostimulant spray or tap water, respectively). We consider this effect to be ascribed mainly to the total nitrogen content, since in the aforementioned studies, the reduced concentration of the other elements did not affect the growth in a negative way. The decrease in nitrate level during nutrient solution replacement has been demonstrated in the past [95], but depending on the length of the treatment, it is possible to negatively affect the resulting yield. In this experiment, a period of 24 h was applied while taking into account the current lighting conditions in an effort to significantly affect the nitrate levels without compromising yield.

Results demonstrated a significant decrease in the leaf nitrate content of 36% and 49%, while benefiting from the extra cultivation day by resulting in a yield of 8.23 and 6.85 kg m^{-2} and a yield/time index of 0.59 and 0.49 kg m^{-2} $days^{-1}$. for plants that received protein hydrolysate biostimulant spray or tap water, respectively. The best results in terms of yield, yield/time index, and nitrate content were observed in the treatment where plants were cultivated with 15 mmol L^{-1} nitrogen for 13 days while being sprayed with protein hydrolysate biostimulant every 3–4 days, followed by an extra day after replacing the nutrient solution with tap water (Sp-N_{15}-TW24).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10040375/s1, Figure S1: (a) Sprouted lettuce cultivated in one layer of the vertical farm on rockwool plug-sheets. Each sheet accommodates 100 seedlings and represents one replicate. In each layer, eight pieces were placed from sowing to harvest and treated accordingly to the experimental design. (b) Baby leaf lettuce with 6–8 leaves at the harvest phase. (c) Sideview of a vertical farm at the harvest phase. Upper layer: $B_{low}R_{high}$; base layer: $B_{high}R_{low}$. (d) Top view of rockwool sheets during harvest.; Table S1: Required amounts of fertilizers for the preparation of stock solutions A and B for the two nutrient solution treatments.

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