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RESEARCH PAPER

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EFFECTS OF NITROGEN DOSE AND N-NH_4:N_{total} RATIO ON GROWTH, YIELD, AND QUALITY OF GREENHOUSE LETTUCE ACROSS SEASONS

Ismet Babaj[1], Veton Haziri[1], Ilmije Vllasaku[1], Edona Lika[1], Leonita Abdyli[1], Glenda Sallaku[2], Astrit Balliu[2]✉

[1] UBT – Higher Education Institution, Lagjja Kalabria p.n., 10 000 Pristina, Republic of Kosovo

[2] Faculty of Agriculture and Environment, Agricultural University of Tirana, Str. Paisi Vodica 1029, Tirana, Albania

ABSTRACT

A factorial experiment testing the effects of different nitrogen dose applications (60, 80, and 120 kg ha^{-1}) provided varying ratios of N-NH_4 to the total amount of nitrogen supplied (0.4, 1.0) was conducted in two successive growing seasons (autumn-winter and spring) with the *Lactuca sativa* Lagarde F1. Phosphorus and potassium fertilizers were kept constant and uniform at all experimental plots, respectively 25 (P_2O_5) and 180 (K_2O) kg ha^{-1}. Root traits, growth parameters, yield, nitrogen use efficiency (NUE), and NO_3 concentration in the lettuce leaves were measured and analyzed. N-NO_3 concentration and NUE were the most sensitive traits to N dose applications and N-NH_4:N_{total} ratio. The remaining traits, yield included, rather than on the N dose and its application forms, were subject to seasonal variation of environmental factors. A range of 60–80 kg ha^{-1} N was the optimum for greenhouse lettuce fertilization. Further increase of N dose applications did not provide a higher yield, whereas it significantly increased the N-NO_3 concentration in the plant and reduced the N use efficiency. The NO_3 concentration in the lettuce leaves was reduced by increasing the ratio of N-NH_4 to total N applied and extending the period of the latest N application before harvesting.

Keywords: *Lactuca sativa*, root traits, nitrogen use efficiency, NO_3 concentration, fertilization

INTRODUCTION

Lettuce (*Lactuca sativa* L.) is one of the most important vegetable crops, occupying a total of 1226,370 ha and reaching a worldwide production of 27,660,187 Mt [Martínez-Moreno et al. 2024]. It is an important source of vitamins and minerals and of bioactive compounds such as polyphenols, carotenoids, and chlorophyll, with significant health benefits [Shi et al. 2022, Martínez-Moreno et al. 2024]. Yet, similar to other raw, salad-type crops, lettuce is considered to be one of the main sources of dietary nitrate intake [Blekkenhorst et al. 2017].

Although there are several benefits of nitrate on human health [Du et al. 2007], its potential harmful effects are a common public concern. Nitrates themselves have low toxicity to humans, but nitrates become reduced to nitrites, which react with other compounds as amines or amino acids contained in food, leading to nitrosamine formation [Urlić et al. 2017b, Martínez-Moreno et al. 2024]. These specific compounds might put humans at risk of gastrointestinal cancer and methemoglobinemia [Liu et al. 2016]. The risk is highlighted in EU Regulation 1258/2011, which sets upper limits on nitrate levels in lettuce depending on the growing season: 5000 mg kg^{-1} fresh mass (FM) in winter and 4000 mg kg^{-1} FM in summer for greenhouse lettuce [European Commission 2011]. Hence,

✉ aballiu@ubt.edu.al

reducing nitrate content in vegetables through modulating the production environment is a major concern of lettuce producers [Liu et al. 2016].

Nitrate is taken up by roots and stored in root vacuoles or is transported to leaves, where it is reduced to ammonium and incorporated into carbon skeletons, organic molecules that act as acceptors for reduced nitrogen during amino acid biosynthesis, or otherwise stored in the leaves [Castaings et al. 2011]. The absorption, translocation, and assimilation of nitrates in vegetables are tightly regulated by the interaction of internal cues and external environmental factors [Bian et al. 2020]. The amount and form of N-fertilizer, light availability and quality, water availability, air and root zone temperature, and CO_2 concentration [Bian et al. 2020, Martínez-Moreno et al. 2024] affect N uptake and accumulation in plants. Among them, nitrogen fertilization and light intensity are the most important factors.

Nitrate accumulation in leafy vegetables is tightly linked to N fertilization practices. Various studies reported a significant positive correlation between the dose of nitrogen supply and NO3 content in leafy vegetables [Fu et al. 2017, Ortega-Blu et al. 2020]. As a common rule, an increase in N supply is followed by a significant increase in N concentration [De Pinheiro Henriques 2000, Cometti et al. 2011, Fu et al. 2017]. Furthermore, N fertilization and the type of N fertilizers impact the fresh yield and might pose some environmental risks. Over-fertilization limits lettuce productivity because of osmotic stress [Albornoz and Lieth 2015], whereas high-dose N applications are responsible for significant N losses and severe environmental impacts [Balliu et al. 2007a, 2008].

Appropriate NH_4:NO_3 ratios can modulate N accumulation in the plant's leaves [Hachiya and Sakakibara 2017]. An increase in NH_4 supply versus the total amount of N supplied to the plants leads to a reduction in N-NO_3 accumulated in the plants [Santamaria et al. 2001]. Next to it, the NH_4:NO_3 ratio impacts the morphology of the root system. It has been proven that ammonium supply increases lateral root initiation and higher-order lateral root branching, whereas the elongation of lateral roots is stimulated mainly by nitrate [Lima et al. 2010, Hachiya and Sakakibara 2017].

Light intensity modulates uptake and nitrate reduction, and is considered the leading environmental factor controlling nitrate accumulation in leafy vegetables [Albornoz and Lieth 2015]. Plants reduce biomass production [De Pinheiro Henriques 2000], but accumulate more nitrate under low light intensities [Lillo and Appenroth 2001]. Furthermore, significant interactions between N supply and light intensity are often reported in lettuce crops and other leafy vegetables. An increase in N supply, combined with reduced light intensity, is the recipe for having the maximum N-NO_3 concentration in the plant [Cometti et al. 2011, Fu et al. 2017]. Still, besides the intensity, the quality of light plays a significant role in the N accumulation in lettuce plants [Chen et al. 2014, Liu et al. 2016].

Considering that background, the goal of this experiment was to estimate the potential interactions between N dose applications and different NH_4-N:total-N ratios as they were modulated by the growing season, on root morphology traits, N use efficiency, yield, and NO_3 concentration of lettuce plants grown under greenhouse conditions.

MATERIALS AND METHODS

Plant materials and experimental design. The study was conducted in plastic, non-heated greenhouses, Vushtrri municipality (42°53'N, 20°52'E) of the Republic of Kosovo. The area is characterized by a continental climate with temperatures varying from 0.6 to 22.4 °C, whereas the irradiation values range from 42 to 214 kWh. Details of the climatic conditions during both growing conditions are provided in the Table 1. Natural soil classified as clay loam soil was used as growing media. Details about soil physical and chemical characteristics are provided in Table 2. Two sequential planting seasons, autumn (October 2023–February 2024) and spring (February–May 2024), have been experimented with.

The lettuce cultivar Lagarde F1 (BASF) was used as a plant material in both seasons. A split-plot experimental design was established with N doses (respectively 60, 80, and 120 kg ha^{-1}), as the main factor, and N-NH_4 to total nitrogen supplied (N_{total}) ratio (0.4, 1.0) as the secondary factor. Phosphorus and potassium were kept constant and uniform at all experimental plots, respectively 25 (P_2O_5) and 180 (K_2O) kg ha^{-1}. An experimental unit (treatment) was represented by a 2 square meter plot (2 m × 1 m). Thirty-day-old seed-

Table 1. The average monthly temperatures and global horizontal irradiation. Data retrieved from PVGIS. European Union. https://re.jrc.ec.europa.eu/pvg_tools/en/tools.html

Month	Average temperature (°C)	Global horizontal irradiation (kWh)
January	0.6	47.8
February	2.3	65.9
March	5.8	107.5
April	10.8	145.7
May	15.0	179.4
June	19.6	198.1
July	22.2	214.3
August	22.4	193.6
September	17.2	134.5
October	11.6	95.6
November	6.6	57.0
December	2.0	41.6

Table 2. The physical and chemical properties of the soil

The parameters and methods				
Volumetric density (g cm^{-3})	Granulometric analysis (%)			
	Sand (2.0–0.2 mm)	Fine sand (0.2–0.02 mm)	Silt (0.02–0.002 mm)	Clay (<0.002 mm)
2.50	3.0	29.8	25.2	27.6
pH (H_2O)	$CaCO_3$ (%)	organic matter (%)	nitrogen (%)	Ass. P_2O_5 (mg 100g)
7.04	2.78	2.94	0.14	14.7
1:2 method S-2.10	Volumetric method	SPWR 2003 Method S-14.10	Soil Kjeldahl Nitrogen Method S-8.10	Mehlich 3
Exchangeable macro nutrients (mg kg^{-1})				
Ca	Mg	K	Na	S
1743	151	134	12	147
Mehlich 3	Mehlich 3	Mehlich 3	Mehlich 3	Mehlich 3
Micronutrients (mg kg^{-1})				
B	Zn	Fe	Mn	Cu
0.19	2.43	0.57	0.12	0.85
Hot water	Mehlich 3	Mehlich 3	Mehlich 3	Mehlich 3

lings were transplanted in the field at a fixed planting density of 24 m^{-2} (0.25 m × 0.25 m). Four randomly distributed replications were applied for each experimental unit, assembling in total a 24-treatment design (3 (N doses) × 2 (NH_4:N_{total} ratios) × 4 (replications)).

Combinations of different fertilizers: Frutta (Adriatica Spa, Strada Dogado, Rovigo, Italia; 8-16-20-13.5-11), K_2SO_4 (0-0-51), NH_4NO_3 (34-0-0), and $CO(NH_2)_2$ (46-0-0) at different amounts were used to compose the specific nutrient formulations for each treatment. Details on the respective fertilizer compositions were provided in Table 3. The whole amount of Frutta (8-16-20-13.5-11) was applied as basic fertilization, providing that the total amount of phosphorus and partial amounts of potassium and nitrogen were applied before transplanting. The rest of the fertilizers were delivered during the vegetation and split into two equal doses for each specific treatment. Common

Table 3. Detailed information on the fertilizer's recipe composition

	Fertilizer's composition	Amount of each fertilizer used (kg ha^{-1})	N-total	N-NO_3	N-NH_4	P_2O_5	K_2O	NH_4:N_{Total} ratio
	A_60-25-180							
A1	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	NH_4NO_3 (34-0-0)	140	47.5	36.4	12.5			
	Total A1		**60**	**36**	**25**	**25**	**180**	**0.4**
A2	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	$CO(NH_2)_2$ (46-0-0)	103	47.5		47.5			
	Total A2		**60**	**0**	**60**	**25**	**180**	**1.0**
	B_80-25-180							
B1	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	NH_4NO_3 (34-0-0)	198	67.5	51.7	17.8			
	Total B1		**80**	**52**	**30**	**25**	**180**	**0.4**
B2	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	$CO(NH_2)_2$ (46-0-0)	147	67.5		67.5			
	Total B2		**80**	**0**	**80**	**25**	**180**	**1.0**
	C_120-25-180							
C1	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	NH_4NO_3 (34-0-0)	316	107.5	82.3	28.3			
	Total C1		**120**	**82**	**41**	**25**	**180**	**0.4**
C2	Frutta (8-16-20-13.5-11)	156	12.5		12.5	25.0	31.0	
	K_2SO_4 (0-0-51)	291					149.0	
	$CO(NH_2)_2$ (46-0-0)	234	107.5		107.5			
	Total C2		**120**	**0**	**120**	**25**	**180**	**1.0**

commercial crop management practices were equally applied to all treatments during the plant life cycle. Harvesting was conducted when the plant's rosette reached marketable size, respectively 107 days after transplanting (DAT) in the autumn season, and 68 DAT in the spring season. The number of marketable heads was counted for each treatment and each replication, and the average head weight was calculated as the ratio of total weight to the number of respective heads for each replication.

Nitrate/nitrite measurements. Mature leaves of ten randomly selected plants in each experimental plot were collected to analyze the nitrate (NO_3) and nitrite (NO_2) concentrations. The leaves were washed out carefully, dried, and analyzed by the UV-VIS spectroscopy method [Cataldo et al. 1975, Zhao and Wang 2017] using a Spectrophotometer (NANOCOLOR VIS II). The analyses were performed only at the harvesting time, in the autumn season, whereas several successive measurements (5, 12, and 34 days after the latest fertilizer application) were performed during the spring season.

Biomass assessment and root analyses. On the harvesting day, six plants were randomly selected and used for biomass assessment and root morphology analyses. For that purpose, the plants were carefully removed from the soil with their root system intact. The roots were dissected from the aboveground organs, washed free of adhering soil particles using a soft water jet, and scanned with an Epson Expression/STD 4800 Scanner. The acquired root images were analyzed with WinRHIZO Arabidopsis 2013 software (Regent Instruments Inc., Quebec, Canada). Root length (RL), root surface area (RSA), average root diameter (AvgD), and root volume (RV) were individually measured and recorded.

The leaves were carefully removed from the rosettes and individually counted for each plant. Ten cm^2 discs were cut from 10 randomly selected leaves of each treatment. They were dried out for 72 h at 65 °C and weighed with an accuracy of ±1 mg. The coefficient of leaf area per unit of dry weight (cm^2 mg^{-1}) was calculated for each treatment, which was then used to calculate the entire leaf area for each treatment.

Following root morphology analyses, the roots and leaves of each plant were dried (65 °C, 72 h), and the dry matter (DM) of the roots and shoots of each plant was determined separately to an accuracy of ±1 mg (TP 303; Denver Instruments GmbH, Göttingen, Germany). The ratio of root dry matter to shoot dry matter weight DM_{root}:DM_{shoot}, and the ratio of shoot dry matter weight to fresh shoot weight ratio (DM_{shoot}:FW_{shoot}) were calculated for each treatment. Subsequently, specific root length (SRL) – root length divided by root dry mass (m g^{-1}) [Bergmann et al. 2020], root tissue density (RTD) – root dry mass divided by fresh root volume (g cm^{-3}), and root length ratio (RLR) – root length divided by whole plant dry mass (m g^{-1}) [Ryser 1996] were calculated for each treatment.

Statistical analysis. A factorial arrangement of 24 treatments (3 nitrogen dose levels × 2 levels of NH_4:N_{total} ratios), 4 replicates each, was employed in a randomized complete block design. Residuals of all variables were tested for equality of variances and normality using Brown-Forsythe and Shapiro-Wilk tests, respectively. Differences regarding harvested yield, biomass indicators, root morphology traits, and nitrate/nitrite concentration were tested by three-way ANOVA, using the PC program SigmaPlot 13 (Systat Software Inc., San Jose, CA, USA). Each significant ANOVA result ($p < 0.05$) was followed by a Holm-Sidak test at $p < 0.05$ as a post-hoc test. Values given throughout the text are means ± SE. Main contributors of diversity regarding root traits, growth parameters, yield, and leaf N concentration under different growing seasons were assessed by principal component analysis (PCA). To produce a graphical evaluation of their relationships the respective heat-map analysis was performed via ClustVis (https://biit.cs.ut.ee/clustvis/, accessed on 14 February 2025) online program package.

RESULTS

The nitrogen (N) supply did not affect root traits. So did the NH_4:N_{total} ratio (Tab. 4). Although root length (RL), root surface area (RSA), and root length ratio (RLR) were slightly higher at the highest N dose (N120) the differences with N60 and N80 were not significant (Tab. 4). The only exception was root tissue density (RTD), where higher N doses were followed by a substantial reduction ($p = 0.044$) in the tissue density (Tab. 4). On the contrary, the planting season significantly affected most of the root traits. The

Table 4. Root length (RL, m), root surface area (RSA, cm^2), average root diameter (AvgD, mm), root volume (RV, cm^3), specific root length (SRL, m g^{-1}), root tissue density (RTD, g cm^{-3}), and root length ratio (RLR, m g^{-1}) of autumn and spring grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N ratio (0.4, 1) conditions, at the harvesting day (respectively 107 and 68 days after transplanting). Different letters indicate significant differences within parameters (Holm-Sidak test, $p < 0.05$; mean ±SE); significant p-values of a three-way ANOVA are noted in bold

Factors		RL	RSA	AvgD	RootV	SRL	RTD	RLR
N_{total} amount	NH_4-N ratio							
60		1190 ±110	178 ±16.1	0.481 ±0.009	2.12 ±0.19	1357.0 ±148	0.455 ±0.03a	62.4 ±6.43
80		1177 ±59	171 ±7.7	0.466 ±0.008	1.99 ±0.09	1951.0 ±363	0.372 ±0.03b	64.9 ±4.81
120		1301 ±54	184 ±8.2	0.451 ±0.004	2.07 ±0.11	1432.4 ±76	0.384 ±0.01b	67.0 ±3.61
	0.4	1206 ±247	185 ±5.8	0.489 ±0.06	2.26 ±0.07	1707.8 ±249	0.406 ±0.02	66.5 ±3.91
	1	1127 ±330	174 ±7.6	0.499 ±0.09	2.13 ±0.09	1452.5 ±105	0.402 ±0.01	63.1 ±4.33
Autumn		1123 ±45	177 ±6.4	0.46 ±0.04b	2.06 ±0.07b	1779.7 ±123	0.373 ±0.01b	82.0 ±3.52a
Spring		1110 ±50	181 ±7.2	0.52 ±0.08a	2.33 ±0.08a	1380.6 ±237	0.435 ±0.02a	47.6 ±2.22b
Autumn								
60	0.4	1307.8 ±132.1	196.0 ±20.2	0.479 ±0.01	2.351 ±0.26	1779.2 ±226.4	0.330 ±0.02	92.8 ±9.1
	1	1073.2 ±176.6	160.0 ±24.6	0.480 ±0.01	1.905 ±0.27	1739.1 ±431.2	0.391 ±0.06	75.0 ±15.1
80	0.4	1235.8 ±62.2	185.3 ±8.79	0.478 ±0.01	2.218 ±0.12	1712.2 ±184.2	0.364 ±0.05	79.2 ±7.5
	1	1118.8 ±100.9	157.8 ±10.5	0.454 ±0.01	1.777 ±0.08	1760.7 ±269.2	0.384 ±0.04	80.6 ±10.5
120	0.4	1241.4 ±49.1	177.0 ±8.34	0.453 ±0.04	2.009 ±0.11	1491.1 ±79.4	0.423 ±0.02	78.9 ±2.6
	1	1361.5 ±95.4	191.2 ±14.4	0.447 ±0.08	2.142 ±0.18	1758.9 ±183.6	0.386 ±0.04	82.1 ±4.8
Spring								
60	0.4	1148.9 ±47.8	175.6 ±9.31	0.486 ±0.01	2.146 ±0.16	926.3 ±111.1	0.626 ±0.07	46.4 ±4.19
	1	789.1 ±105	132.8 ±10.9	0.551 ±0.03	1.812 ±0.13	983.3 ±166.1	0.473 ±0.03	35.2 ±4.28
80	0.4	1124.1 ±128	178.8 ±21.4	0.508 ±0.01	2.280 ±0.31	1712.1 ±90.1	0.364 ±0.04	79.2 ±5.36
	1	1282.3 ±132	201.1 ±19.5	0.502 ±0.01	2.518 ±0.24	1390.0 ±111.2	0.375 ±0.02	52.7 ±5.26
120	0.4	1183.3 ±155	197.9 ±15.8	0.529 ±0.02	2.580 ±0.11	1397.0 ±114.6	0.326 ±0.02	54.2 ±6.37
	1	1136.9 ±94.6	204.0 ±14.9	0.559 ±0.01	2.656 ±0.23	1082.8 ±82.4	0.402 ±0.03	52.7 ±4.61
Significance								
N_{total} dose (A)		0.159	0.069	0.426	0.099	0.157	**0.044**	0.668
NH_4:N_{total} ratio (B)		0.228	0.250	0.307	0.242	0.349	0.884	0.423
Growing season (C)		0.092	0.679	**<0.001**	**0.018**	0.145	**0.033**	**<0.001**
A × B		0.072	0.079	0.110	0.187	0.435	0.578	0.180
A × C		0.298	0.110	**0.025**	**0.030**	0.489	**0.006**	0.297
A × B × C		0.322	0.341	0.587	0.361	0.803	0.068	0.751

Table 5. Dry matter of roots (DM_{root}, g plant^{-1}), dry matter of shoots (DM_{shoot}, g plant^{-1}), fresh weight of shoot (FW shoot, g plant^{-1}), dry matter of roots:dry matter of shoots ratio (DM_{root}:DM_{shoot}), dry matter of shoots:fresh weight of shoots ratio (DM_{shoot}:FW_{shoot}), leaf number (LN, leaves plant^{-1}), leaf area (LA, cm^2 plant^{-1}), and yield (kg variant^{-1}) of autumn and spring grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N ratio (0.4, 1) conditions, at the harvesting day (respectively 107 and 68 day after transplanting). Different letters indicate significant differences within parameters (Holm-Sidak test, $p < 0.05$; mean ±SE); significant p-values of a three-way ANOVA are indicated in bold

Factors									
N_{total} amount	NH_4-N ratio	DM_{root}	DM_{shoot}	FW_{shoot}	DM_{root}:DM_{shoot}	DM_{shoot}:FW_{shoot}	LN	LA	Yield
60		0.709 ±0.04	18.38 ±1.15	8.83 ±0.96b	0.049 ±0.001	0.067 ±0.003	30.5 ±0.58	5602 ±352	8.83 ±0.96b
80		0.688 ±0.06	19.16 ±1.13	9.37 ±0.98a	0.043 ±0.002	0.065 ±0.003	29.7 ±0.58	5839 ±344	9.37 ±0.98a
120		0.827 ±0.05	17.98 ±0.59	9.09 ±0.81ab	0.051 ±0.002	0.064 ±0.003	30.2 ±0.31	5481 ±181	9.09 ±0.81ab
	0.4	0.885 ±0.05	18.76 ±0.84	9.05 ±0.75	0.048 ±0.002	0.066 ±0.003	30.66 ±0.41a	5718 ±263	9.05 ±0.75
	1	0.832 ±0.03	18.25 ±0.75	9.14 ±0.73	0.046 ±0.001	0.065 ±0.003	29.63 ±0.40b	5564 ±229	9.14 ±0.73
Autumn		0.741 ±0.03b	14.39 ±0.28b	5.57 ±0.11b	0.051 ±0.01a	0.082 ±0.001a	28.69 ±0.36b	4385 ±87.7	5.57 ±0.11b
Spring		0.976 ±0.04a	22.62 ±0.51a	12.6 ±0.14a	0.042 ±0.01b	0.048 ±0.001b	31.61 ±0.31a	6896 ±156	12.6 ±0.14a
Autumn									
60	0.4	0.758 ±0.07	13.36 ±0.70b	5.01 ±0.15c	0.056 ±0.002	0.085 ±0.003	28.83 ±0.83b	4074.4 ±214b	5.01 ±0.15c
	1	0.661 ±0.04	14.03 ±0.41b	5.29 ±0.25bc	0.047 ±0.002	0.084 ±0.003	28.83 ±0.70b	4278.1 ±125b	5.29 ±0.25bc
80	0.4	0.703 ±0.12	14.50 ±0.74b	5.54 ±0.22bc	0.048 ±0.007	0.083 ±0.003	28.33 ±1.05b	4420.1 ±225b	5.54 ±0.22bc
	1	0.672 ±0.06	13.82 ±1.14b	5.58 ±0.23bc	0.049 ±0.005	0.079 ±0.002	27.16 ±1.22b	4213.7 ±349b	5.58 ±0.23bc
120	0.4	0.839 ±0.03	14.89 ±0.43b	5.81 ±0.08bc	0.056 ±0.002	0.082 ±0.003	30.00 ±0.57b	4540.6 ±133b	5.81 ±0.08bc
	1	0.814 ±0.09	15.70 ±0.25b	6.17 ±0.081b	0.051 ±0.005	0.081 ±0.003	29.00 ±0.73b	4787.1 ±78b	6.17 ±0.081b
Spring									
60	0.4	1.305 ±0.11	24.16 ±1.71a	448.2 ±11.8a	0.053 ±0.003	0.051 ±0.002	33.66 ±0.88a	7364.8 ±522a	12.55 ±0.33a
	1	0.857 ±0.09	21.95 ±1.72a	446.4 ±6.8a	0.039 ±0.002	0.047 ±0.001	30.83 ±1.10a	6691.9 ±524a	12.50 ±0.19a
80	0.4	0.86 ±0.15	24.83 ±0.69a	480.3 ±11.8a	0.034 ±0.006	0.049 ±0.001	32.16 ±0.16a	7569.6 ±211a	13.20 ±0.21a
	1	0.919 ±0.03	23.48 ±1.00a	469.6 ±8.9a	0.039 ±0.001	0.049 ±0.001	31.16 ±0.60a	7156.3 ±305a	13.15 ±0.25a
120	0.4	0.844 ±0.07	20.80 ±0.48a	436.6 ±9.4a	0.040 ±0.003	0.045 ±0.001	31.00 ±0.36a	6341.1 ±146a	12.22 ±0.26a
	1	1.069 ±0.11	20.53 ±0.47a	433.9 ±19.4a	0.051 ±0.004	0.048 ±0.001	30.83 ±0.40a	6257.2 ±143a	12.15 ±0.54a
Significance									
N_{total} dose (A)		0.189	0.207	**0.015**	0.057	0.323	0.324	0.171	**0.025**
NH_4:N_{total} (B)		0.328	0.355	0.851	0.465	0.514	**0.027**	0.355	0.580
Grow. season (C)		**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
A × B		0.016	0.595	0.758	0.027	0.645	0.751	0.456	0.915
A × C		0.175	0.002	**0.001**	0.540	0.740	0.065	0.012	**0.001**
A × B × C		0.068	0.682	0.994	0.216	0.617	0.219	0.654	0.894

spring-grown lettuce plants had substantially higher average root diameter (AvgD), root volume (RootV), and root tissue density (RTD) than autumn-grown lettuce plants, but significantly smaller root length ratio (RLR). Yet, no differences were found regarding RL, RSA, and specific root length (SRL) due to the planting season (Tab. 4). Although no effects of N supply or NH_4:N ratio were found within each planting season for most root traits, the significant interactions between N supply and planting season regarding AvgD, RootV, and RTD make it difficult to identify the impacts of N supply on these specific parameters.

The amount of nitrogen (N) supplied affected the average shoot fresh weight (FW_{shoot}), and the yield of lettuce plants. A significant increase in FW_{shoot} and total harvested yield followed the rise of N dose from 60 to 80 kg ha^{-1} but the further N dose rise to 120 kg ha^{-1} was not followed by any further increase of either yield or FW_{shoot} (Tab. 4). Importantly noting, the N dose effects were season-dependent. The rise of N supply was followed by an increase in the FW_{shoot} and the yield in the autumn plantings, but not in the spring. Yet significantly higher FW_{shoot} and yield were received in spring plantings than in autumn plantings for the same amount of N supply (Tab. 4).

Neither the N_{total} dose, or NH_4:N_{total} ratio affected the dry matter weight of roots (DM_{root}), the ratio of root and shoot dry matter weight (DM_{root}:DM_{shoot}), the ratio of dry to fresh shoot weight (DM_{shoot}:FW_{shoot}), the number of leaves per plant (LN), and total leaf area per plant (LA). As an exception, the only trait affected by the NH_4:N_{total} ratio was LN. A smaller number of leaves per plant was recorded when the total amount of N supplied was in the NH_4 form (Tab. 5). On the contrary, significant differences were noted in all the above parameters due to the planting season. Spring-grown lettuce plants were characterized by a higher number of leaves per plant and larger plant leaf area than autumn-grown plants. They also had higher DM_{root}, DM_{shoot}, and FW_{shoot}, but significantly lower root-to-shoot-dry matter weight (DM_{root}:DM_{shoot}), and dry-to-fresh shoot weight (DM_{shoot}:FW_{shoot}) – as in Table 5.

In addition to the impacts on plant fresh weight and harvested yield, significant N-dose effects were found regarding the nitrogen use efficiency (NUE) from the lettuce plants. In either autumn or spring plantings, the increase in the N supply was followed by a steady decrease in NUE (Tab. 6). Yet, significant differences exist between the autumn and spring plantings, with the latter almost double the NUE values of autumn plantings. As expected, the increase in the N supply was followed by an increase in NO_3 and NO_2 concentrations in the plant. Remarkably, if no yield increase was found when shifting from N80 to N120, a significant increase was found regarding NO_3 or NO_2 concentration. Again, a strong planting season effect was noticed; significantly smaller NO_3 concentrations were found in spring lettuce plants for each respective N dose.

There was no effect of the NH_4:N ratio in NUE either in autumn or spring plantings (Tab. 6). On the contrary, the NH_4:N ratio significantly affected NO_3 and NO_2 concentrations in lettuce plants at harvest time. The increase in the ratio of N-NH_4 versus total N supplied from 40% to 100% was followed by a significant decrease in NO_3 and NO_2 concentration (Tab. 6). That was common to each N dose, either in autumn or spring plantings. Yet, a significant seasonal effect was strongly present. Significantly smaller NO_3 concentrations were found in spring-grown plants (317 mg kg^{-1}) compared to autumn-grown plants (1244 mg kg^{-1}). In contrast, the opposite results were found regarding the planting season effect on the NO_2 concentrations (4.92 mg kg^{-1} – autumn season *vs.* 10.9 mg kg^{-1} – spring season) at the harvesting time (Tab. 6).

The NO_3 and NO_2 concentrations in the lettuce plants were significantly falling as the fertilizer withholding period (WHP) was extended. Both reached the peak values immediately after the N supply and were gradually reduced (Tab. 7). Moving from day 5 after N supply (WHP 5) to WHP 12, the NO_3 concentration was reduced from nearly 1500 mg L^{-1} to nearly 600 mg L^{-1}. It was further reduced until the day of harvest (DAT 68, WHP 34) to less than 300 mg L^{-1} (Tab. 7). NO_2 concentration has followed the same course, falling from nearly 30 mg L^{-1} by WHP 5 to less than 15 mg L^{-1} by WHP 34 (Tab. 7).

The principal component analyses (PCA) have shown that the variability of lettuce plants in response to N dose, NH_4:N_{total} ratio, and planting season was largely (97%) determined by the first principal component (PC1). LA, SRL, RL, and NO_3 concentration (in diminishing order) show the highest (positive)

Table 6. Nitrogen use efficiency (NUE, kg kg^{-1}), NO_3 concentration (NO_3, mg kg^{-1}), and NO_2 concentration (NO_2, mg kg^{-1}) of autumn and spring-grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N_{total} ratio (0.4, 1) conditions. NO_3 and NO_2 concentrations are measured on the harvesting day (107 and 68 days after transplanting). Different letters indicate significant differences within parameters (Holm-Sidak test, $p < 0.05$; mean ±SE); significant p-values of a three-way ANOVA are noted in bold

Factors		NUE	NO_3	NO_2
N_{total}	NH_4:N			
60		736 ±79a	628 ±104c	5.63 ±0.84c
80		585 ±61a	732 ±136b	8.21 ±1.09b
120		378 ±33b	983 ±182a	10.2 ±1.17a
	0.4	564 ±58	820 ±129a	9.03 ±1.04a
	1	569 ±57	742 ±112b	6.98 ±0.78b
Autumn		342 ±16b	1244 ±66a	4.92 ±0.38b
Spring		791 ±46a	317 ±10b	10.9 ±0.76a
Autumn				
60	0.4	417.4 ±14.7d	1036 ±2.59d	3.36 ±0.017j
	1	441.5 ±14.7d	906 ±3.75e	2.59 ±0.037k
80	0.4	346.7 ±9.51e	1320 ±11.5c	5.54 ±0.066h
	1	349.2 ±9.51e	1027 ±3.46d	5.12 ±0.009i
120	0.4	242.3 ±11.6f	1625 ±4.61a	6.77 ±0.066f
	1	257.1 ±11.6f	1553 ±7.21b	6.22 ±0.063g
Spring				
60	0.4	1045.8 ±12.4a	282 ±9.16g	9.46 ±0.08d
	1	1041.6 ±12.4a	287 ±7.23g	7.10 ±0.26e
80	0.4	825.0 ±9.41b	290 ±5.81g	13.66 ±0.20b
	1	821.8 ±9.41b	292 ±4.05g	7.50 ±0.28e
120	0.4	509.3 ±3.57c	368 ±1.15f	15.40 ±0.23a
	1	506.2 ±3.57c	387 ±6.17f	12.73 ±0.08c
Significance				
N_{total} dose (A)		**<0.001**	**<0.001**	**<0.001**
NH_4:N_{total} (B)		0.584	**<0.001**	**<0.001**
Grow. season (C)		**<0.001**	**<0.001**	**<0.001**
A × B		0.903	**<0.001**	**<0.001**
A × C		**<0.001**	**<0.001**	**<0.001**
A × B × C		0.884	**<0.001**	**<0.001**

scores associated with PC1. The remaining traits show uniform (negative) values. Interestingly, having the smallest (negative) value, NUE was also clearly distinguished from the remaining traits. PC2 was responsible for only 3% of variability, with NO_3 concentration, RL, and SRL (in diminishing order) showing the highest (positive) associated scores. Overall, SRL, RL, leaf NO_3 concentration, and NUE appear to be the most important traits that express plant variability (Tab. 8). As such, they can potentially be developed as assisted markers to evaluate the sensitivity of lettuce cultivars to nitrogen supply.

The respective heat map visualizes the differences (Fig. 1). A clear distinction exists between autumn and spring-grown lettuce plants. Within the growing season, a clear separation of N60 with N80 and N120 variants exists in spring-grown plants, with the last two being grouped. On the contrary, the picture is mixed in autumn-grown plants with no clear separation among N60, N80, and N120 variants. Furthermore, the NH_4:N_{total} ratios (0.4, 1) were orderly arranged in the spring-grown but not in autumn-grown lettuce plants (Fig. 1), indicating significant N dose-growing season and NH_4:N_{total} ratio-growing season interactions. Overall, the map confirms that SRL, RL, NO_3 concentration (grouped), and NUE are the most sensitive traits re-

Table 7. NO_3 concentration (mg kg^{-1}), and NO_2 concentration (mg kg^{-1}) of spring-grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N_{total} ratio (0.4, 1) conditions on different fertilizer withholding intervals (WHP; 5, 12, 34) after N application. Different letters indicate significant differences within parameters (Holm-Sidak test, $p < 0.05$; mean ±SE); significant p-values of a two-way ANOVA are noticed in bold

N_{total} amount	NH_4-N_{total} ratio	NO_3 concentration (mg kg^{-1})	NO_2 concentration (mg kg^{-1})
WHP 5			
60	0.4	1474 ±41.3c	27.210 ±0.38b
	1	1274 ±13.4d	25.987 ±0.18b
80	0.4	1532 ±19.6c	26.667 ±0.88b
	1	1218 ±13.5e	25.777 ±0.77b
120	0.4	1860 ±41.7a	35.443 ±0.43a
	1	1604 ±16.2b	33.377 ±0.44a
WHP 12			
60	0.4	560 ±26.1g	11.653 ±0.20c
	1	307 ±13.5i	11.000 ±0.23c
80	0.4	615 ±9.95g	12.143 ±0.38c
	1	311 ±6.08i	10.467 ±0.29c
120	0.4	729 ±16.2f	13.967 ±0.43c
	1	444 ±11.5h	12.487 ±0.24c
Harvest day (WHP 34)			
60	0.4	282 ±9.16j	9.467 ±0.08d
	1	287 ±7.23j	7.100 ±0.26d
80	0.4	290 ±5.8j	13.667 ±0.20c
	1	292 ±4.05ij	7.500 ±0.28d
120	0.4	368 ±1.15hi	15.400 ±0.23c
	1	387 ±6.17h	12.733 ±0.08c
Significance			
N_{total} dose (A)		<0.001	<0.001
NH_4:N_{total} ratio (B)		<0.001	<0.001
Withhold timespan of N application (C)		<0.001	<0.001
A × B		0.044	0.006
A × C		<0.001	<0.001
A × B × C		0.342	0.002

Table 8. The principal components score of root morphology, growth, yield, and fruit quality traits of autumn and spring--grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N ratio (0.4, 1) conditions

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
DM_{root}	–1.482	–0.179	–0.010	0.014	–0.017	0.002	–0.001	0.000
DM_{shoot}	–1.438	–0.181	–0.010	0.014	–0.017	0.002	–0.001	0.000
DM_{root}:DM_{shoot}	–1.484	–0.179	–0.010	0.014	–0.017	0.002	–0.001	0.000
DM_{shoot}:FW_{shoot}	–1.484	–0.179	–0.010	0.014	–0.017	0.002	–0.001	0.000
RL	**1.549**	**0.565**	0.030	0.148	0.165	0.003	0.000	0.000
RSA	–1.021	–0.091	0.000	0.046	0.010	–0.016	0.000	0.000
AvgD	–1.483	–0.179	–0.010	0.014	–0.017	0.001	–0.001	0.000
RootV	–1.478	–0.179	–0.010	0.014	–0.017	0.001	–0.001	0.000
SRL	**2.439**	**1.156**	0.452	–0.036	–0.055	0.000	0.000	0.000
RTD	–1.483	–0.179	–0.010	0.014	–0.017	0.002	–0.001	0.000
RLR	–1.303	–0.109	0.006	0.013	–0.011	0.002	0.012	0.000
LN	–1.407	–0.169	–0.009	0.013	–0.015	–0.001	–0.001	0.003
LA	**12.397**	–0.619	–0.079	0.014	–0.028	0.000	0.000	0.000
Yield	–1.462	–0.184	–0.010	0.015	–0.017	0.001	–0.001	0.000
NUE	**–0.138**	–0.515	0.090	–0.240	0.106	0.000	0.000	0.000
NO_3 concentration	**0.742**	**1.405**	–0.398	–0.090	–0.016	0.000	0.000	0.000
NO_2 concentration	–1.465	–0.183	–0.012	0.019	–0.020	0.001	0.000	–0.001

garding N doses and NH_4:N_{total} ratio. The remaining traits, yield included, rather than on the N dose and its application forms, were subject to the growing season.

DISCUSSION

Commonly, an increase in N dose application is followed by a significant enhancement in plant growth [Balliu et al. 2007b, 2009]. However, within the 60 to 120 kg^{-1} ha total nitrogen range, we did not find significant effects regarding lettuce root parameters. Only slightly higher root length (RL), root surface area (RSA), and root length ratio (RLR) values were recorded at the maximum nitrogen (N) supply rate. Within a certain range of N applications, Babaj et al. [2021] have reported similar results for pepper seedlings grown in small containers. The root development is restrained under N supply limitations [Kiba and Krapp 2016]. Hence, since there was no difference in RL and RSA as the N supply was increased, we conclude that even the 60 kg ha^{-1} N supply regime provides enough N for the greenhouse-cultivated lettuce plants. The following discussion on the impact of N dose on the harvested yield confirms that conclusion. Although a common decrease followed the increase in the NH_4:N_{total} ratio from 0.4 to 1.0, no significant effects of the NH_4:N_{total} ratio were found regarding root morphology traits. Similar results were reported by Wang and Shen [2011] that analyzed root length and root surface area of hydroponically grown lettuce within the range of 0.25 to 0.5 NH_4:N_{total} ratio.

The amount of nitrogen supplied did not affect the dry matter of either roots (DM_{root}) or shoots (DM_{shoot}), but a significant effect was noticed regarding the average shoot fresh weight (FW_{shoot}). The increase in N application dose within the range from 60 to 80 kg ha^{-1} led to an increase in the harvested yield, but the further increase to 120 kg ha^{-1} was not followed by any further increase in the yield. By itself, our results are almost identical to a previous report of Thouraya et al. [2022]. They tested the effect of several N dose applications (0, 40, 60, 80, and 120 kg ha^{-1}) in different types of

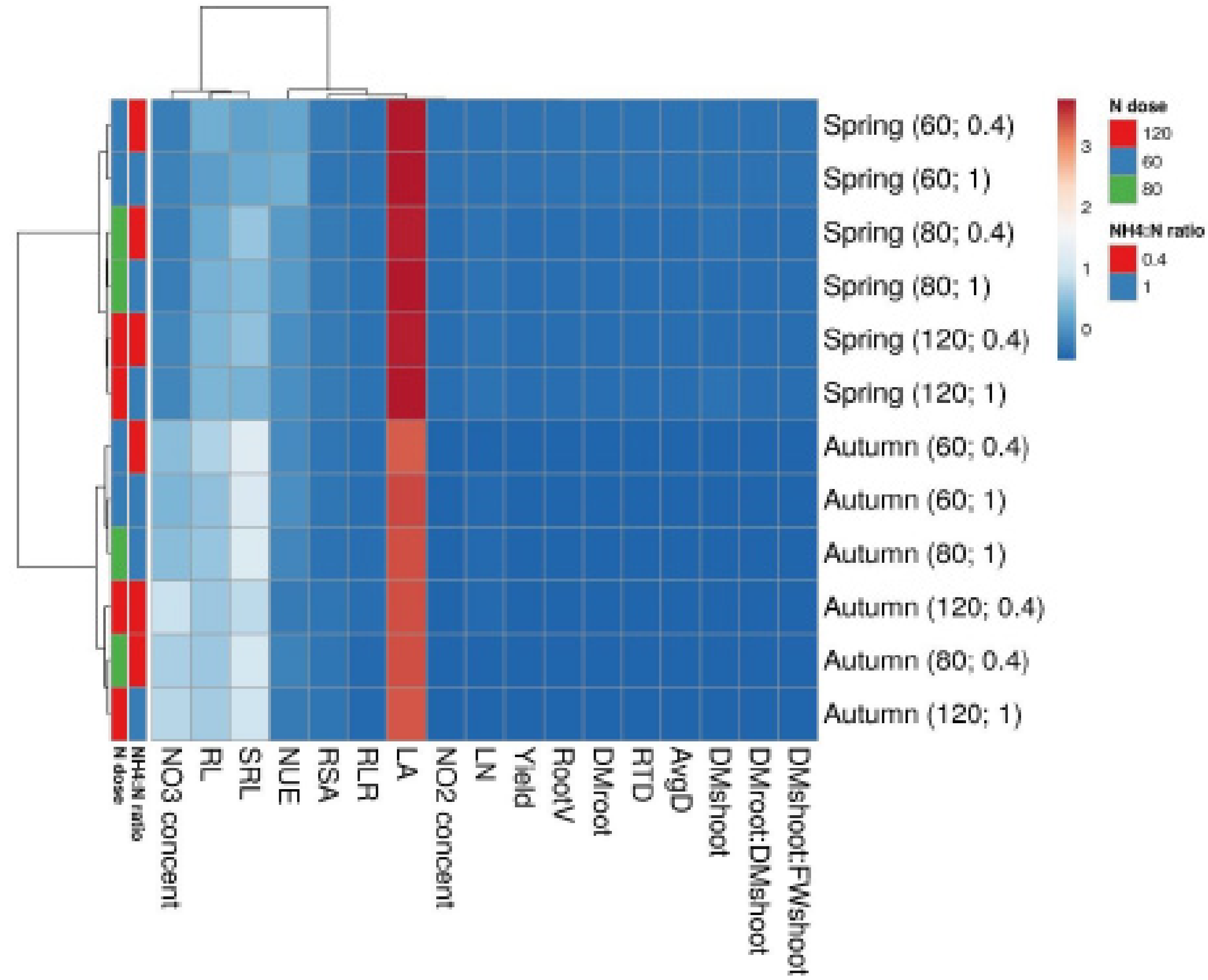

Fig. 1. The heatmap of root morphology, yield, and fruit quality traits of autumn and spring-grown lettuce plants under different N supplies (60, 80, 120 kg ha^{-1}) and NH_4-N_{total} ratio (0.4, 1) conditions. Rows are centered; unit variance scaling is applied to rows. Both rows and columns are clustered using correlation distance and average linkage

lettuce and found that the highest yields were obtained in the range from 60 to 80 kg ha^{-1}. The excessive additions of N fertilizers might even reduce the productivity of lettuce crops because of limitations on the K uptake and the reduction of the stomatal conductance [Albornoz and Lieth 2015]. Yet, it is important to note that the differences we found exist only in the autumn season. No differences were found regarding the yield in the spring season concerning N dose applications.

While there was almost no increase in the harvested yield, a significant, steady decrease in nitrogen use efficiency (NUE) followed the increase in the N dose application. It fell from nearly 1000 kg kg^{-1} to 500 kg kg^{-1} in the spring season and from slightly above 400 kg kg^{-1} to almost 250 kg kg^{-1} in the autumn season. Very similar to us, Saah et al. [2022], have reported an increase in lettuce NUE following an increase in N dose application from 31.3 to 62.5 kg ha^{-1} and later a steady decrease following a further increase in N dose application to 93.8 and 125 kg ha^{-1}.

Opposite to NUE, the increase in the N supply was followed by a steady increase in NO_3 and NO_2 concentrations in the plant. Both reached the peak values at the highest N dose applications (120 kg ha^{-1}). Many authors

[Cometti et al. 2011, Urlić et al. 2017a, Thouraya et al. 2022] have reported similar findings. The imbalance between nitrate absorption and reduction, i.e., the plants absorb more nitrates from the soil than is required for their growth, is supposed to be the reason for nitrate accumulation in the plants [Bian et al. 2020].

Different from El-Ghany et al. [2022] who reported higher yields when all N was supplied as NH_4 versus the combined application of NO_3 and NH_4, we did not find any significant effect of the N-NH_4:N_{total} ratio in lettuce yield. Although toxicity symptoms are reported in cases when the total amount of N supplied was in NH_4 form [Guo et al. 2002, Martínez-Moreno et al. 2024], we did not notice any. Apart from the fact that the different crops express different levels of susceptibility, other factors such as rooting medium (soil, soilless substrate, pure nutrient solution) influence the response of plants to the N-NH_4:N_{total} ratio, mostly due to the cation exchange capacity which alters NH_4^+ availability in the root zone and the rhizosphere pH [Savvas et al. 2006]. Natural soil, as in our case, offers higher protection capabilities than soilless production regarding any potential toxicity issues regarding high N-NH_4 dose applications. In addition, we used urea as a source of N-NH_4, which is only gradually converted to NH_4, and the fertilizers were supplied in two doses, avoiding high NH_4 concentrations in the soil that might potentially negatively affect the lettuce plants.

Interestingly, the increase in the N-NH_4:N_{total} ratio from 0.4 to 1.0 was followed by a significant decrease in NO_3 and NO_2 concentration. That was common to each N dose, either in autumn or spring plantings. Similar results were reported by Burns et al. [2011] and Martínez-Moreno et al. [2024] in lettuce and Zhu et al. [2021] in flowering Chinese cabbage. An explanation for this phenomenon is provided by Kronzucker et al. [1999]. According to them, the exposure of plant roots to NH_4^+ imposes a reduction in NO_3^- influx and the enhancement of NO_3^- efflux. The result will be a lower N-NO_3 concentration in the plant tissues. Furthermore, similar to Borgognone et al. [2016] we found that moving from day 5 after N supply (WHP 5) until the harvest day (WHP 34) of spring-grown lettuce, the NO_3 concentration was reduced from nearly 1500 mg L^{-1} to less than 300 mg L^{-1}. The NO_2 concentration followed the same course, falling from nearly 30 mg L^{-1} by WHP 5 to less than 15 mg L^{-1} by WHP 34. Tabaglio et al. [2020] reported a similar trend of decreasing NO_3 concentration in NFT, spring-grown lettuce plants when they withheld fertilization in periods from 2 to 10 days.

The impacts of the growing season were more significant than the N dose application and N-NH_4:N_{total} ratio. It heavily impacted root morphology traits, the dry and fresh plants' weight, N use efficiency, and N concentration in plant tissues. Spring-grown lettuce plants tend to have thicker roots, which leads to a larger root volume and a significantly higher root tissue density. Interestingly, although there were no differences in total root length (RL), the spring-grown lettuce plants had a significantly smaller root length ratio (RLR). Since RLR (m g^{-1}) represents the ratio of root length (RL) to total plant weight, a reduction in RLR indicates a significantly lower investment of photosynthates of the spring-grown lettuce plants towards the root system. That is further supported by a greater root tissue density (RTD), indicating an increased root longevity of spring-grown lettuce plants [Ryser 1996].

The spring-grown lettuce plants showed significantly enhanced nutrient uptake capabilities. Overall, the N use efficiency in spring-grown plants was twice as high as in autumn-grown plants. Was that a consequence of improved environmental conditions, i.e., higher soil and air temperatures [Balliu and Sallaku 2021], improved radiation [Fu et al. 2017], or enhanced plant symbiotic activities with soil microorganisms facilitated by thicker roots? This remains an open question. Not to forget, a significantly larger AvgD of spring-grown lettuce plants might indicate a higher colonization rate from the arbuscular mycorrhizal fungi [Bergmann et al. 2020, Ma et al. 2018] than the autumn-grown plants and potentially higher exudation rates of the root enzymes [Sallaku et al. 2022, Williams et al. 2022]. Both options have significantly boosted the plant's uptake capabilities.

The photosynthates saved in root system construction were invested in the above-ground organs. The spring-grown lettuce has shown significantly lower root-to-shoot dry matter weight (DM_{root}:DM_{shoot}), and lower dry-to-fresh shoot weight (DM_{shoot}:FW_{shoot}) than the autumn-grown plants. In addition, the spring-grown lettuce plants did have a higher $FW_{shoot,}$ a higher number of leaves per plant, and a larger plant leaf area than autumn-grown plants. Finally, there was a higher yield than autumn-grown lettuce plants for the same amount of N supply. Seasonal effects on lettuce production are not unknown. Similar effects were previously reported by Konstantopoulou et al. [2010], and El-Ghany et al. [2022].

Significantly smaller NO_3 concentrations were found in spring-grown plants than in autumn-grown plants for each respective N dose. Similarly, Savvas et al. [2006] and Konstantopoulou et al. [2010] reported lower N-NO_3 concentrations in autumn-grown lettuce plants compared to the winter-grown plants. Among many factors affecting NO_3 uptake and accumulation in vegetable tissues, N fertilization and light intensity have been identified as the major factors [Santamaria 2006, Bian et al. 2015]. The seasonal differences, particularly solar radiation, which strongly impacts nitrate reductase activity, and temperature, which accelerates both nitrification and plant metabolism [Savvas et al. 2006] enforce the differences between the spring and autumn-grown lettuce plants. A greater accumulation of nitrates was recorded in autumn-winter-grown leafy vegetables due to lower natural radiation values, which led to reduced nitrate reductase activity [Urlić et al. 2017b]. That's why different lettuce nitrate maximum levels (limits) are imposed by European Commission Regulation (EC) No. 563/20027, respectively 4000–4500, and 2500–3500 (mg kg^{-1}) for the autumn-winter (October 1–March 31) and spring-summer (April 1–September 30) period [European Commission 2011]. From the qualitative point of view, the nitrate content in our experiment, in both growing seasons and within all tested nitrogen doses, was quite low and always under the limits imposed by European regulations.

CONCLUSIONS

N-NO_3 concentration and NUE were the most sensitive traits to N dose applications and N-NH_4:N_{total} ratio. The remaining traits, yield included, rather than on the N dose and its application forms, were subject to seasonal variation of environmental factors. A range of 60–80 kg ha^{-1} N is recommended for the fertilization of greenhouse-grown lettuce. Further increase of N dose applications does not provide a higher yield. On the other hand, an increase in N dose applications was followed by a significant drop in N use efficiency and a significant increase in N-NO_3 concentration in the plant. The NO_3 concentration in the lettuce leaves can be reduced by increasing the ratio of N-NH_4:N_{total} applied and/or by extending the period of the latest N application before harvesting.

Although the impacts of N dose applications on yield, quality, and leaf NO_3 concentrations are largely studied, the picture is not yet fully completed. The influence of cultivar variation, fertigation method (quantitative *vs*. proportional fertigation), and the impacts of slow-release fertilizers on NUE and leaf NO_3 concentration at various environmental conditions remain open questions.

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