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Effects of Nitrogen Nutrition on the Nutraceutical and Antinutrient Content of Red Beet (*Beta vulgaris* L.) Baby Leaves Grown in a Hydroponic System

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Abstract

Efficient nitrogen fertilization is critical for maximizing crop productivity while minimizing environmental and health risks. Red beet baby leaves are valued for their vibrant color, flavor, and antioxidant content, particularly betalains, but they are also prone to accumulating antinutritional compounds such as nitrate and oxalate. Excessive nitrogen supply can exacerbate this accumulation, highlighting the need to optimize nitrate input to balance yield, nutritional quality, and safety. This study examined how different nitrate concentrations (1 mM and 10 mM NO_3^-) in hydroponic systems influence red beet baby leaf yield, quality, and levels of beneficial and harmful compounds. The plants were sampled at 10 and 17 days after planting (DAP), and the effects of the treatments in relation to plant age were assessed. Both sampling time and nitrate concentration significantly influenced red beet baby leaf growth and quality. Extending cultivation to 17 days improved yield and antioxidant levels (phenols, flavonoids, betalains) but also increased soluble oxalates. Low nitrate (1 mM) reduced both yield and antioxidant content, regardless of harvest time. However, after 17 days, low nitrate also lowered total oxalate levels, likely due to increased oxalate oxidase activity. Although 1 mM nitrate reduces fertilizer input, it compromises yield and quality. Therefore, intermediate nitrate levels should be explored to optimize both fertilizer use and product quality.

Keywords: betalains; fertilization; leaf vegetables; oxalate; nitrate

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1. Introduction

Implementing precise and efficient fertilization strategies is essential for balancing crop productivity with environmental and health concerns [1]. Excessive use of nitrogen can cause water pollution, eutrophication, and greenhouse gas emissions, all of which contribute to environmental degradation [2]. Moreover, excessive nitrogen fertilization can lead to nitrate accumulation in leaves, which may pose a risk to consumers' health [3].

Bull's Blood red beet (*Beta vulgaris* L.) is a leafy vegetable, characterized by red leaves and stems; due to its mild flavor and attractive appearance, its baby leaves are used in mixed salads and as garnishes [4]. The color of red beet is due to its high level of betalains, which includes betacyanins and betaxanthins [5]. These pigments are found typically in species of the *Amaranthaceae* family and possess great antioxidant properties that help protect cells from oxidative damage [6]. Red beet leaves also have a high nutritional value

due to their content of many minerals and vitamins (e.g., K, A, and C) [7]. These nutrients support overall health, including immune function, bone health, and cardiovascular well-being [8]. Red beet also contains nitrate (NO_3^-), which can be harmful to human health, as it can lead to the formation of nitrosamines, as well as oxalate (Ox) [9].

Oxalic acid (OxA) is present in many species including species belonging to the *Amaranthaceae* and other botanical families, in both soluble and insoluble forms [10]. Oxalates can negatively impact human health by acting as antinutrients as they interfere with the absorption of calcium, magnesium, and iron from food, and can cause hyperoxaluria and oxalosis [11].

In plant cells, Ox synthesis plays a role in maintaining the equilibrium between inorganic cations (K^+, Na^+, NH_4^+, Ca^{2+}, Mg^{2+}) and anions (NO_3^-, Cl^-, $H_2PO_4^-$, SO_4^{2-}) [12]. The primary way plants regulate Ox levels is through its degradation, which involves enzymes such as oxalate oxidase (OxO), oxalate decarboxylase, oxalyl-CoA synthetase, and oxalyl-CoA decarboxylase/formyl-CoA transferase [13,14]. Additionally, nitrate availability in the growth medium has a positive influence on leaf OxO concentration [14]. Baby leaves are immature, tender leaves harvested at an early growth stage (typically 20–40 days after sowing), before full plant development. They are usually consumed fresh, often in mixed salads, and are valued for their delicate texture, mild flavor, and elevated levels of health-promoting compounds, such as vitamins and antioxidants, which are frequently higher than in mature leaves of the same species [15]. Planting density for baby-leaf production varies considerably, ranging from about 150 to 800 plants m^{-2} [16,17]. Among closed-loop hydroponic systems, the floating system is widely used for cultivating short-cycle leafy vegetables at high densities, including many baby-leaf crops [18,19] such as chard [20].

This study aimed to evaluate how varying nitrate concentrations in the nutrient solution affect the yield, quality, antioxidant content (betalains), and antinutritional factors (nitrate and oxalate) of hydroponically grown red beet (*Beta vulgaris* L.) baby leaves to explore the potential for reducing nitrogen fertilizer inputs.

2. Materials and Methods

2.1. Plant Material and Growing Conditions

The experiment was conducted in a glasshouse at the Department of Agriculture, Food, and Environment, University of Pisa. The daily mean air temperature and solar radiation inside the glasshouse were 22.6 °C and 4.25 MJ m^{-2} d^{-1} (approximately 9.50 mol m^{-2} d^{-1} of photosynthetically photon fluence density, PPFD), respectively. T The recorded minimum and maximum air temperatures were 17.9 °C and 31.5 °C, respectively. Supplemental lighting was provided by high-pressure sodium (HPS) lamps, delivering 100 µmol m^{-2} s^{-1} PPFD for 12 h daily, from 07:00 to 19:00, totaling about 4.32 mol m^{-2} d^{-1}.

Seeds of Bull's Blood red beet were purchased from "RB sementi" (www.rbsementi.com (accessed on 1 July 2023), sown in rockwool cubes on 25 September 2023, and germinated in a growth chamber at 25 °C for four days before being moved to the glasshouse. Transplantation into 13 L hydroponic tanks occurred on 9 October 2023 (14 days after sowing, two-leaf stage). Each tank contained 80 seedlings with a crop density of approximately 720 plants per m^2 (inter-plant spacing approximately 3.7 cm^2). The experiment lasted 17 days after planting (DAP).

2.2. Experimental Design and Nutrient Solutions

Two nutrient solutions with 1 (N_1) or 10 (N_{10}; control) mM NO_3^-, were compared in a randomized design with three replicates. Each replicate consisted of an individual hydroponic tank. A nitrate concentration of 10 mM, previously used by Puccinelli et al. [21–23]

as optimal for *Beta vulgaris* grown as baby leaves, was selected as the reference level and reduced tenfold to assess the effects of low nitrogen availability. Moreover, 1 mM of NO_3^- has been previously used as low nitrogen treatment in *Beta vulgaris* [21,22]. The mineral composition of both nutrient solutions is shown in Table 1. The nutrient solutions were prepared by mixing fertilizers to obtain the desired concentration of NO_3^- (1 and 10 mM) and as similar concentration as possible of the other mineral elements, between the two solutions. The treatments started at 3 DAP and lasted 14 days.

Table 1. Electrical conductivity (EC) and mineral composition of the nutrient solutions used in the experiments with red beet plants grown hydroponically in a glasshouse. Mineral nutrients were provided as calcium nitrate, calcium chloride, magnesium sulfate, potassium phosphate, potassium sulfide, potassium nitrate, nitric acid, Fe-EDDHA, Cu-EDTA, Zn-EDTA, Mn-EDTA, sodium molybdate, and sulfuric acid.

	Nutrient Solution	
	10 mM NO_3^-	**1 mM NO_3^-**
EC (mS/cm)	2.4	2.3
N-NO_3 (mM)	10.0	1.0
P-PO_4 (mM)	1.5	1.5
K (mM)	9.0	8.0
Ca (mM)	4.5	4.5
Mg (mM)	2.0	2.0
S-SO_4 (mM)	7.0	9.0
Na (mM)	0.8	0.8
Cl (mM)	0.7	0.7
Fe (µM)	40.0	40.0
B (µM)	40.0	40.0
Cu (µM)	3.0	3.0
Zn (µM)	10.0	10.0
Mn (µM)	10.0	10.0
Mo (µM)	1.0	1.0

2.3. Growth Analysis

Thirty plants were sampled at 10 and 17 DAP from each hydroponic tank for the determination of leaf fresh (FW) and dry (DW) weight, root DW, and leaf area. Due to the short cultivation period, additional intermediate sampling points were not included, as the resulting intervals would have been too brief to yield reliable information. Plants sampled from a single tank constituted one replicate, with a total of three replicates per treatment. Dry weight was obtained by placing fresh samples in a ventilated oven at 70 °C until their weight stabilized. Leaf area was assessed using a digital planimeter (DT Area Meter MK2, Delta T-Devices, Cambridge, UK), and the leaf area index (LAI) was determined by multiplying the leaf area per plant by the crop density. Leaf succulence was calculated as the ratio of leaf fresh weight to leaf area.

2.4. Mineral Elements

Leaf samples were dried and ground before being either mineralized using a mixture of 65% nitric acid (HNO_3) and 30% hydrogen peroxide (H_2O_2) in a 5:2 volume ratio at 240 °C for one hour or extracted with distilled water at room temperature for two hours [23]. Mineralized samples were analyzed for potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) using atomic absorption spectroscopy (Varian Spectra AA240 FS, Agilent Technologies Australia [M] Pty Ltd., Mulgrave, Australia). Phosphorus (P) was measured using UV/VIS spectrometry following the Olsen method [24]. Nitrate concentration in leaf water extracts was determined

spectrophotometrically using the salicylic sulfuric acid method, as described by Puccinelli et al. [21].

2.5. Secondary Metabolites

The antioxidant capacity and concentrations of total chlorophylls, carotenoids, flavonoids, and phenols were determined spectrophotometrically. Fresh leaf samples, each composed of leaves from four individual plants, were extracted using 99% (*v*/*v*) methanol. The extracts were sonicated for 60 min and stored at −18 °C for 24 h prior to analysis.

The concentrations of chlorophyll a, chlorophyll b, and carotenoids were quantified spectrophotometrically using a Shimadzu UV-1280 (Shimadzu Italy, Milano, Italy) spectrophotometer at wavelengths of 662.5 nm, 652.4 nm, and 470 nm, respectively. Total chlorophylls were obtained by adding chlorophyll a and b. Calculations were performed based on the equations described by Wellburn and Lichtenthaler [25].

For the determination of total flavonoid content, a 0.1 mL aliquot of the methanol extract was mixed with 0.06 mL of 5% $NaNO_2$ and 0.04 mL of 10% $AlCl_3$. After a 5 min incubation, 0.4 mL of NaOH and 0.2 mL of H_2O were added, and the absorbance was measured at 510 nm using the same spectrophotometer. Quantification was achieved via a calibration curve prepared with catechin standards [26].

Total phenolic content was measured at 765 nm using the Folin–Ciocâlteu method and expressed relative to a gallic acid standard calibration curve [27].

Total antioxidant capacity was assessed using two different assays. The ferric reducing ability of plasma (FRAP) assay was performed spectrophotometrically at 593 nm and the results were expressed as μmol Fe(II) mg^{-1} FW [28]. Additionally, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was measured by mixing 0.1 mL of the methanol extract with 2.9 mL of a 20 mg L^{-1} methanolic DPPH solution. The mixture was incubated in the dark at room temperature for 45 min, and the absorbance was recorded at 515 nm against a methanol blank. The percentage of scavenging activity was calculated relative to a DPPH solution without the sample, and the antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (TEAC) using a Trolox standard calibration curve [29].

2.6. Ascorbic and Oxalic Acid

For ascorbic acid (AsA) extraction, the method reported by Kim and Kim [30] was followed with minor modifications. The samples (500 mg) stored at −80 °C were extracted immediately before analysis with 5 mL of cold 50 mM KH_2PO_4 buffer (pH 7.0) using a mortar and pestle under dim light. The extracts were filtered using Chromafil® Xtra 20/25 H-PTFE syringe filters (Macherey–Nagel, Duren, Germany) and analyzed directly for AsA content. To determine total ascorbic acid (the sum of AsA and dehydroascorbic acid DAsA), 50–500 μL of 2 mM dithiothreitol (pH 7.0) were added to 500 μL of the filtered extract, and the solution was kept in the dark at room temperature for 20 min before HPLC injection.

The total Ox concentration in leaves was measured using dried leaf samples extracted with 0.25 M HCl (50 mg DW in 6 mL) at 100 °C for 30 min. After cooling, the mixture was diluted to a final volume of 10 mL with 0.25 M HCl and filtered as described above [31]. Soluble Ox was determined similarly, using distilled water instead of 0.25 M HCl for sample extraction.

Ascorbic acid and Ox were determined simultaneously by HPLC [30,32]. The equipment (Jasco, Tokyo, Japan) consisted of a PU-2089 four-solvent low-pressure gradient pump and an MD-4010 diode array detector, and separation was carried out on a C18 250/4.6 Atlantis® T3 5 μm 4.6 × 250 mm column (Waters, Milford, MA, USA). The analysis was

performed in isocratic mode using 50 mM KH_2PO_4 buffer (pH 2.8) with a flow rate of 0.8 mL min^{-1}. The column was washed with 95% CH_3CN for 5 min, followed by 5 min of re-equilibration. The injection volume was 20 µL, and chromatograms were recorded at 243 nm for AsA and 214 nm for oxalic acid. Calibration was performed using AsA and oxalic acid (OxA) standard solutions. The detection limit (LOD) and the quantification limit (LOQ) were 0.0008 and 0.0027 mg kg^{-1} FW, respectively, for AsA, and 0.5 and 1.6 g kg^{-1} FW, respectively, for Ox.

2.7. Oxalate Oxidase

Fresh leaf samples (0.5 g each) were homogenized in phosphate buffer (0.4 M, pH 7.0) and centrifuged to obtain crude extracts. The assays were conducted in foil-wrapped tubes containing succinate buffer (0.05 M, pH 5.0), $CuSO_4$, and OxA, incubated at 40 °C for 5 min. A 4-aminophenazone-based reagent was then added to each tube to develop color; after 30 min in the dark, absorbance was measured at 520 nm using a hydrogen peroxide reference curve for comparison. A sample blank with all reagents except the enzyme extract was maintained. According to Sathishraj and Augustin [33], one unit of OxO corresponds to the production of 1 µmol of H_2O_2 per 5 min under standard conditions. The specific activity of OxO was expressed as µmol of H_2O_2 per mg of proteins, which were quantified according to Bradford method, using bovine serum albumin standards for calibration [34].

2.8. Color Variation

Leaf color parameters (L *, a *, and b * values) were measured using a Konica Minolta CR-400 colorimeter with D65 illuminant (Konica Minolta, Chiyoda, Japan), following calibration with a white standard tile. Lightness was measured directly, and HUE angle and Chroma Index were calculated using a * and b * data [35].

2.9. Ethylene Evolution

Ethylene production was measured by sealing approximately 1 g of fresh leaf tissue in airtight 30 mL containers. Following a 1 h incubation at room temperature, a 2 mL gas sample was withdrawn from the container headspace.

Ethylene concentration was determined using a gas chromatograph (HP 8890, Hewlett-Packard, Menlo Park, CA, USA) equipped with a flame ionization detector (FID). The instrument utilized a stainless-steel column (150 × 0.4 cm diameter, packed with Hysep T) and helium as the carrier gas at a flow rate of 30 mL/min. The temperatures for the column and detector were maintained at 70 °C and 350 °C, respectively.

Ethylene concentration was quantified using an external standard, and the results were expressed on a fresh weight basis as nanoliters per hour per gram of fresh weight (nL h^{-1} g^{-1} FW).

2.10. Statistical Analysis

Prior to analysis, data were assessed for normal distribution and homogeneity of variance using the Shapiro–Wilk test and Levene's test, respectively. Data were then subjected to a two-way analysis of variance (ANOVA) to evaluate the effects of NO_3^- level and sampling time. Mean separation was performed using Tukey's HSD post hoc test at a significance level of $p < 0.05$. A principal component analysis (PCA) was also conducted to explore relationships among leaf production and quality parameters. All statistical analyses were performed using JMP statistical software (JMP 17 Pro, Statistical Discovery LLC, Cary, NC, USA).

3. Results

3.1. Leaf Production and Quality

As expected, leaf fresh weight (FW) and dry weight (DW), total DW and leaf area index (LAI) were greater at 17 DAP than at 10 DAP and significantly decreased in N_1 plants compared to N_{10} plants (Table 2). Root DW was significantly lower in the control plants than in N_1 plants. Leaf dry matter content showed a slight decrease over time (Table 3). Leaf succulence was higher 10 DAP than 17 DAP (Table 3).

Table 2. Leaf fresh (FW) and dry (DW) weight, root and total DW, and leaf area index (LAI) in red beet plants grown hydroponically with different nitrate concentrations in the nutrient solution. The measurements were taken 10 and 17 days after planting (DAP).

Sampling Time (DAP)	NO_3^- (mM)	Leaf FW (g m^{-2})	Leaf DW (g m^{-2})	Root DW (g m^{-2})	Total DW (g m^{-2})	LAI
10	10	936.0 ± 50.7	38.4 ± 2.7	2.97 ± 0.39	41.3 ± 3.1	1.24 ± 0.16 c
	1	741.3 ± 26.8	33.3 ± 1.8	4.44 ± 0.47	37.8 ± 1.6	1.19 ± 0.02 c
17	10	2764.0 ± 225.3	146.7 ± 14.4	10.44 ± 0.90	157.1 ± 15.2	5.97 ± 0.64 a
	1	2264.0 ± 343.0	115.6 ± 14.9	13.98 ± 2.22	129.6 ± 15.3	4.04 ± 0.47 b
MAIN EFFECT						
10		838.6 ± 50.5 b	25.9 ± 1.8 b	3.70 ± 0.43 b	39.6 ± 1.7 b	1.21 ± 0.07 b
17		2514.0 ± 214.9 a	127.8 ± 11.6 a	12.21 ± 1.33 a	143.3 ± 11.4 a	5.01 ± 0.56 a
	10	1850.0 ± 421.6 a	92.5 ± 25.1 a	6.70 ± 1.73 b	99.2 ± 26.8 a	3.61 ± 1.10 a
	1	1502.6 ± 373.6 b	74.5 ± 19.6 b	9.21 ± 2.36 a	83.7 ± 21.6 b	2.61 ± 0.67 b
ANOVA						
DAP		***	***	***	***	***
NO_3^-		*	*	*	*	*
DAP x NO_3^-		ns	ns	ns	ns	*

Mean values (n = 3; ± SE) flanked by the same letter are not statistically different at the 5% level after Tukey's post hoc test. *** $p \leq 0.001$; * $p \leq 0.05$; ns = not significant.

Table 3. Leaf moisture content, succulence, and concentration (on a fresh weight basis) of total phenols, flavonoids, and total ascorbic acid, and antioxidant capacity (as measured using FRAP and DPPH assay) in red beet plants grown hydroponically (floating raft system) with different nitrate (1 and 10 mM NO_3^-) concentrations in the nutrient solution. The measurements were taken 10 and 17 days after planting (DAP).

Sampling Time (DAP)	NO_3^- (mM)	Moisture Content (%)	Succulence (kg m^{-2} FW)	Total Ascorbic Acid (g kg^{-1} FW)	Phenols (g kg^{-1})	Flavonoids (g kg^{-1})	FRAP (mmol Fe (II) kg^{-1})	DPPH (mmol TE kg^{-1})
10	10	95.9 ± 0.1	0.737 ± 0.056	0.132 ± 0.009	1.84 ± 0.04 b	0.552 ± 0.035 b	28.0 ± 0.8 b	7.96 ± 0.27 b
	1	95.5 ± 0.1	0.597 ± 0.018	0.108 ± 0.007	1.59 ± 0.05 b	0.487 ± 0.039 b	21.4 ± 1.3 c	6.38 ± 0.39 bc
17	10	94.7 ± 0.1	0.441 ± 0.011	0.051 ± 0.002	2.76 ± 0.08 a	0.854 ± 0.033 a	45.0 ± 0.2 a	11.54 ± 0.62 a
	1	94.9 ± 0.1	0.549 ± 0.1214	0.58 ± 0.002	1.63 ± 0.18 b	0.518 ± 0.025 b	24.5 ± 0.4 bc	5.37 ± 0.22 c
MAIN EFFECT								
10		95.7 ± 0.07 a	0.667 ± 0.037 a	0.120 ± 0.007 a	1.71 ± 0.06 b	0.520 ± 0.028 b	24.7 ± 1.6 b	7.17 ± 0.41 b
17		94.8 ± 0.04 b	0.495 ± 0.054 b	0.0546 ± 0.002 b	2.19 ± 0.27 a	0.686 ± 0.077 a	34.8 ± 4.6 a	8.46 ± 1.41 a
	10	95.3 ± 0.3	0.589 ± 0.071	0.091 ± 0.019	2.30 ± 0.21 a	0.703 ± 0.071 a	36.5 ± 3.8 a	9.75 ± 0.86 a
	1	95.2 ± 0.2	0.573 ± 0.056	0.088 ± 0.012	1.61 ± 0.09 b	0.503 ± 0.022 b	23.0 ± 0.9 b	5.88 ± 0.30 b

Table 3. *Cont.*

Sampling Time (DAP)	NO_3^- (mM)	Moisture Content (%)	Succulence (kg m^{-2} FW)	Total Ascorbic Acid (g kg^{-1} FW)	Phenols (g kg^{-1})	Flavonoids (g kg^{-1})	FRAP (mmol Fe (II) kg^{-1})	DPPH (mmol TE kg^{-1})
ANOVA								
DAP		***	*	***	**	**	***	*
NO_3^-		ns	ns	ns	***	***	***	***
DAP x NO_3^-		ns	ns	ns	**	**	***	***

Mean values (n = 3; ± SE) flanked by the same letter are not statistically different at 5% level after Tukey's post hoc test. *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns = not significant.

Ethylene production was not affected by plant age, but decreased slightly, yet significantly, in N_1 plants compared to N_{10} plants (Figure 1).

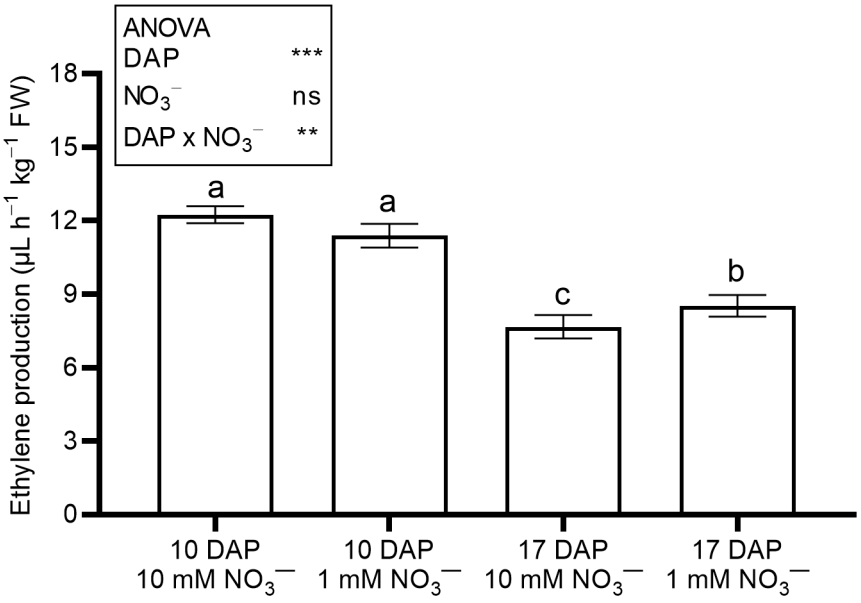


Figure 1. Leaf ethylene production of red beet plants grown hydroponically with different nitrate concentrations in the nutrient solution. The measurements were taken 10 and 17 days after planting (DAP). Mean values (n = 3; ± SE) flanked by the same letter are not statistically different at 5% level after Tukey's post hoc test. *** $p \leq 0.001$; ** $p \leq 0.01$; ns = not significant.

On average, the leaf concentration of both DAsA and total AsA was much higher at 10 DAP than at 17 DAP (Table 3). In contrast, the leaf concentrations of total phenols and flavonoids, and antioxidant capacity, as measured by FRAP and DPPH assays, were higher in plants sampled at 17 DAP (Table 3). A significant regression (R^2 = 0.927) between the antioxidant capacity measured by FRAP and DPPH assays was found. Reducing nitrate concentration in the hydroponic solution resulted in a significant decrease in the leaf concentration of total phenols and flavonoids, and antioxidant capacity with no significant difference between N_1 and N_{10} plants regarding AsA (Table 3).

Leaf carotenoid concentration did not change in response to the NO_3^- level in the nutrient solution and was significantly affected by plant age only in N_{10} plants, which showed a higher content of these pigments at the end of the experiment (Table 4). Leaf concentration of total chlorophylls and betalains was significantly higher at 17 DAP than at 10 DAP, as well as in the N_{10} plants than in the N_1 plants, but only at the first sampling (Table 4). Differences in betalain content were mainly due to the higher concentration of betacyanins, whose content was much higher than that of betaxanthins (Table 4).

Table 4. Leaf concentration (on a fresh weight basis) of total chlorophylls, carotenoids and betalains, and leaf color parameters in red beet plants grown hydroponically with different nitrate concentrations in the nutrient solution. The measurements were taken 10 and 17 days after planting (DAP).

Sampling Time (DAP)	NO_3^- (mM)	Chlorophylls (g kg^{-1})	Carotenoids (g kg^{-1})	Betacyanins (g kg^{-1})	Betaxantins (g kg^{-1})	Total Betalains (g kg^{-1})	Lightness	Chroma	Hue Angle
10	10	0.794 ± 0.046 b	0.182 ± 0.012 b	0.211 ± 0.006 a	0.053 ± 0.005	0.263 ± 0.006 a	26.4 ± 1.1	7.50 ± 0.12	15.6 ± 1.3
	1	0.721 ± 0.039 b	0.252 ± 0.015 ab	0.135 ± 0.008 b	0.039 ± 0.003	0.174 ± 0.005 b	27.9 ± 0.9	8.93 ± 0.29	24.5 ± 2.0
17	10	1.069 ± 0.013 a	0.258 ± 0.025 a	0.210 ± 0.002 a	0.065 ± 0.004	0.275 ± 0.002 a	27.5 ± 0.4	7.88 ± 0.31	37.9 ± 0.8
	1	0.743 ± 0.002 b	0.210 ± 0.011 ab	0.185 ± 0.006 a	0.075 ± 0.008	0.260 ± 0.002 a	29.1 ± 1.1	8.73 ± 0.23	41.5 ± 0.5
MAIN EFFECT									
10		0.758 ± 0.032 b	0.217 ± 0.018	0.173 ± 0.018 b	0.046 ± 0.004 b	0.219 ± 0.020 b	27.1 ± 0.7	8.22 ± 0.35	20.1 ± 2.2 b
17		0.906 ± 0.073 a	0.234 ± 0.016	0.197 ± 0.006 a	0.070 ± 0.004 a	0.268 ± 0.004 a	28.3 ± 0.6	8.30 ± 0.26	39.7 ± 0.9 a
	10	0.932 ± 0.065 a	0.220 ± 0.021	0.210 ± 0.003 a	0.059 ± 0.004	0.269 ± 0.004 a	26.9 ± 0.6	7.69 ± 0.17 b	26.8 ± 5.0 b
	1	0.732 ± 0.018 b	0.231 ± 0.012	0.160 ± 0.012 b	0.057 ± 0.009	0.217 ± 0.019 b	28.5 ± 0.7	8.83 ± 0.17 a	33.0 ± 3.9 a
ANOVA									
DAP		**	ns	**	**	***	ns	ns	***
NO_3^-		***	ns	***	ns	***	ns	**	**
DAP x NO_3^-		**	**	**	ns	***	ns	ns	ns

Mean values (n = 3; ± SE) flanked by the same letter are not statistically different at 5% level after Tukey's post hoc test. *** $p \leq 0.001$; ** $p \leq 0.01$; ns = not significant.

No significant effect of plant age or NO_3^- level in the nutrient solution was observed on leaf lightness. In contrast, Chroma, which reflects color intensity, and the hue angle, which represents the specific color shade or tone, were significantly higher in N_1 plants than in N_{10} plants (Table 4). The Hue angle also increased considerably with plant age (Table 4).

Leaf nitrate concentration did not change significantly with plant age and as expected, was slightly but significantly lower in the N_1 compared to the controls (Figure 2).

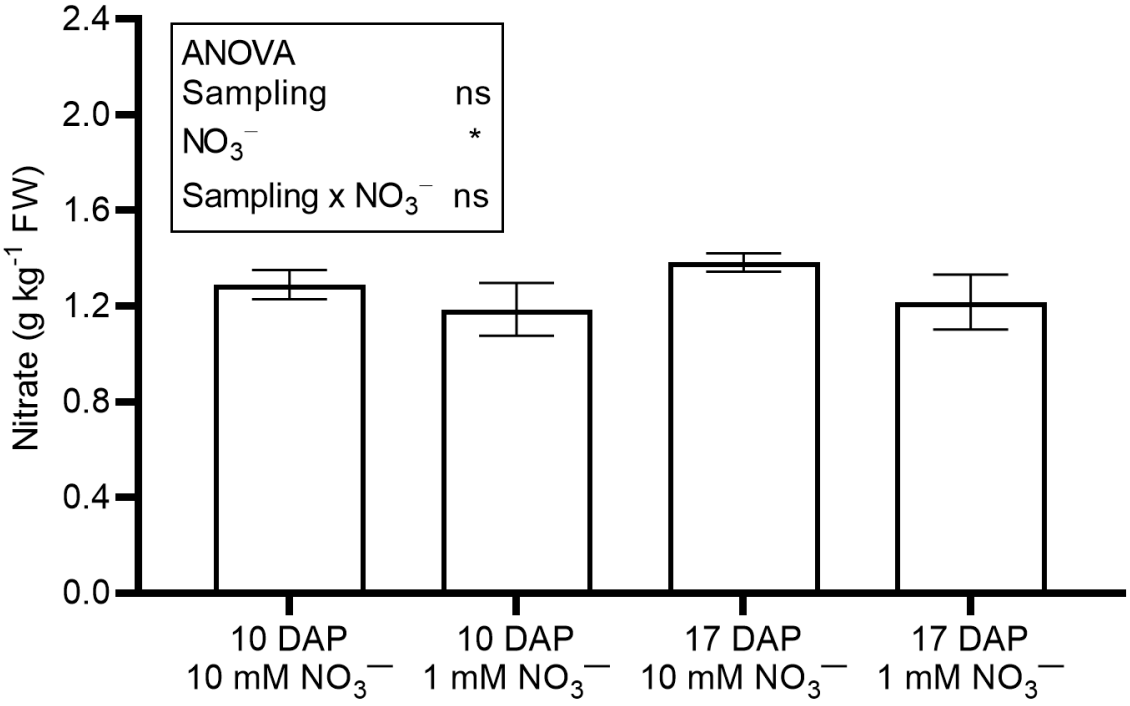


Figure 2. Leaf concentration (on a fresh weight basis) of nitrate of red beet plants grown hydroponically with different nitrate concentrations in the nutrient solution. The measurements were taken 10 and 17 days after planting (DAP). Mean values (n = 3; ± SE) flanked by the same letter are not statistically different at 5% level after Tukey's post hoc test. * $p \leq 0.05$; ns = not significant.

Leaf concentration of soluble Ox was not significantly affected by the NO_3^- level in the nutrient solution, while the concentration of total Ox was significantly lower in N_1 plants than in N_{10} plants, but only at 17 DAP (Figure 3). The activity of OxO was significantly higher at 17 DAP than at 10 DAP, as well as being higher in N_1 plants than in the controls (Figure 3).

On average, the concentration of N, Ca and Mg decreased in leaves of N_1 plants. In contrast, the average leaf concentration of Mn and Cu increased in N_1 plants. Leaf concentration of N, P, K and Na decreased 17 DAP. Meanwhile, Mn, Zn and Cu concentrations increased at this point. The leaf concentration of K and Na increased in N_1 plant at 10 DAP but decreased at 17 DAP. The leaf concentration of P and Fe decreased in N_1 plants only at 10 DAP (Table 5).

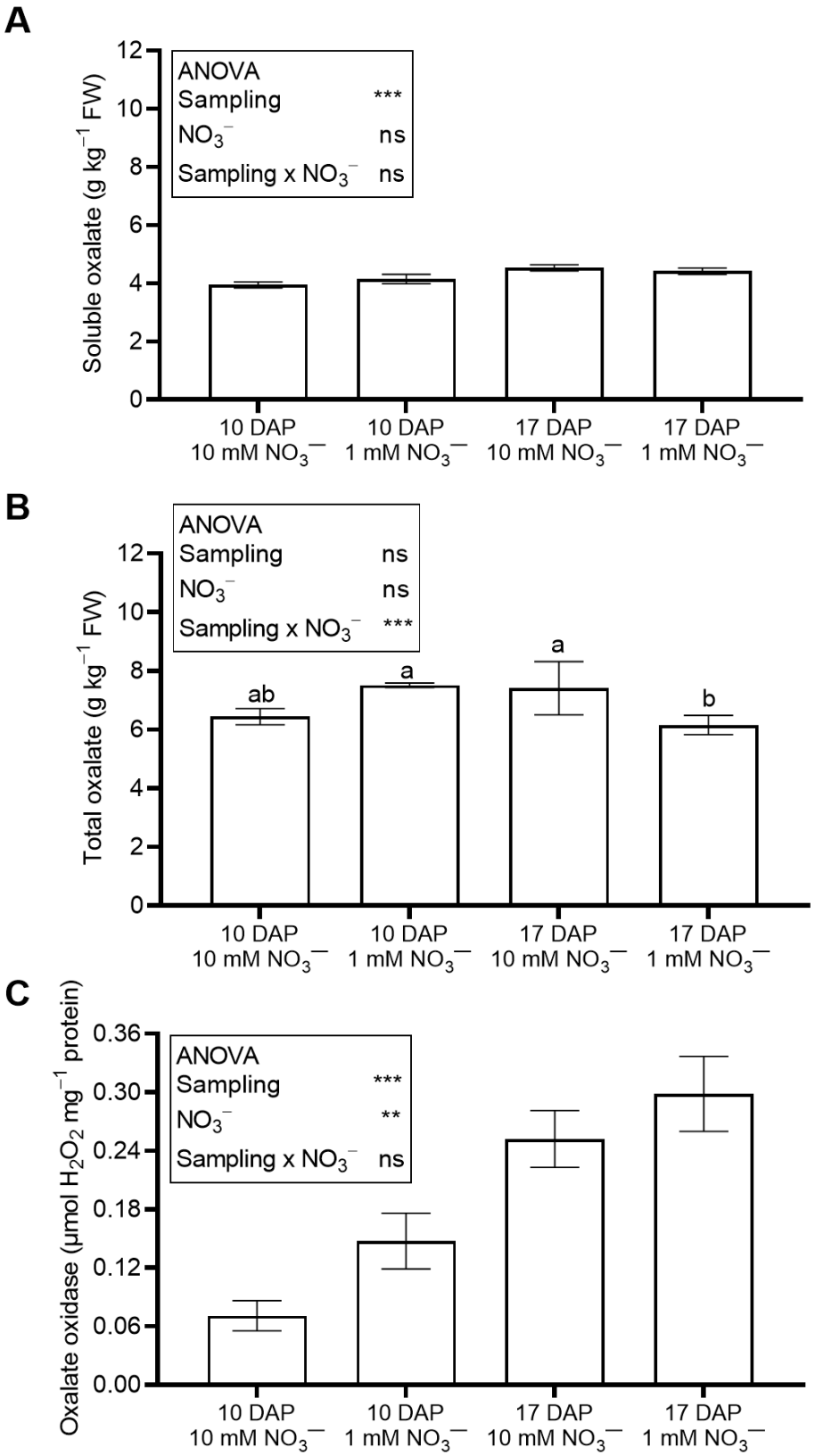


Figure 3. Leaf concentration of soluble (**A**) and total oxalates (**B**), and oxalate oxidase activity (**C**) of red beet plants grown hydroponically with different nitrate concentrations in the nutrient solution. The measurements were taken 10 and 17 days after planting (DAP). Mean values (n = 3; ± SE) flanked by the same letter are not statistically different at 5% level after Tukey's post hoc test. *** $p \leq 0.001$; ** $p \leq 0.01$; ns = not significant.

Table 5. Leaf concentration (on a dry weight basis) of mineral elements in red beet plants grown hydroponically with different nitrate concentrations in the nutrient solution. The measurements were taken 10 and 17 days after planting (DAP).

Sampling Time (DAP)	NO_3^- (mM)	N (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	Na (g kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})	Cu (mg kg^{-1})
10	10	57.1 ± 0.3	21.01 ± 1.09a	167.7 ± 5.9 b	6.25 ± 0.10 a	10.16 ± 0.16 a	70.6 ± 3.7 b	347.4 ± 9.8 a	91.6 ± 6.0	68.3 ± 4.4	22.9 ± 2.3
	1	51.1 ± 0.3	9.82 ± 0.45 b	249.7 ± 7.5 a	3.75 ± 0.09 c	8.11 ± 0.09 c	166.8 ± 1.9 a	196.9 ± 11.6b	115.5 ± 3.1	67.7 ± 1.7	26.1 ± 0.5
17	10	55.3 ± 0.0	7.03 ± 0.15 b	138.0 ± 2.6 c	5.28 ± 0.18 b	9.33 ± 0.24 b	13.0 ± 0.6 d	249.4 ± 4.2 b	142.7 ± 5.1	96.1 ± 5.9	33.3 ± 1.3
	1	50.8 ± 0.6	9.18 ± 0.43 b	99.5 ± 2.4 d	3.47 ± 0.10 c	6.74 ± 0.30 d	51.8 ± 1.9 c	262.0 ± 30.1b	167.5 ± 3.0	103.0 ± 3.5	38.7 ± 1.7
MAIN EFFECT											
10		54.1 ± 1.1 a	15.42 ± 2.56a	208.7 ± 18.8 a	5.00 ± 0.56 a	9.14 ± 0.46 a	118.7 ± 21.6 a	272.2 ± 34.3a	103.5 ± 6.2 b	68.0 ± 2.1 b	24.5 ± 1.3 b
17		53.1 ± 0.9 b	8.11 ± 0.52 b	118.8 ± 8.8 b	4.37 ± 0.41 b	8.04 ± 0.60 b	32.4 ± 8.7 b	255.7 ± 13.9b	155.1 ± 6.1 a	99.6 ± 3.4 a	36.0 ± 1.6 a
	10	56.2 ± 0.4 a	14.20 ± 3.16a	152.9 ± 7.2 b	5.76 ± 0.23 a	9.74 ± 0.23 a	41.8 ± 13.0 b	298.4 ± 22.4	117.2 ± 12.0b	82.2 ± 7.1	28.1 ± 2.6 b
	1	51.0 ± 0.4 b	9.50 ± 0.31 b	174.6 ± 33.8 a	3.61 ± 0.09 b	7.43 ± 0.34 b	109.3 ± 25.7 a	229.4 ± 20.5	141.5 ± 11.8a	85.4 ± 8.1	32.4 ± 2.9 a
ANOVA											
DAP		*	***	***	***	***	***	ns	***	***	***
NO_3^-		**	***	**	***	***	***	**	***	ns	*
DAP x NO_3^-		ns	***	***	*	ns	***	**	ns	ns	ns

Mean values (n = 3; ± SE) flanked by the same letter are not statistically different at 5% level after Tukey's post hoc test. *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns = not significant.

3.2. Principal Component Analysis

The results of a Principal Component Analysis (PCA) on yield, antioxidant capacity, and the concentrations of total ascorbic acid, nitrate, betacyanin, betaxanthin, total betalains, chlorophyll, phenols, flavonoids, and soluble Ox are presented in Figure 4.

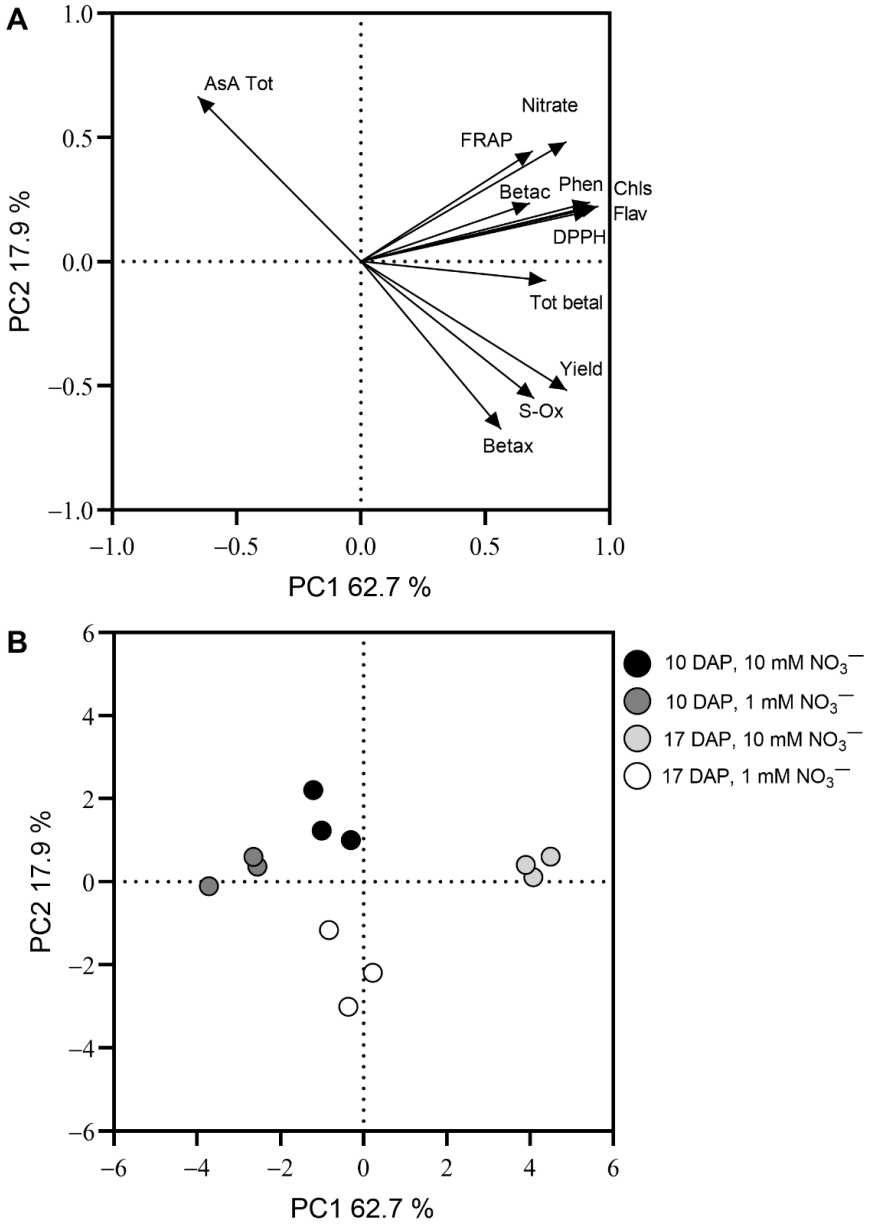


Figure 4. Loading (**A**) and score plot (**B**) for principal component (PC) 1 and 2, describing the variation in yield and leaf qualitative characteristics in red beet plants grown hydroponically (floating raft system) with different nitrate concentrations in the nutrient solution. The measurements were taken 10 and 17 days after planting (DAP). Abbreviations: AsA Tot, total ascorbic acid; Chls, chlorophylls; Phen, phenols; Flav, flavonoids; FRAP, FRAP index; LS, leaf succulence; DPPH, DPPH index; Betac, betacyanin, Betax, betaxanthin, Tot betal, total betalains, S-Ox, soluble oxalate.

A principal component analysis revealed that the first two components were sufficient to explain 80.6% of the total variance, a result supported by the scree plot. PC1 accounted for 62.7% of the total variance and was positively correlated with leaf FW, antioxidant capacity, and concentration of nitrate, betacyanin, betaxanthin, total betalains, chlorophylls, phenols, flavonoids, and soluble Ox, and negatively with the leaf concentration of total

AsA. PC2 explained an additional 17.9% of the variance and was positively correlated to the leaf antioxidant capacity and concentration of total AsA, nitrate, betacyanins, chlorophyll, phenols, and flavonoids, and negatively with leaf FW, as well as the concentration of betaxanthin and soluble Ox (Figure 4A).

The loadings show the correlations between the factors assessed in this investigation (Figure 4A). A strong covariance existed between parameters that were close to each other, and the further a parameter was from the origin, the more it contributed to the PCs. In the loading plot's top-right side, a cluster of leaf concentration of nitrate, betacyanins, chlorophylls, phenols, flavonoids and antioxidant capacity measured by DPPH assay, indicated a strong covariance and, along with total betalains concentration, strongly contributed to PC1 (positive side). Another cluster that strongly contributed to PC1 is in the bottom-right side of the plot (positive side of PC1); it is composed of leaf FW, and concentration of soluble Ox and betaxanthin. The concentration of total AsA, nitrate and antioxidant capacity measured by FRAP assay (on the loading plot's positive side) and betaxanthin (on the negative side) were the most significant factors affecting PC2.

The relationships between the treatments are shown in the score plot (Figure 4B). The leaves of 10 mM NO_3^- plants sampled at 17 DAP, showed the highest leaf antioxidant capacity and concentration of nitrate, betacyanins, total betalains, phenols, flavonoids, chlorophyll, were in the right half of the plot. Conversely, the leaves sampled at 10 DAP with higher concentration of total AsA, fell in the left side of the plot. In general, leaf FW, and concentration of soluble Ox and betaxanthins were greater at 17 DAP.

4. Discussion

4.1. Crop Growth and Yield

Regardless of the NO_3^- level in the hydroponic solution, the baby leaf production of beet plants after 17 DAP was consistent with previous findings for the same species grown for 16 days in a floating raft system during autumn [36,37]. Leaf production was much lower at 10 DAP, suggesting that cultivation should last at least two weeks.

As expected, a reduction in plant growth was observed in plants grown with lower concentrations of NO_3^- in the nutrient solution. This reduction has previously been observed in baby-leaf spinach [38], sea beet [21] and wild Swiss chard [21,39]. Nitrogen is an essential nutrient for plants, and the amount of nitrogen available for plant uptake contributes to photosynthesis, resulting in biomass production [40]. The relative reduction in biomass production of red beet in our experiment was consistent at both 10 and 17 DAP. Thus, low NO_3^- fertilization affects growth from the early days, up to 17 DAP, suggesting that delaying harvest could lead to higher production with the same relative reduction in yield due to low NO_3^- fertilization. Additionally, the increased ethylene production was detected only at 17 DAP, indicating stress. This suggests that a longer period of cultivation with low NO_3^- concentration may be necessary to trigger an increase in ethylene production as a response to stress. Indeed, ethylene acts as a crucial cellular signal, regulating root system architecture adaptation, nitrogen uptake and translocation, thus adjusting plant growth and development in response to external nitrogen conditions [41]. According to our results, ethylene is typically produced at moderate levels from seed germination through the early stages of seedling development. However, as the plant progresses into later stages of vegetative growth, ethylene production tends to decrease [42].

The lower NO_3^- concentration in the nutrient solution likely led to increased root biomass, as plants may attempt to maximize nitrogen uptake under nutrient-scarce conditions. One key adaptive strategy that crops use in response to nitrogen deficiency is root elongation [41].

4.2. Leaf Organoleptic and Nutraceutical Quality

The decrease in leaf succulence and moisture content 17 DAP compared to 10 DAP is probably due to changes occurring in plants during growth. Indeed, younger leaves are generally thinner and more tender than older ones, resulting in less resistance to handling and post-harvest storage [43]. Leaf succulence influences texture, which is a key factor in sensory perception [44].

According to previous studies, a longer period of exposure to low nitrogen concentrations led to a reduction in leaf area in sugar beet [45].

The antioxidant capacity measured in our experiment was positively correlated with the leaf concentration of betalains, flavonoids, and phenolic compounds. These compounds are known to have antioxidant properties [46], thus the variation in antioxidant capacity between samplings and treatments is probably due to the variation in leaf concentration of these compounds.

The increase in phenolic and flavonoid leaf concentration at 17 DAP compared to 10 DAP is in agreement with previous findings where an increase in phenol content was found with aging in tomato leaves [47]. Moreover, in our experiment, the observed increase in leaf concentrations of betacyanin, betaxanthin, and total betalains at 17 DAP aligns with the general trend that betacyanin levels tend to rise as leaves age [48]. According to our results, a higher chlorophyll concentration was also detected in leaves aged 2–3 weeks [49]. The reduction in leaf AsA concentration at 17 DAP is consistent with prior findings indicating an age-related decline in AsA levels in leafy vegetables [50].

A higher hue angle indicates a less red color of leaves, which is in agreement with the lower concentration of betalains detected in our experiment in plants treated with 1 mM NO_3^-. Conversely, the increase in chlorophyll concentration in leaves of plants harvested at 10 DAP may have induced a higher hue angle even in the presence of a higher betalain concentration.

There are only a few studies on the effect of nitrogen fertilization on betalain concentration in the leaves of red beet. The reduction in betacyanin, and consequently total betalain, concentration in leaves of plants grown with low NO_3^- in the nutrient solution at 10 DAP contrasts with previous findings on red beet, where an increase in betacyanin concentration was detected in leaves of beet plants subjected to nitrogen deprivation compared to adequately fed plants [51]. Betalains are nitrogen-containing pigments found in plants and a limited nitrogen supply during the initial days of growth may have led to a reduced synthesis of these compounds [52]. However, in subsequent stages of cultivation, this effect could have been mitigated by the plants' adaptation to the lower nitrogen concentration in the solution, along with the typical increase in betacyanin levels observed in plants under nutritional stress [51].

The reduction in phenol, flavonoid, and chlorophyll concentrations in leaves due to low NO_3 treatment observed at 17 DAP may be attributed to stress from low NO_3^- levels. This reduction was not seen at 10 DAP, suggesting that a longer exposure period is needed to induce stress and alter leaf concentrations of these compounds. This finding contrasts with previous studies, such as those on beet [51] and *Moringa oleifera* [53], which showed increased phenol concentrations under nitrogen deprivation. Other studies have been conducted, including those on strawberry [54], tomato [55], parsley and shoot [56], and kale [57].

The reduction in chlorophyll concentration detected in our experiment in leaves of plants treated with low NO_3^- concentration is in agreement with previous work conducted on maize [58] and blackgram [59], where chlorophyll concentrations increased significantly with increasing nitrogen rate. Chlorophyll and nitrogen contents in plants are strongly linked, as around 70% of the nitrogen in leaves is stored in chloroplasts, where chlorophyll

pigments are produced [60]. As noted by Fathi et al. [61], many plant species, including beet [62], have shown a strong correlation between chlorophyll levels and nitrogen concentration.

4.3. Leaf Antinutrient Content

Nitrate accumulation poses a well-documented risk to human health, and leafy vegetables are a major contributor to dietary intake of the compound. Consequently, the European Union has established specific limits on the nitrate content of various leafy greens, such as lettuce, spinach, and rocket salad [63]. In this study, conducted in the fall season, nitrate levels in plants were consistently much lower than the maximum level established for spinach (another species in the Amaranthaceae family) by the European Union (3.5 mg kg^{-1} fresh weight), and the nitrate concentration generally found in different types of Swiss chard [64]. Moreover, as expected, the leaf nitrate concentration was significantly lower in plants grown with a 1 mM NO_3^- nutrient solution.

Oxalate is deemed an antinutritional component in diets because excessive intake can lead to health issues [65]. While there are no official guidelines for daily Ox consumption, it is generally recommended that adults limit their intake to 50–200 mg per day to prevent kidney stones, with a stricter limit of 50 mg per day for those at higher risk [66]. Soluble Ox can bind to calcium ions (Ca^{2+}) in the bloodstream, potentially reducing calcium availability [67]. Thus, consuming plants with a high oxalate-to-calcium ratio requires an increased calcium intake to offset the calcium loss caused by soluble Ox. For example, the maximum total Ox concentration detected in our experiment in red beet leaves is 7.51 g kg^{-1}, meaning that consuming approximately 27 g of leaves could reach the daily intake limit of 200 mg [66]. Red beet accumulated higher levels of Ox (6.44–7.51 g kg^{-1}) than those reported by Simpson et al., [68] in young leaves of silver beet, with a lower percentage of soluble Ox. In our work, leaf soluble and total Ox concentration increased significantly 17 DAP, while treatment with 1 mM NO_3^- reduced the leaf concentration of total oxalate 17 DAP. The increment of total Ox concentration in older leaves is in agreement with a previous study conducted on silver beet where a higher concentration was detected in developed leaves compared to the young ones [68], as well as in spinach, where a higher Ox concentration was found in older leaves of the same plant [69].

In plant cells, the synthesis of Ox is associated with regulating the balance between inorganic cations (K^+, Na^+, NH_4^+, Ca^{2+}, Mg^{2+}) and anions (NO_3^-, Cl^-, $H_2PO_4^-$, SO_4^{2-}) [12], and the primary mechanism for controlling Ox levels involves Ox breakdown [13], which is mediated by enzymes such as OxO, oxalate decarboxylase, oxalyl-CoA synthetase, and oxalyl-CoA decarboxylase/formyl-CoA transferase [14]. Oxalate is broken down into CO_2 and H_2O_2 by the manganese-dependent enzyme OxO, which is present in bacteria, mosses, monocotyledons, and dicotyledons [14]. Oxalic acid plays a role in various metabolic processes, including ion homeostasis, pH regulation, and resistance to both biotic and abiotic stress [14]. The observed reduction in total Ox levels and increase in OxO activity in plants grown with 1 mM NO_3^- is consistent with the biochemical model that links Ox accumulation to both de novo synthesis and degradation by OxO [14]. The rise in enzyme activity resulted in a reduction in total Ox concentration, albeit only 17 DAP. It is likely that a longer period of higher OxO activity is required to observe significant effects on total Ox concentration in leaves.

Despite several studies being available about the effect of nitrogen on growth and quality of beet baby-leaf [70–72], to the best of our knowledge, there are no studies published about the effect of nitrogen level on Ox accumulation in *Beta vulgaris*. Several studies have examined how different types and amounts of nitrogen affect Ox buildup in plants. Generally, there is a positive correlation between nitrate levels in the growth medium and

Ox accumulation. Increased NO_3^- fertilization enhances root absorption of this anion as well as the activities of nitrate reductase and glutamine synthetase in leaves, promoting cation accumulation and stimulating Ox biosynthesis to maintain intracellular pH [14]. Moreover, NO_3^- can induce the biosynthesis of organic acids, including Ox [73], and inhibit OxO by binding to its active site, thus leading to increased Ox accumulation [74]. For example, in hydroponically grown spinach, increasing nitrogen levels from 4 to 12 mM significantly raised soluble and total Ox in leaves [35]. Conversely, reducing the NO_3^-/NH_4^+ ratio decreased Ox levels [57]. These findings are consistent across various species such as *Pennisetum purpureum* [75], *Portulaca oleracea* [76], and *Atriplex nummularia* [77], where higher nitrate concentrations in nutrient solutions led to increased oxalic acid content. Nitrate must be reduced in the shoots before plants can utilize it, leading to the production and accumulation of organic acids like oxalic acid [12]. Studies have shown nitrogen's role in increasing Ox production through its association with organic acids [78] and its impact on the glycolytic and tricarboxylic acid cycles [79]. High nitrogen levels trigger cytoplasmic OH^- release, speeding up glycolysis and the tricarboxylic acid cycle to maintain pH balance, thus increasing organic acid synthesis [80]. Çalişkan [12] suggested that oxalic acid accumulated in leaves and stems as a result of nitrate ions' inhibition of OxO action. Adjusting hydroponic fertilizer solutions can lower Ox levels, which is beneficial for individuals prone to calcium oxalate kidney stones [81]. However, this adjustment may come at the cost of reduced plant biomass, highlighting a trade-off that must be considered in agricultural practices.

5. Conclusions

This study highlights the critical role of both nitrate availability and harvest timing in modulating the phytochemical profile and nutritional quality of red beet baby leaves. Late harvests consistently enhanced the accumulation of pigments (chlorophylls and betalains) and antioxidant compounds (phenols and flavonoids), although they were also associated with increased soluble oxalate levels—an antinutrient of dietary concern. Notably, while low nitrate supply (1 mM) reduced the overall antioxidant content irrespective of harvest time, it also activated oxalate detoxification mechanisms, as evidenced by increased oxalate oxidase activity and reduced total oxalate concentrations after 17 days of cultivation. From a sustainability standpoint, the use of low nitrate input represents a potential strategy to limit fertilizer use and environmental impact. However, it comes at the cost of reduced yield and phytochemical quality.

These findings point toward a need to redefine fertilization strategies that move beyond yield maximization to include nutritional optimization and antinutrient management. Future studies should explore intermediate nitrate concentrations and dynamic fertilization regimes that respond to plant developmental stages or physiological signals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture15181914/s1. Table S1. Leaf concentration (on a dry weight basis) of mineral elements in red beet plants grown hydroponically with different nitrate concentrations in the nutrient solution. The measurements were taken 10 and 17 days after planting (DAP).

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