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Effects of Plant-Derived Protein Hydrolysates on Yield, Quality, and Nitrogen Use Efficiency of Greenhouse Grown Lettuce and Tomato

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Abstract: Plant-derived protein hydrolysates (PHs) are gaining global interest for their sustainability and positive effects on crops under abiotic stresses. However, the long-term effects of PHs on the yield and quality of greenhouse crops have not been described. Romaine lettuce (*Lactuca sativa* L.) and Micro-Tom tomato (*Solanum lycopersicum* L.) were grown with commercial growing media in 1 L pots and fertigated with four N levels (2, 5, 10, and 15 mM). PH (0 or 3 g/L) was applied as a foliar spray (PH-F) or root drench (PH-R) once every week. Compared to PH-F, PH-R effectively stimulated the yield, photosynthesis, water-use efficiency, chlorophyll contents, and antioxidant activities, and compounds regardless of N levels and species. Increasing the N level led to a total dry weight gain, and PH-R enhanced the lettuce shoot dry weight (+31%) and tomato fruit dry weight (+22%). PH-R also increased the fresh marketable yield of lettuce (+21%) and tomato (+32%). The increasing N level decreased antioxidant parameters in lettuce leaves, not in tomato fruits, whereas PH-R improved them in both species. PH-R significantly enhanced the N use and uptake efficiency. Taken together, our results suggested that the addition of PH-R effectively increased the N uptake and subsequently increased the lettuce and tomato yield and quality regardless of N levels.

Keywords: biostimulants; protein hydrolysate; nitrogen use efficiency; nitrogen uptake efficiency; *Solanum lycopersicum* L.; *Lactuca sativa* L; antioxidant activity

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1. Introduction

The world's population is continuously growing and therefore so is the demand for fresh and nutritious food. In addition, the world's arable land has been reduced due to erosion and excessive chemical use. Controlled environment agriculture has the potential to provide a solution due to its high crop production per area and the easy management of crop growth and quality compared to traditional open field crop production [1–4]. In addition, a widespread trend has seen agriculture move towards sustainable crop production because conventional agricultural practices are considered harmful to the environment and greenhouse crop production also been encouraged to adopt sustainable compromises [5,6]. The excessive use of nitrogen (N) fertilizers which are often applied to ensure the high yields of agricultural products, in addition to leaching and runoff from the production facilities could lead to the significant contamination of water bodies [7,8]. Under this pressure, farmers must optimize fertilizer management to reduce nutrient pollution and preserve the economic margin by following sustainable production practices.

Plant biostimulants are a large group of substances and microorganisms derived from natural and biological sources that improve crop growth and quality, nutrition uptake,

and/or abiotic and biotic stress tolerance with a small amount of application [9,10]. Particularly, protein hydrolysates (PHs) are one emerging category of biostimulants due to its positive effects on crop growth and yield under various abiotic stresses [11]. PHs are largely achieved by heat, chemicals (acid or alkaline), and/or proteolytic enzymes using various source of proteins such as those of animal origin (animal epithelial tissue, collagen, blood, feather [12–15]) and plant origin (legume, alfalfa, soybean [16–18]). Various PHs products derived from plant and animal origins have been released to the market. Compared to animal-derived PHs, plant-derived PHs are more eco-friendly and cost-effective because they are produced from agricultural byproducts through mainly enzymatic hydrolysis, while animal-derived PHs require chemical and thermal hydrolysis with high pressure [16].

In greenhouse crop production, soilless growing media, also known as root substrates, are commonly used to provide a pathogen-free root environment and proper physical and chemical properties such as enough air space, water-holding capacity, and pH [19,20]. Originally, growing media were formulated by growers with their own recipes, but today, most growers purchase these from commercial growing media companies [20]. Particularly, the chemical properties of a growing media are important because the pH of the growing media affects nutrient solubility and the forms of the nutrient, subsequently changing the N uptakes [21]. The effects of plant-derived PH under different N levels were investigated in spinach, lettuce, rocket, and tomato grown in either field soils or quarziferous sand, with nitrate being the main N source for the treatments [22–24]. Interestingly, increasing N fertilization increased the growth and yield of field soil-grown spinach, lettuce, and rocket but lowered the quality by significantly reducing the antioxidant activities. Meanwhile, at low N fertilization, the foliar application of PH (only foliar application was tested) alleviated growth and yield loss while securing antioxidant activities, which underlines the potential benefits of PHs to enhance crop growth and N uptake under low N input. Although Sestili et al. (2018) compared the root application and foliar application of PH in tomato seedlings, the most effective method for improving the yield and quality of tomato fruits remains unknown. In addition, the above studies grew crops with field soils or quarziferous sand, despite the fact that the effects of different PH application methods on greenhouse crops grown with commercial growing media under various N levels remain unknown.

The aims of the current study were (1) to investigate the effects of the different PH application methods on the growth, physiology, and the quality of greenhouse lettuce and tomato under four different N levels; and (2) to evaluate the effects of the root application of PH on the nitrogen use, uptake, and utilization efficiencies of plants.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The experiment was conducted in a glass-glazed greenhouse situated at Purdue University, West Lafayette, IN (40° N, 86° W). Seeds of romaine lettuce (*Lactuca sativa* L. var. longifolia Lam.-cv. Dragoon) and dwarf tomato (*Solanum lycopersicum* L.-cv. Micro-Tom) were purchased from Johnny's Selected Seeds (Winslow, ME, USA) and Totally Tomatoes Company (Randolph, WI, USA), respectively, and germinated in a commercial germination mix (Berger BM2, Saint-Modeste, QC, Canada) in a climate room under 150 μmol m^{-2} s^{-1} using full-spectrum LEDs (320-W "VYPRx", Fluence Bioengineering, Inc., Austin, TX, USA) for 18 h d^{-1}. The germination mix with seeds was irrigated with tap water, and a 1/4-strength Hoagland solution was provided once the seeds germinated and until the development of true leaves [25]. After 14 days and 21 days for lettuce and tomato, respectively, uniform seedlings were randomly selected and transferred to a glass-glazed greenhouse. The seedlings were transplanted in 1 L pots filled with commercial growing media (Berger BM8, Saint-Modeste, QC, Canada) and fertigated with a half-strength Hoagland solution using drip system. Lettuce and tomato were grown until 4 weeks and 8 weeks after transplant, respectively. A photoperiod of 14 h (8:00 a.m. to 10:00 p.m.) was provided with the combination of natural daylight and supplemental lighting using high-pressure sodium lamps (600 W, P.L. Light Systems Inc., Beamsville,

ON, Canada). Day/night temperatures were set at 24/18 °C with an hour transition between them. The greenhouse temperature was regulated as needed using a pad-and-fan evaporative-cooling system, retractable shade curtains, and radiant hot-water-pipe heating by an environmental control system (Maximizer Precision 10, Priva Computers Inc., Vineland Station, ON, Canada).

2.2. Treatments

N treatments consisted of four N levels: 2 mM, 5 mM, 10 mM, and 15 mM. The composition of all nutrient solutions is shown in Table 1. Each N treatment consisted of half-strength modified Hoagland's solution [26] to provide the same amount of nutrients except N, and $Ca(NO_3)_2 \cdot 4H_2O$, and NH_4NO_3 were used as a N source. The nutrient solution was replaced every week and the pH of the nutrient solution was adjusted within a range of 5.6–5.9 throughout the experiment by adding H_2SO_4 or NaOH. The biostimulant used in the experiment was a plant-derived protein hydrolysate (PH) (Trainer® manufactured by Hello Nature Inc., Anderson, IN 46016, USA) extracted through an advanced enzymatic hydrolysis of proteins from legume seeds. The PH contained 35.5% organic matter, 5% total nitrogen, and 27% amino acids and soluble peptides. The PH application started right after transplanting the plants into the hydroponic system. PH solution was freshly prepared at a concentration of 3 mL L^{-1} and applied once a week. Two application methods, foliar spray (PH-F) or root application (PH-R), were employed to apply PH to the plants. For PH-F, 50 mL of the solution was sprayed to cover the entire surface of the plant shoots while protecting roots from drip. Plants were sprayed in the afternoon and held overnight to allow leaves to dry. For PH-R, 50 mL of the solution was applied to each plant over growing media onto the roots. For both treatments, plants were not watered for 24 h after the application.

Table 1. Chemical composition of nutrient solutions used in this study which were set at four different total nitrogen concentrations of 2, 5, 10, or 15 mM.

N Level	Chemical Composition (mM)						
	KNO_3	$Ca(NO_3)_2$	NH_4NO_3	KH_2PO_4	KCl	$CaCl_2$	$MgSO_4$
2 mM	1	0.1	0.4	1	2	2.9	0.5
5 mM	1	1	1	1	2	2	0.5
10 mM	2	2	2	1	1	1	0.5
15 mM	3	3	3	1	0	0	0.5

The other micronutrients were the same for all treatments (half-strength of a modified Hoagland solutions, after Epstein 1972).

2.3. Plant Growth and Yield Measurements

At 4 and 8 weeks after transplanting the lettuce and tomato, respectively, leaf samples were harvested and placed on a black cloth without overlapping for leaf area measurements. A piece of red paper (4 cm × 4 cm) was placed next to the leaf samples to calibrate the image processing steps. Photographs were taken with a Canon EOS Rebel T6 (Canon Inc., Tokyo, Japan) and the images were used to analyze the leaf area using the image analysis software ImageJ [27]. Some portions of the leaf and tomato fruit samples were quickly frozen with liquid N and stored at −80 °C for future analysis. The rest of the leaves were dried in a forced-convection oven at 70°C (Heratherm OMH400, Thermo Scientific Inc., Waltham, MA, USA) for at least 3 days to obtain a constant weight. The total shoot dry weight (DW) was estimated by multiplying a total fresh weight (FW) with the ratio of the (total shoot DW–subsample DW) and (total shoot FW–subsample FW). All dry samples were ground through a 40-mesh sieve with a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ, USA) and kept in plastic vials at room temperature for future nutrient analysis. All growth parameters and yield traits, including dry biomass (shoot, root, and fruit), fresh yield, fruit number, fruit mean weight, and harvest index (dry weight basis), were also measured and presented in Table 2.

Table 2. Effects of plant-derived protein hydrolysates (PHs) application on growth parameters and yield traits of lettuce and tomato. Lettuce and tomato plants were grown in a 1 L pot filled with commercial growing media and fertigated with 4 different N levels (2 mM, 5 mM, 10 mM, and 15 mM). PHs was applied as either foliar spray (PH-F) or root drench (PH-R) every week at a rate of 3 mL L^{-1}.

Treatments		Dry Biomass (g $plant^{-1}$)				Fresh Yield (g $plant^{-1}$)	Fruit Number (n $plant^{-1}$)	Fruit Mean Weight (g $fruit^{-1}$)	Leaf Area (cm^2 $plant^{-1}$)	Harvest Index (g g^{-1} Dry Weight Basis)
		Total	Shoot	Root	Fruit					
Plant Species: Tomato						**(Fruit)**				**(Fruit/Total Biomass)**
N level	2 mM	12.7 c	4.2 c	0.28 b	8.2 c	97.1 c	46 b	0.19 b	405 b	0.645
	5 mM	17.2 b	5.7 b	0.33 ab	11.2 b	126.5 b	46 b	0.26 a	529 ab	0.647
	10 mM	20.7 a	7.0 a	0.39 a	13.3 a	146.7 a	57 a	0.24 a	597 a	0.645
	15 mM	21.0 a	6.8 ab	0.38 a	13.4 a	147.2 a	59 a	0.25 a	517 ab	0.659
PH	Control	15.9 b	5.2 b	0.32 b	10.3 c	117.8 b	42 b	0.25	468	0.649
	PH-F	18.2 ab	6.1 ab	0.36 a	11.8 b	124.8 ab	56 a	0.22	502	0.651
	PH-R	19.4 a	6.5 a	0.37 a	12.6 a	142.9 a	58 a	0.23	564	0.648
Significance	N level	***	***	**	***	***	*	**	**	-
	PH	*	*	*	*	***	**	-	-	-
	N × PH	-	-	-	-	-	-	-		-
Plant Species: Lettuce						**(Shoot)**				**(Shoot/Total Biomass)**
N level	2 mM	5.1 c	4.8 c	0.33 a		84.2 c			1199 c	0.934 b
	5 mM	5.7 c	5.5 c	0.24 c		138.6 b			1589 b	0.958 a
	10 mM	6.9 b	6.6 b	0.25 bc		173.6 a			1744 ab	0.963 a
	15 mM	8.1 a	7.8 a	0.31 ab		178.8 a			1813 a	0.960 a
PH	Control	5.4 b	5.1 b	0.29		118.4 b			1418 b	0.946 b
	PH-F	6.6 a	6.3 a	0.27		150.5 ab			1589 ab	0.957 a
	PH-R	7.0 a	6.7 a	0.27		156.8 a			1709 a	0.957 a
Significance	N level	***	***	***		***			***	***
	PH	**	**	-		**			*	*
	N × PH	-	-	-		-			-	-

Data are the average of 5 replicates. ANOVA results, -, *, **, *** means not significant or significant at $p \leq 0.05$, 0.01, 0.001, respectively. The difference letters indicate significant difference according to the Tukey test, $p \leq 0.05$.

2.4. Leaf Chlorophyll and Carotenoid Concentrations, and Leaf Gas Exchange

Chlorophyll a (Chl_a), chlorophyll b (Chl_b), carotenoid contents in leaves were determined as previously described by Lichtenthaler and Wellburn (1983) [28], with a small modification. Freeze-dried powder (10 mg) of each sample was extracted with 1.5 mL 80% acetone at 4 °C overnight and centrifuged at 14,000× *g* for 3 min. The optical density of the supernatant was determined at a wavelength of 470, 646, and 663 nm using an Epoch Microplate spectrophotometer (Epoch, Biotek, Winooski, VT, USA). The contents were determined by the equations below.

$$Chl_a\ (\mu g/mg) = 12.21\ (A_{663}) - 2.81\ (A_{646})$$

$$Chl_b\ (\mu g/mg) = 20.13\ (A_{646}) - 5.03\ (A_{663})$$

$$\text{Carotenoids}\ (\mu g/mg) = (1000A_{470} - 3.27Chl_a - 104Chl_b)/229$$

Gas-exchange measurements was conducted as described by Yang and Kim. (2020) [29] with a portable gas-exchange system (LI-6400XT; LI-COR Biosciences, Lincoln, NE, USA) by clipping young fully expanded leaves inside the 6 cm^2 leaf chamber with built-in LEDS (470 and 665 nm peak wavelengths for the blue and red LEDs, respectively). Illumination was supplied at a photosynthetic photon flux of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ by the light source under ambient temperature conditions. The reference CO_2 concentration was 400 $\mu mol \cdot mol^{-1}$ and the flow rate through the chamber was 500 $\mu mol \cdot s^{-1}$. The measurements of net CO_2 assimilation rate (*Pn*), stomatal conductance (gs), transpiration rate, and internal CO_2 concentration were performed between 9:00 a.m. and 14:00 p.m. The readings were recorded after the total coefficient of variation (the sum of variations of air-flow rate, CO_2, and water-vapor differentials) was less than 0.2%. The intrinsic water-use efficiency (WUE) was calculated by dividing *Pn* by gs.

2.5. Use Efficiency and Uptake Efficiency of N

Total N contents were determined for oven-dried samples, ground through a 40-mesh sieve with a Wiley mini-mill (Thomas Scientific, Swedesboro, NJ, USA). Thirty milligrams of each sample was placed into an empty sample tin and carefully wrapped to prevent sample loss. Total N was determined using the FlashEA (C/N machine, Swedesboro, NJ, USA) as described by Bhattacharyya et al. (2015) [30].

Nitrogen-use efficiency (NUE), nitrogen-uptake efficiency (NUpE), and nitrogen-utilization efficiency (NUtE) were calculated from the following equations:

$$\text{NUE} = \frac{T}{N_A},\ \text{NUpE} = \frac{N_T}{N_A},\ \text{NUtE} = \frac{T}{N_T}$$

where T is the total dry weight of tomato fruits or lettuce shoot (g), N_A is the total N applied (g) to plants throughout the production period, and N_T is the total N contents in tomato fruits or lettuce shoot (g).

2.6. Determination of Antioxidant Activities, Total Phenolic and Flavonoid Compounds, and Lycopene Concentration

2.6.1. Extract Preparation for Phytochemical Analysis

Frozen lettuce leaf and tomato fruit samples were lyophilized in a freeze-dryer (Labconco Freeze Dry System, Kansas City, MO, USA). Sample extraction and phytochemical analysis were conducted as previously described by Ku et al. (2013) [31] with minor modifications. Twenty milligrams of fine powder of freeze-dried samples were extracted in 1.4 mL of 70% methanol at 95 °C for 10 min. The extract was cooled on ice and centrifuged at 3000× *g* for 10 min. The supernatant was transferred in a 2 mL microcentrifuge tube (Fisher Scientific, Waltham, MA, USA) and centrifuged at 4700× *g* for 2 min. Then, the supernatant was used for the antioxidant activity and total phenolic content.

2.6.2. Determination of Antioxidant Activity via DPPH Free Radical Scavenging Assay

The sample extract (10 µL) was combined with 190 µL of a 200 µM DPPH in ethanol and incubated at room temperature for 30 min in the 96-well plates. The absorbance of the DPPH free radical was measured at 515 nm and various concentrations of vitamin C were used to generate a standard curve.

2.6.3. Determination of Total Phenolic Compounds (TPCs)

The sample extract (10 µL) and 100 µL of 2 N Folin–Ciocalteu reagent was added in the 96-well microplate and incubated for 3 min at room temperature. Then, 90 µL of Na_2CO_2 (7.5%) was added and incubated for 60 min in the dark at room temperature. Absorbance was obtained at 735 nm and 6.25 to 200 µg/mL of gallic acids was used for a standard curve.

2.6.4. Determination of Total Flavonoid Compounds (TFCs)

The sample extract (20 µL) was added in the 96-well. Then, 40 µL of distilled water and 6 µL of 5% (w/v) $NaNO_2$ were added to each well. After 5 min, 12 µL of 10% (w/v) $AlCl_3$ was added to each well. After 6 min, 40 µL of 1 M NaOH with 42 µL of distilled water were added to each well. Absorbance was obtained at 515 nm, and 0–100 µg/mL of quercetin in 80% methanol was used for a standard curve. The optical density was measured using an Epoch Microplate spectrophotometer (Epoch, Biotek, Winooski, VT, USA).

2.6.5. Determination of Lycopene Concentration

Lycopene concentration of tomato fruits was determined as previously described by [32]. Tomato fruits were homogenized, and 5 g of sample was extracted by adding 50 mL of hexane/acetone/ethanol (2:1:1, v/v/v) for 30 min. The extract was used to determine the total lycopene content using an Epoch Microplate spectrophotometer (Epoch, Biotek, Winooski, VT, USA). Absorbance was obtained at 472 nm, and pure lycopene (Sigma-Aldrich, St. Louis, MO, USA) was used for a standard curve. The total lycopene content was presented in mg 100 g^{-1} FW.

2.7. Experimental Design and Statistical Analysis

Each treatment consisted of 5 replicates, and plants were arranged in a randomized complete block design. All data were subjected to two-way analysis of variance using JMP for Windows, Version 15.0 (SAS Institute Inc., Cary, NC, USA). Mean separation within each measured parameter was conducted by Tukey's honestly significant difference (HSD) test at $p < 0.05$. Regression analysis was carried out to look for trends in response to different N concentrations.

3. Results

3.1. Effects of N Level and PH Application on Yield and Growth of Lettuce and Tomato

Different N levels and PH applications had significant effects on the growth parameters of lettuce and tomato plants (Table 2). In tomato, both the N level and PH application significantly changed all the dry weights and the number of fruits. However, no interaction between the N level and PH application was detected for any of the growth parameters. The shoot DW increased as the N level increased, and 10 mM N was optimal for the tomato growth and fruit yield. PH-R significantly enhanced the shoot DW while PH-F showed an intermediate effect. Particularly, the shoot DW increase in PH-R-treated plants was correlated with the improved leaf surface area (Table 2). The fruit mean weight increased as the N level increased, but both PH applications demonstrated that the fruit number enhancement led to improved fresh yield while there was no change in fruit mean weight. In romaine lettuce, both the N level and PH application had significant effects on the shoot DW, fresh yield, leaf area, and harvest index (Table 2). Increasing the N level increased the shoot DW, fresh yield, leaf area, and harvest index regardless of the PH application, and the PH application improved them compared to control regardless of the N levels. However,

root DW was not affected by PH but by the N level, with 2 mM N being the highest root DW compared to other N treatments. Like the tomatoes, no interaction between the N level and PH application was observed under all lettuce growth parameters (Table 2).

3.2. Effects of N Level and PH Application on Chlorophyll, Photosynthesis, and Water Use Efficiency

The chlorophyll a (Chl_a) concentration was much higher than that of chlorophyll b (Chl_b) and carotenoid in lettuce and tomato leaves (Table 3). The different N levels and PH application methods significantly affected all chlorophyll and carotenoid concentrations, while no interaction between the N level and PHs was observed. Chl_a, Chl_b, and carotenoids were positively affected by increasing the N levels from 2 mM N to 10 mM N. This indicates that the 10 mM N is an optimal N level for greenhouse romaine lettuce and Micro-Tom tomato production. PH-R significantly enhanced the Chl_a, Chl_b, and carotenoid concentrations while PH-F showed intermediate effects with no N level and PH application interaction (Table 3).

Table 3. Effects of the plant-derived protein hydrolysates (PHs) application on chlorophyll a, chlorophyll b, carotenoid concentrations, photosynthesis rate (*Pn*), and water use efficiency (WUE) of tomato and lettuce leaves. Lettuce and tomato plants were grown in a 1 L pot filled with commercial growing media and fertigated with 4 different N levels (2 mM, 5 mM, 10 mM, and 15 mM). PHs were applied as either foliar spray (PH-F) or root drench (PH-R) every week at a rate of 3 mL L^{-1}.

Treatments		Total Chlorophyll (μg mg^{-1})	Chlorophyll a (μg mg^{-1})	Chlorophyll b (μg mg^{-1})	Carotenoid (μg mg^{-1})	*Pn* (μmol CO_2 m^{-2} s^{-1})	WUE (μmol CO_2 mol^{-1} H_2O)
Tomato Leaves							
N level	2 mM	28.1 b	20.0 b	7.6 b	3.5 b	8.9 b	87.5 ab
	5 mM	31.4 ab	22.8 a	9.3 a	4.1 a	6.9 c	100.5 a
	10 mM	33.8 a	24.3 a	9.5 a	4.2 a	8.3 b	87.4 ab
	15 mM	33.4 a	24.2 a	9.2 ab	4.1 a	10.8 a	77.7 b
PH	Control	28.6 b	21.2 b	7.8 b	3.8 b	7.7 b	78.7 b
	PH-F	31.9 ab	22.9 ab	9.0 ab	4.0 a	8.7 ab	90.8 ab
	PH-R	34.0 a	24.2 a	9.8 a	4.1 a	9.8 a	96.2 a
Significance	N level	**	***	*	***	***	*
	PH	**	**	**	*	**	*
	N × PH	-	-	-	-		
Lettuce Leaves							
N level	2 mM	26.8 b	20.3 b	6.5 b	4.1 b	9.8 b	27.4
	5 mM	32.2 a	23.5 a	8.7 a	4.9 a	10.0 b	24.4
	10 mM	34.7 a	25.2 a	9.5 a	5.0 a	11.3 ab	26.7
	15 mM	34.6 a	25.3 a	9.4 a	4.8 a	12.9 a	28.8
PH	Control	29.7 b	22.1 b	7.6 b	4.4 b	9.9 b	24.2 b
	PH-F	31.9 ab	23.5 ab	8.4 ab	4.8 ab	11.0 ab	26.7 ab
	PH-R	34.1 a	24.7 a	9.4 a	4.9 a	12.0 a	29.2 a
Significance	N level	***	***	***	***	***	-
	PH	**	*	**	*	**	*
	N × PH	-	-	-	-	-	-

Data are the average of 5 replicates. ANOVA results, -, *, **, *** means not significant or significant at $p \leq 0.05$, 0.01, 0.001, respectively. The difference letters indicate significant difference according to the Tukey test, $p \leq 0.05$.

The net CO_2 assimilation rate (*Pn*) and water-use efficiency (WUE) of the tomato and lettuce were measured at 20 days after transplanting and are presented in Table 3. The N level and PH had significant effects on the *Pn* and WUE of the lettuce and tomato. No interaction between the N level and PH application was observed for these parameters. The *Pn* of lettuce increased as the N level increased, but the WUE of lettuce showed no difference between the N levels. Similarly, the *Pn* of the tomato increased from the 5 mM N to a 15 mM N with the lowest value at 5 mM N. The opposite trend was observed for WUE in tomato, with 5 mM N being the highest and 15 mM N being the lowest. When it

comes to PH application, PH-R enhanced the *Pn* and WUE by 21% and 21%, respectively, in lettuce, and 28% and 20%, respectively, in tomato compared to control plants. PH-F presented intermediate effects on these variables. Taken together, the findings suggest that PH-R stimulates the *Pn* and WUE of lettuce and tomato plants after transplanting.

3.3. Effects of N Level and PH Application on Antioxidant Activity and Bioactive Compounds

The PH-R significantly improved the DPPH radical scavenging activity, total phenolic compounds (TPCs), and total flavonoid compounds (TFCs) by 77%, 22%, and 36%, respectively, in romaine lettuce leaves; and 28%, 27%, and 21%, respectively, in tomato fruit. Consistently, the PH-F presented intermediate effects on these parameters (Table 4). The lycopene concentration in PH-R-treated tomato fruit was also enhanced by 26% compared to PH-untreated control. No N level × PH interaction was observed for all antioxidant parameters. The values of antioxidant parameters decreased as the N level increased in romaine lettuce leaves ($p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively) while no difference was observed in tomato fruits.

Table 4. The DPPH radical scavenging activity (%), total phenolic compounds (TPCs) (GAE, gallic acid equivalent), total flavonoid compounds (TFCs) (QE, quercetin equivalent), and lycopene (mg 100 g-1 FW) concentrations in lettuce leaves and tomato fruit. Lettuce and tomato were grown in a 1 L pot filled with commercial potting mix and fertigated with 4 different N levels (2 mM, 5 mM, 10 mM, and 15 mM). PH was applied as either root drench (PH-R) or foliar spray (PH-F) every week at a rate of 3 mL L^{-1}.

Treatments		DPPH Radical Scavenging Activity (%)	TPC (mg GAE g^{-1} DW)	TFC (mg QE g^{-1} DW)	Lycopene (mg 100 g^{-1} FW)
Tomato Fruits					
N level	2 mM	44.1	226.0	208.4	1.96
	5 mM	39.9	200.6	174.5	2.01
	10 mM	41.1	216.7	195.6	2.18
	15 mM	35.9	207.7	196.6	2.19
PH	Control	36.5 b	193.2 b	175.0 b	1.83 b
	PH-F	37.6 b	200.4 b	191.6 ab	2.08 ab
	PH-R	46.7 a	245.2 a	212.6 a	2.30 a
Significance	N level	-	-	-	-
	PH	***	***	***	**
	N×PH	-	-	-	-
Lettuce Leaves					
N level	2 mM	49.2 a	194.7 a	759.9 a	
	5 mM	34.7 b	168.3 ab	593.7 ab	
	10 mM	28.0 b	155.6 b	551.7 b	
	15 mM	26.3 b	139.1 b	523.5 b	
PH	Control	26.2 b	151.5 b	541.3 b	
	PH-F	33.9 ab	162.4 ab	561.8 b	
	PH-R	46.5 a	185.5 a	737.1 a	
Significance	N level	***	***	**	
	PH	**	*	*	
	N × PH	-	-	-	

Data are the average of 5 replicates. ANOVA results, -, *, **, *** means not significant or significant at $p \leq 0.05$, 0.01, 0.001, respectively. The difference letters indicate significant difference according to the Tukey test, $p \leq 0.05$.

3.4. Effects of N Level and PH Application on Nutrient Use Efficiency and Uptake Efficiency

The effects of PH-R on N-use efficiency (NUE), N-uptake efficiency (NUpE), and N-utilization efficiency (NUtE) in tomato and lettuce were evaluated and it was found that they were highly correlated ($R^2 > 0.78$) as a function of N concentration, except the NUtE of tomato (Figure 1). Increasing N levels tended to decrease NUE and NUpE. PH-R significantly increased all parameters compared to the PH-untreated plants. When

averaged over N treatments, the PH-R significantly improved the NUE and NUpE by 35% and 43% in tomatoes and 21% and 39% in lettuces (Figure 1A,B,D,E). Notably, the greater differences in NUpE between PH-R and PH-untreated plants were found at lower N levels, presenting the PH-R as more effective under a low N supply (Figure 1B,E). When averaged over N treatments, control plants tended to decrease the NUtE, thus showing the greatest differences at 2mM-N (Figure 1C,F).

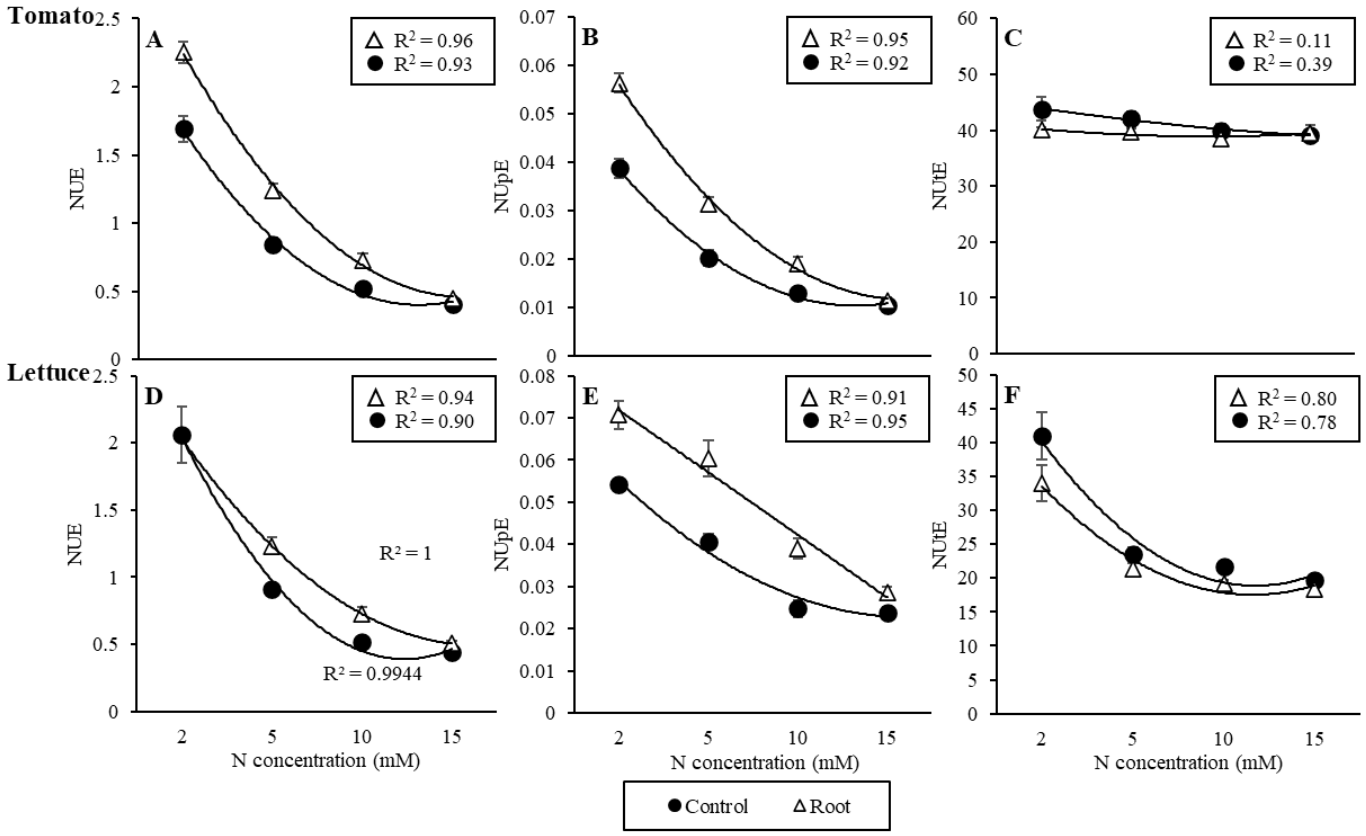


Figure 1. Nitrogen-use efficiency (NUE; (**A**,**D**)), nitrogen-uptake efficiency (NUpE; (**B**,**E**)), and nitrogen-utilization efficiency (NUtE; (**C**,**F**)). Lettuce and tomato plants were grown in 1 L pots filled with commercial growing media and fertigated with 4 different N levels (2, 5, 10, and 15 mM). PH was applied as either the root drench or foliar spray every week at a rate of 3 mL L^{-1} (50 mL pot^{-1}). The results were presented as means ± SE (n = 5). Significant p value presents ANOVA result. The difference letters indicate significant difference according to the Tukey test, $p \leq 0.05$.

4. Discussion

Our results show that the PHs significantly increased the lettuce and tomato growth compared to in the PH-untreated plants, and PH-R was more effective than PH-F if plants were grown with a commercial growing medium under greenhouse conditions. The positive effects of PHs on the crop yields under different N levels were reported for spinach, lettuce, rocket, and tomato. Despite involving plant species-specific response, there is a general agreement that PHs provide potential advantages on crop growth and N uptake under various N levels [22–24,33]. These results are well in line with our study, in which PHs improved the romaine lettuce and tomato growth and yield regardless of the N level. A possible mechanism behind the significant effects of PHs could be associated with hormone-like activities because the PHs used in the current study induced auxin, brassinosteroid, and gibberellin-like activities leading to the morphology and metabolic changes of plants, consequently improving plants growth and yield [16,34]. Particularly, the hormone-like activities of PHs might be derived from the fact that PHs contain various small peptides such as signaling molecules that may modulate a pathway involved in

an endogenous phytohormone synthesis [35,36]. The increase in fruit number was previously illustrated in other PH studies with tomatoes [37–39]. Notably, Rouphael et al. [38] presented that PH application increased the number of cultivar of one fruit and the fruit mean weight of another, showing a significant cultivar × PH interaction. The increase in fruit number by PHs could be due to multiple effects. For example, a direct effect of PH might be due to better pollen vitality from the hormone-like activity and amino acids in PH [37]. Alternatively, the indirect effects of PHs can be due to the enhanced photosynthetic capacity and, therefore, the better source–sink ratio with more photo-assimilates to fruit sets. Our findings agree with Sestili et al. [24] who reported that PH-R was more effective than PH-F in tomato seedling growth at both N levels (low N: 7 mg L^{-1} and high N: 112 mg L^{-1}). They showed that the PH-R differentially changed the gene expressions of nitrate, ammonium, amino acid transporters, and N-metabolism depending on the N levels, consequently increasing the tomato seedling performance regardless of the N level. The author concluded that PH stimulated root growth and induced a "nutrient acquisition response" that promotes N uptake. Indeed, PHs increased the root growth of tomato plants in our study but not in lettuce, demonstrating a species-specific effects. This is in contrast to some PH studies where PHs improved root growth in lettuce [40,41]. It is well known that root growth varies in function of plant species and environmental conditions such as abiotic and/or biotic conditions of the environment including soil type [42,43]. While the cause of such a discrepancy is not clear, it may be partly due to the growing system which directly impacted root growth. Unlike the aforementioned studies where field soil was used, our study employed commercial growing media of limited volume in a pot and the roots were subjected to mechanical impedance caused by a rigid wall. Nonetheless, we observed species-specific responses of root growth in tomato and lettuce.

Our findings on the positive effects of PHs on the chlorophylls and carotenoids are in line with those of other PH studies, wherein leaf greenness represented by the Soil Plant Analysis Development (SPAD) index was increased in leafy vegetables such as spinach and lettuce [22,23]. Our study is unique in that we examined different PH application methods on the greenhouse crops using commercial media and showed different levels of efficacy of the application method on chlorophylls, photosynthesis, and WUE. Additionally, the chlorophyll concentration in PH-untreated plants was increased by PH application, resulting in improved photosynthesis and WUE. The enhanced photosynthesis by PHs could be associated with the direct action of amino acids on a photosynthetic apparatus [44] and improved essential macronutrient uptake such as magnesium, participating in various biochemical and physiological activities such as photosynthetic carbon fixation and the formation of chlorophylls' porphyrin ring [45].

Antioxidant activity is essential for evaluating the nutraceutical qualities of foods, including leafy vegetables and tomato fruits, because antioxidant compounds provide beneficial effects to human health by playing a central role in inhibiting oxidative damage and cancer diseases [46–48]. Various biostimulants are available on the market, and the effect of different biostimulant types showed diverse results in the nutraceutical quality of crops [11,49–51]. For example, Mannino et al. (2020) [52] demonstrated that although the seaweed biostimulant improved the growth and antioxidant capacities such as DPPH free radical scavenging activity in Micro-Tom tomato fruits, bioactive compounds (lycopene, carotenoids, polyphenols, and flavonoids) were not significantly different from control plants. However, our study showed that the effects of PHs on tomato fruits were distinct from a seaweed biostimulant presenting PHs which enhanced not only antioxidant capacity (DPPH) but also bioactive compounds (TPCs, TFCs, and lycopene). The excess use of nitrogen fertilizers negatively affects the nutraceutical qualities of food crops [53,54]. We also demonstrated that a high N fertilization leads to the reduction in antioxidant activities in lettuce leaves. Our finding is in agreement with that of Zhou et al. (2019) [55] who reported that decreasing N inputs enhanced bioactive compounds of lettuce by partially upregulating genes involved in the phenolic synthesis pathway. Our findings of the improved nutraceutical quality of plants by PHs were in line with previous PH studies

with rocket and lettuce [23,33] in which weekly PH application improved the antioxidant compounds of rocket and lettuce compared to the non-treated control. However, the previous studies grew rocket and lettuce using field soils and only tested the effects of PH-F. The difference between our study and other studies is that we compared two PH application methods, PH-R and PH-F, in greenhouse vegetable and fruit production using a commercial growing media, and PH-R effectively enhanced the beneficial compounds in lettuce leaves and tomato fruit compared to PH-F. Possible mechanisms behind the beneficial effects of PHs on antioxidant activities could be: (1) the change of antioxidant homeostasis by stimulating the enzyme activities of antioxidant homeostasis; and (2) better plant nutrient assimilation of PH-treated plants leading to the increase in amino acids synthesis [11,56].

Notably, greater differences in NUpE between PH-R and PH-untreated plants were found at lower N levels, supporting our findings that PH-R is more effective under low N supply (Figure 1B,E). Our results are in line with those of Hirel et al. [57], in which the amount of N fertilizers applied to crops increased the yield by 7.4 times from 1960 to 2000, but the total yield only increased 2.4 times, indicating that NUE and NUpE are lower at high N fertilization. Because 15 mM-N did not significantly increase the fresh yield, NUE, and NUpE of tomato and lettuce compared to 10 mM-N (Table 2 and Figure 1), 10 mM-N is the optimal N supply for efficient N use for greenhouse-grown tomato and lettuce. Our findings also proved that PH-R could be an effective tool to minimize N fertilizer inputs and leaching in greenhouse crop production, subsequently reducing the fertilizer cost and adverse effects of leached N on the environment [58]. The positive effects of PH-R can be attributed to changes in the gene expressions of nitrate, ammonium, amino acid transporters, and N -metabolism.

5. Conclusions

Greenhouse crop production has been gaining popularity but have represented a challenge for commercial growers and researchers in terms of sustainability due to their intensive inputs. Plant-derived protein hydrolysates may be an effective means of reaching sustainability goals by improving nutrient use efficiency and uptake efficiency, subsequently enhancing the yield and quality of romaine lettuce and Micro-Tom tomato. Compared to PH-F, PH-R effectively increased the yield, *Pn*, WUE, chlorophyll concentrations, and antioxidant activities regardless of N levels in lettuce and tomato. Such improvements were associated with increased N use and uptake efficiencies which may be attributed to the changes in the gene expressions of nitrate, ammonium, amino acid transporters, and N-metabolism. Overall, our findings suggested that the root application of PHs can be a sustainable method in controlled greenhouse crop production with commercial growing media to enhance crop yield, quality, and N use and uptake efficiencies under both high and low N fertilization.

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