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Manipulation of contents of nitrate, phenolic acids, chlorophylls and carotenoids in lettuce (*Lactuca sativa* L.) via contrasting responses to nitrogen fertilizer when grown in a controlled environment.

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ABSTRACT

This study aimed to use different nitrogen fertilizer regimes to produce Butterhead lettuce with such large differences in nitrate content that they could be used as treatment and placebo to study the effect of inorganic nitrate on human health. Plants were grown under controlled conditions at 27/23°C day/night with a relatively low Photosyntetically Active Radiation (PAR) of 150µmol m^{-2} s^{-1} for 14 hours day^{-1} and nitrogen supplies ranging from 26 to 154ppm N as ammonium nitrate in the fertigation solution. This resulted in contrasting high (~1078 mg nitrate $100g^{-1}$ FW) or low (~6 mg $100g^{-1}$) nitrate contents in the leaves. Contents of carotenoids and chlorophylls in fresh weight did not differ significantly between highest and lowest N-supply levels. However, increased nitrogen supply reduced contents of phenolic compounds from 154 to 22mg $100g^{-1}$ FW, dry matter content from 8.9 to 4.6% and fresh weight per plant from 108.52 to 47.57 g/plant FW (all $P<0.001$). So while fertilizer treatments can provide lettuce with substantially different nitrate contents, maintaining similar pigment contents (color), they also strongly influence the contents of phenolic acids and flavones.

Keywords

***Lactuca sativa var capitata*, plant secondary metabolites, light intensity, bioactive compounds, quality management**

INTRODUCTION

Plant growing conditions involve a broad range of factors that affect chemical compositions of vegetables. One of these is the use of fertilizers, which may influence the nutrient composition of plants, including secondary metabolites as observed in kale [1] and tobacco [2]. Similarly, different availability of nitrogen resulting from organic and conventional crop management practices may cause differences in the contents of plant secondary metabolites between organic and conventional vegetables and fruit [3], in addition to the more intuitive effects on the plant nitrate content [4].

Nitrate is taken up from fertilizers as a key plant nutrient, and excess may be stored in plant tissues, primarily in leaves, while hardly any nitrate is found in seeds and mature fruits [5]. Recent studies show that vegetables provide approximately 40–92% of the average daily intake of nitrate by humans, where nitrate-rich leafy vegetables such as rocket, spinach and lettuce are some of the richest sources of nitrate in the human diet [6]. Therefore, lettuce as the main vegetable in salad and sandwich fillings is responsible for much of the population's intake of nitrate, and this is of interest due to the effects of nitrate and its metabolite nitrite on human health: While previous research focused mainly on potentially harmful effects on human health [5], other studies have instead focused on benefits, e.g. that a diet rich in leafy vegetables is correlated with a significantly reduced incidence of high blood pressure, myocardial infarction and stroke [7].

To resolve the question of whether nitrate in vegetables is beneficial or harmful to the consumer, it is necessary to investigate to what extent the effect of lettuce consumption on human health is due to nitrate or to other bioactive phytochemicals in this vegetable. The best way to do this is to carry out a randomized placebo-controlled human intervention trial, to assess effects of lettuce with high (treatment) or low (placebo) nitrate content on nitrate bioavailability in humans and on a marker of human health (blood pressure). This requires a reliable supply of this vegetable with reproducible differences in the composition, to control as many confounding factors as feasible.

Previous studies have shown that various growing conditions such as light intensities, temperature and nitrogen fertilizer as well as interactions between genotype and environment all

affect nitrate accumulation in lettuce [8]. Most of the studies were done in greenhouse environments, and in those where other phytochemical compounds than nitrate were measured, it was found that nitrogen fertilization generally tended to reduce contents of phenolic compounds while increasing the contents of nitrate, carotenoids and chlorophylls. However, absolute values differed substantially among the studies [6, 8-11], and also whether contents were reported based on fresh weight or dry matter. In addition to variety differences [10], the compositions are probably affected by specific climatic conditions, in particular light and humidity are difficult to reproduce across different seasons.

The aim of this work was therefore to develop two nitrogen fertilization regimes to provide radically different concentrations of nitrate in plants with a similar healthy appearance, and then determine the effects on other potentially health relevant compounds such as phenolic acids, flavonoids, chlorophylls and carotenoids under identical completely controlled growing conditions.

MATERIAL AND METHODS

Growth condition and plant materials. Butterhead lettuce (*Lactuca sativa* L. var. *capitata* cv Egery) was sown in 11x10x10 cm square pots in a growth cabinet (Plant Growth/Environmental Chambers - MLR-351, SANYO) with five shelves. Pots contained 1litre of peat moss (White sod peat with structure fine (0-10mm), KLASMAN code 822) with 33.3g CaCO3 l^{-1}. Three seeds were sown and irrigated with deionized water at 20 °C for three days, then reduced to 1 plant per pot after germination. For plant growth, the PAR of 150μmol m^{-2} s^{-1} for 14 hours day^{-1}, including 12 hours at 27°C and 2 hour for transitions at 23°C a day at end of the light period, then the dark was for 10 hours day^{-1} at 23°C. During plant growth, the location of shelves and plants were changed every ten days to even out any differences in irradiance among shelves and positions on a shelf. The humidity was 64%, recorded by humidity meter (Ebro-a Xylem brand, LEBI-20TH, Klipspringer). Plants were irrigated every day, from germination until one day before harvesting, with 50ml of nutrient solution per pot per day for the first four weeks and then increased to 100ml for the last three weeks.

Nutrient solution. The nutrient solution was based on Hoagland's nutrient solution and designed to allow the nitrogen concentrations to differ at five levels 26, 39, 51, 103 and 154 ppm of N, corresponding to 0.089, 0.135, 0.18, 0.36 and 0.54 g N/plant for the entire growing period or 7.21, 10.8, 14.4, 28.2, and 43.2 Kg Ha^{-1}. Values were calculated using 8 plants m^{-2}, while keeping all other nutrients constant. This was done by changing the source of macronutrients (N, P, K) to ammonium nitrate (NH_4NO_3), potassium hydroxide (KOH) and Doff Single Super Phosphate (7.6%P) instead of ammonium phosphate $(NH_4)_3PO_4$, potassium nitrate (KNO_3) and calcium nitrate ($Ca(NO_3)_2$) respectively, see Table 1 (additional calcium was provided from the $CaCO_3$ in the peat moss mixture).

Position of Table 1, Modified Hoagland's nutrition solutions

The plants were grown for seven weeks, with each treatment provided to six plants. For each plant, after weighing the harvested above-ground material, half of the fresh plant was used for nitrate analysis and the second half was individually frozen at -20°C and freeze-dried in a VirTis GPFD 24DX48 (SP Scientific) freeze-drier. The freeze-dried samples were weighed and broken into a coarse powder, which was stored at -20°C for up to 6 months until analysis.

Nitrate analyses. 14 to 16g of fresh lettuce was mixed with 15-25ml of de-ionized water, homogenized using an Ultra Turrax T-25 for at least 1 minute and then centrifuged (2000rpm, 10 min). Nitrate concentration in the aqueous extract was analyzed by gas chromatography-mass spectrometry (GC–MS) as previously described [12].

Extraction of samples for phenolic acid analysis. The method was as described by Alarcón-Flores et al. [13] with some modifications. 150 mg of lyophilized sample were weighed into a 15 mL polypropylene centrifuge tube and ten mL of methanol: water (80:20, v/v) were added. The mixture

was agitated for 30 min with a shaker, centrifuged for ten min at 4000rpm, and 1.5 ml of supernatant was transferred into an HPLC vial.

Phenolic acid compositions and flavonoids analysis. The HPLC column was a Kinetex EVO Reverse phase (C18, 100A, 250×4.6mm, 5um), and the column oven was set at 25°C. The injection volume was 20 µL, and the HPLC, system was equipped with a Shimadzu 2 LC-10AD pump, SiL-10A system Autosampler, SPD-M 10A photodiode array UV-VIS detector set to collect all data from 200 to 600 nm, and a CTO-10AD column oven (Shimadzu Corporation. Kyoto, Japan). The mobile phase was 0.1% v/v trifluoroacetic acid in ultra-pure water (solvent A), 0.1% v/v trifluoroacetic acid in HPLC- grade acetonitrile (solvent B) with a flow rate of 1 ml/minute. The solvent gradient (A:B) was 0 min (100: 0), 5 mins (100:0), 15 mins (83:17), 17 mins (83:17), 22 mins (75:25), 30 mins (65:35), 35mins (50:50), 40 mins (0:100), 50mins (0:100) followed by re-equilibration as 55mins (100:0) and 65 mins (100:0).

Quantification of phenolic acids. The identification and quantification of phenolic acids was based on the retention times and absorption spectra of authentic standards, selected based on [14-15]. The diode-array detector was set at 320nm for quantification of phenolic acids and 354nm for flavonols. The concentrations of unidentified phenolic acids were expressed as chlorogenic acid equivalents as recommended by Clifford and Madala [16] and unidentified quercetin derivatives were quantified as rutin equivalents (see Figure 1, shows a HPLC chromatogram of phenolic compounds in lettuce).

Position of Figure 1, HPLC Chromatogram of phenolic compounds in lettuce:

Extraction of samples for determination of chlorophylls and carotenoids. The method was as described by Rashed [17] with some modifications. 70mg of the freeze-dried sample was put in a 10ml screw glass tubes with 3ml of ethyl acetate. Samples were covered with aluminum foil to keep them

dark. Samples were vortexed for a few minutes and left in the fridge overnight. The samples were centrifuged for 10 minutes at 4000 rpm and the supernatant transferred to an HPLC vial.

Chlorophylls and Carotenoids analyses. The analysis was carried out on the same HPLC and column as the phenolic acids. The HPLC oven was set at 40°C with the flow at 1ml/min, and 20 µL samples were injected. The detection was at 450nm. The solvents of the mobile phase were pure water (A), methanol (B), and ethyl acetate (C). The gradient (A:B:C) was: 0 min (50:50:0), 6 mins (50:50:0), 11 mins (30:70:0), 30 mins (15:85:0), 35 mins (0:100:0), 38 mins (0:90:10), 56 mins (0:60:40), 62 mins (0:0:100), 64 mins (0:0:100), 70 mins (0:100:0), followed by re-equilibration as 75 mins (50:50:0), 95 mins (50:50:0).

Identification and quantification of carotenoids and chlorophylls. Retention times and peak areas of carotenoids and chlorophylls were measured by comparing to authentic standards. The concentrations of unidentified carotenoids were expressed as β-carotene equivalents (see Figure 2, shows a HPLC chromatogram of carotenoids and chlorophylls in lettuce).

Position of Figure 2. HPLC Chromatogram of carotenoids and chlorophylls in lettuce:

Chemicals and Reagents. Chlorogenic acid (3-Caffeoylquinic acid) was purchased from Chemstrong scientific Co Ltd, Neochlorogenic acid (5-Caffeoylquinic acid), Cynarin (1,3-Dicaffeoylquinic acid), Isochlorogenic acid A (3,5-Dicaffeoylquinic acid), Isochlorogenic acid B (3,4-Dicaffeoylquinic acid), Isochlorogenic acid C (4,5-Dicaffeoylquinic acid), Cichoric acid (Dicaffeoyltartaric acid), Caftaric acid (Caffeoyltartaric acid) were purchased from Biopurify Phytochemicals Ltd, Rutin (Quercetin-3-rutinoside) was purchased from Sigma-Aldrich Company Ltd. All standards were prepared as stock by concentration 0.1 mg /mL in 70% Methanol; the standards were stored in dark in refrigerant.

Statistical analysis. One-way analysis of variance (ANOVA) and linear regression were used to determine the main effects of nitrogen levels on contents of nitrate and other phytochemical compounds. All the measurements were done separately for each individual plant. For dry matter, nitrate content and plant biomass, for each N level, data were recorded from all 6 plants, while the phenolic compounds, carotenoids and chlorophylls were analyzed in 3 randomly selected plants from each treatment. Statistical significance was determined at the $P<0.05$ level and significantly different values distinguished using Tukey's test. All statistical analyses were completed using Minitab, version 17 Statistical software.

RESULTS

Effects of nitrogen levels on nitrate concentration. Increasing the nitrogen concentration from 26 to 154ppm in the nutrient solution dramatically increased the nitrate content in the lettuce, from 6.02 to 1078mg $100g^{-1}$ FW, respectively (Figure 3).

Position of Figure 3. Effect of nitrogen levels on nitrate content in Butterhead lettuce

Effects of nitrogen supply on plant biomass. The mass of each lettuce plant was reduced with high nitrogen supply (Figure 4.) for both fresh weight and dry matter content of Butterhead lettuce leaves.

Position of Figure 4. Effect of nitrogen levels on weight of plant (g) and dry matter (% and g/plant) in Butterhead lettuce.

Effects of nitrogen supply on phytochemical contents in lettuce. Figure 5 shows the contents of three types of phytochemicals per 100 g fresh weight of Butterhead lettuce: chlorophylls, carotenoids and phenolic compounds. As N-supply increased, the content of phenolic compounds fell dramatically, while the concentrations remained almost unchanged for both of the pigments.

In contrast, when expressed on dry matter basis, a definitive increase of chlorophyll contents in the lettuce leaves was observed, chlorophyll ***b*** (P=0.005) and ***a*** (P=0.008) and for the total chlorophylls (P=0.006) from 26 to 154ppm N concentration in the nutrient solution (Table 2). Similarly, high nitrogen treatments increased carotenoid contents which was significantly different for both β-carotene (P=0.014) and total carotenoids (P=0.014) between 26 and 154ppm. This contrasts with the situation for phenolic acids and flavonoids listed in Table 3, which shows that increasing N supply led to decreases of every type of phenolic compounds. The total phenolic acids and total flavonols of 26ppm N were significantly higher than the other N levels, (P=0.002) and (P<0.001), respectively. Altogether, the total phenolic compounds of 26ppm N was also significantly higher than the other N levels (P=0.001).

Position of Table 2. Carotenoids and chlorophylls in Butterhead lettuce

Position of Table 3. Phenolic compounds in Butterhead lettuce

DISCUSSION

Effect of N-supply on nitrate accumulation. The present study was designed to determine the effect of nitrogen fertilization on concentrations of nitrate and other potentially health relevant phytochemical compounds such as phenolic acids, flavonoids, chlorophylls and carotenoids in controlled growing conditions. Specifically, it aimed to define and control nitrate accumulation in lettuce sufficiently well to develop a set of treatments that could be used as a placebo control tool together with a high nitrate lettuce treatment in an intervention trial study. The results showed as expected substantial differences of nitrate content in Butterhead lettuce among the five nitrogen supply levels, ranging from almost nothing to a level so high that it can provide more than twice the recommended daily intake of approx. 250mg in one 50g portion (Figure 3). It is well known that several plant species have the ability to accumulate nitrate if supplied in excess of existing demands,

e.g. in white cabbage, green cabbage, spinach and rape [18-19]. However in a study using natural soil, but otherwise very similar to the present experiment, the observed differences in nitrate contents in lettuce were much smaller [6], demonstrating the importance of using an N-free substrate like peat moss.

While the results of the present study give information on general aspects of how fertilizer nitrogen supply affects plant composition when everything else is held constant, the measured values cannot be directly applied to nitrate accumulation under field conditions or even in greenhouse, because the processes of absorption, translocation and assimilation of nitrogen are all strongly affected by other factors such as light, temperature and moisture [8, 20-21]. For example, at 14/6 °C (day/night) the nitrate content was significantly higher than at 6/6°C (day/night) in Butterhead lettuce grown in growth chambers [22].

Effect of N-supply on dry matter content. The results showed that dry matter content and fresh weight decreased with increased inorganic nitrogen supply from 26 to 154ppm of nitrogen as ammonium nitrate, however a similar effect of excess N supply on plant fresh weight has also been observed in another study [11] which aimed to reduce nitrate accumulation in lettuce. The effect on dry matter content has been observed in studies of several vegetable species comparing organic and conventional production systems [3], indicating that this may be a general phenomenon.

Effect of N-supply on contents of phytochemicals. The key findings of the present study were to determine how the nitrogen supply affected not only nitrate accumulation and plant growth, but also the contents of other types of phytochemical compounds, resulting in several other consistent differences in plant composition in addition to simply the nitrate content. As summarized in Figure 5, increased N supply strongly reduced the phenolic compounds while the contents of carotenoids and chlorophylls were much less affected if at all. However, when measured on dry matter basis the contents of carotenoids and chlorophylls increased with increasing nitrate accumulation (Table 2).

As summarized in tables 2 and 3, these effects had similar impact on every measured compound within each class of compounds, it was not just one dominant compound that was affected,

supporting the notion that these effects are manifestations of general aspects of plant metabolism rather than specific processes related to particular roles of individual compounds. Several previous studies have shown similar effects of increased nitrogen uptake on each class of phytochemicals. For example it decreased phenolic content in plants [23], increased chlorophyll contents in Butterhead lettuce leaves [9, 24-26], levels of carotenoids [26-29], and nitrogen fertilization reduced contents of soluble phenolics in rocket leaves [30] and of contents of quercetin and total flavonols in tomatoes [31].

Comparison with effects of stress. Regarding phytochemical contents, it is often stated that environmental stresses such as insect infestation or light intensity determine the levels of phenolic compounds [32-33]. carotenoids [34], and chlorophyll [35]. The plants in the present study showed similar levels of carotenoids [29], chlorophylls [36] and flavonols [37] in agreement with the literature on Butterhead lettuce in general, even though they were grown in a fully controlled environment, protected against all the environmental stresses such as excessive light, pests and diseases, drought etc. From this perspective, it is remarkable that N supply as the only variable factor resulted in these very substantial differences in phytochemical composition, particularly for phenolic compounds. This indicates that the ability of plants to adapt to their growing conditions (as in this case nitrogen supply) is much more flexible and fundamental to the plant metabolism than a simple 'stress response' concept. It is quite possible that these effects still reflect adaptations to potential stress, allowing the plants to allocate resources optimally in response to the most likely challenges that may occur in environments with different levels of nutrient supply, as a 'stress anticipation' concept [38]. However it strongly contradicts the widespread view that the presence of pests and diseases [39] or abiotic stresses such as UV light and drought [40] are the dominant non-genetic determinants of plant phytochemical composition. This outcome is for example of interest regarding the effect (or not) of using organic or conventional crop production systems on the phytochemical composition of crops for human consumption [3].

Development of material for human intervention study. The initial aim of testing these different nitrogen application rates on lettuce was to develop a procedure to produce two sets of lettuce materials with controlled low and high nitrate content, to use as placebo and treatment, respectively,

in order to investigate the effects of nitrate in lettuce on blood pressure. The study was fully successful in producing visibly similar plant materials with the very large intended differences in the nitrate contents. However, the results showed that it was not possible to control only the nitrate content in the leaves without affecting other potentially bioactive phenolic compounds, so such treatment and placebo will be different in contents of both nitrate and phenolics. Also, while pigment concentrations and thus leaf colors were similar across all treatments, the effects on plant growth rates was reflected in differences in leaf shape (see TOC graphic), so the visual appearances of lettuce from different treatments were slightly different. This means that when using this method to produce material for human intervention studies to compare the effects of intake of low and high nitrate vegetables, the different phytochemical contents, color shades and leaf sizes must be taken into account in the study design, to ensure that they do not invalidate the outcomes. For example, the plant material can be provided in opaque bags to provide double-blind treatments, and the study outcomes be controlled for effects that could be caused by the other phytochemicals. Overall, the high and low N supply lettuce plants will be particularly appropriate for types of studies where the differences in phenolic compounds do not interfere with the outcomes, for example to determine whether nitrate in a solid food such as lettuce has the same or different effects and bioavailability as nitrate-rich juices.

In the context of the specific research project, the large differences in contents of phenolic compounds was an unintended effect, which must be compensated for. However, the results in Figures 3 and 5 show how the same method could also be used to produce plant material for testing the effect of phenolic compounds on human health. In this case the 'treatment' lettuce should be fertilized with 26 ppm N (or even lower) and the 'placebo' lettuce with 39 ppm N, to produce a 2.5-fold difference in content of phenolic compounds, while keeping the nitrate level in both treatments well within the range of 'low nitrate' vegetables [5].

In addition to the aim of producing material for intervention experiments, the present results also provide or confirm useful general information for quality management of vegetable composition, especially for production of leafy vegetables under controlled growing conditions.

These general effects could be summarized as follows:

1- The nitrate content in lettuce can be accurately controlled by the supply of N from fertilizer, when light, temperature, and other nutrients are kept constant.

2- Increasing nitrogen fertilizer application above the minimum required for highest fresh weight yield under otherwise constant conditions show other consistent effects:

- Decrease in both fresh weight accumulation and dry matter percentage.
- No effect on accumulation of terpenoid plant pigments, both chlorophylls and carotenoids, unless expressed on dry matter basis.
- Decrease in contents of phenolic compounds, both phenolic acids and flavonols, whether measured on the basis of fresh weight or dry matter.

Abbreviations used

N: nitrogen; NO_3: nitrate; DM: dry matter; FW: fresh weight; HPLC: high performance liquid chromatography.

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Supporting Information

1- Figure S1. Effect of nitrogen levels on nitrate content (mg/g dry matter and mg/plant) in Butterhead lettuce.

2- Figure S2. Effect of nitrogen levels on total phenolic compounds, total chlorophylls and total carotenoids contents (mg/g DW) in Butterhead lettuce.

3- Figure S3. Effect of nitrogen levels on total phenolic compounds, total chlorophylls and total carotenoids contents (mg/plant) in Butterhead lettuce.

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Figure captions

Figure 1. HPLC Chromatogram of phenolic compounds in lettuce: Peaks identified by comparison with authentic standards: 6 Caftaric acid (Caffeoyltartaric acid), 7 Neochlorogenic acid (5-Caffeoylquinic acid), 8 Chlorogenic acid (3-Caffeoylquinic acid), 9 Cynarin (1,3-Dicaffeoylquinic acid), 17 Isochlorogenic acid B (3,4-Dicaffeoylquinic acid), 18 Isochlorogenic acid A (3,5-Dicaffeoylquinic acid), 26 Rutin (Quercetin-3-rutinoside), 28 Cichoric acid (Dicaffeoyltartaric acid), 29 Isochlorogenic acid C (4,5-Dicaffeoylquinic acid). 20, 21, 27, 30, 31 and 32: Unknown phenolic acids; 23 and 24 Unknown quercetin derivatives.

Figure 2. HPLC Chromatogram of carotenoids and chlorophylls in lettuce: 10 Lutein, 22 Chlorophyll b, 23 *cis*-Chlorophyll b, 24 Chlorophyll a, 32 β-carotene; 8, 9, 11, 12, 13, 14, 15, 16 and 17: Unknown carotenoids.

Figure 3. Effect of nitrogen levels on nitrate content (FW) in Butterhead lettuce grown at 27/23°C, $P<0.0001$ for trend (linear regression). Values are the mean ± SEM of six plants for each N level (n=6). Bars with different letters indicate significant differences at $P < 0.05$.

Figure 4. Effect of nitrogen levels on fresh weight (g/plant FW) and dry matter (% and g/plant) in Butterhead lettuce ($P<0.001$, $P=0.002$ and $P<0.0001$ respectively). Values are the mean ± SEM of six plants for each N level (n=6). Bars with different letters indicate significant differences at $P < 0.05$.

Figure 5. Effect of nitrogen levels on total phenolic acids, total chlorophylls and total carotenoids contents (FW) in Butterhead lettuce. Linear regression showed $P < 0.004$, $P = 0.037$ and $P = 0.73$, respectively. Values are the mean ± SEM of three plants for each N level (n=3). Bars with different letters indicate significant differences at $P < 0.05$ between different N levels.

Table 1. Modified Hoagland's nutrition solutions, providing five levels of nitrogen for lettuce.

nitrogen levels	chemical	stock solution g L^{-1}	stock solution mL L^{-1} of final solution	element	final solution ppm
nitrogen source					
N1	NH_4NO_3	14.706	5mL L^{-1}	N	26
N2	NH_4NO_3	14.706	7.5mL L^{-1}	N	39
N3	NH_4NO_3	14.706	10mL L^{-1}	N	51
N4	NH_4NO_3	14.706	20mL L^{-1}	N	103
N5	NH_4NO_3	14.706	30mL L^{-1}	N	154*
macronutrients					ppm
for all nitrogen levels	$Ca(H_2PO_4)_2$ + 2 $CaSO_4$	15	10mL L^{-1}	P	11.4
	KOH	20	10mL L^{-1}	K	141**
micronutrients					ppm
for all nitrogen levels	KCl	3.728	in 1 liter mixed stock solution (storable)	Cl	1.77
	H_3BO_3	1.516		B	0.27
	$MnSO_4 H_2O$	0.338		Mn	0.11
	$ZnSO_4 7H_2O$	0.575		Zn	0.131
	$CuSO_4 5H_2O$	0.125		Cu	0.032
	H_2MoO_4 (85% MoO_3)	0.081		Mo	0.05
	Fe-EDTA[3]	6.922	prepared fresh	Fe	1.12

*The lettuce received a total 3.5L of nutrient solution per plant, so the 154 ppm N treatment provided 0.54 g N/plant, which corresponds to 1.54 g ammonium nitrate or 2.39 g nitrate to each lettuce plant.
**Includes the 2 ppm from KCl.

Table 2. Carotenoids and chlorophylls (DW) in Butterhead lettuce [a]

compounds	nitrogen levels (ppm in nutrient solution)					
	26	39	51	103	154	*P-value*
β-carotene (µg/g DW)	680 ± 94 b	879 ± 184 ab	771 ± 263 b	948 ± 42 ab	1310 ± 223 a	0.014
Lutein (µg/g DW)	291 ± 34 a	382 ± 89 a	367 ± 155 a	397 ± 70 a	502 ± 59 a	0.159
unknown carotenoids (µg β-carotene equivalents/g DW)	695 ± 119 b	779 ± 176 b	1037 ± 492 ab	1280 ± 51 ab	1626 ± 277 a	0.010
total carotenoids (mg/g DW)	1.7 ± 0.2 b	2.0 ±0.4 b	2.2 ± 0.9 ab	2.6 ± 0.1 ab	3.4 ± 0.5 a	0.014
Chlorophyll b (mg/g DW)	1.9 ± 0.2 b	2.1 ± 0.4 b	5.0 ± 3.5 ab	6.8 ± 0.2 a	7.5 ± 1.0 a	0.005
Chlorophyll a (mg/g DW)	11.8 ± 1.4 c	14.1 ± 3.1 bc	16.8 ± 2.7 abc	2.9 ± 0.5 ab	3.2 ± 3.9 a	0.008
total chlorophylls (mg/g DW)	16.2 ± 1.3 c	18.7 ± 4.7 bc	24.4 ± 17.4 abc	39.7 ± 0.8 ab	44.8 ± 4.3 a	0.006

[a] Values are the mean ± SD of three plants for each N levels (n=3). In a row, different letters (a, b and c) indicate significant differences at $P < 0.05$ among nitrogen levels.

Table 3. Phenolic compounds (DW) in Butterhead lettuce [a]

compounds	nitrogen levels (ppm in nutrient solution)					
	26	39	51	103	154	*P-value*
Cichoric acid (mg/g DW)	10.2 ± 9 a	4.4 ± 2. b	5.3 ± 2.7 b	2.5 ± 0.7 b	2.7 ± 1.3 b	0.002
Caftaric acid (µg/g DW)	1530 ± 60 a	1180 ± 270 a	960 ± 540 ab	330 ± 80 b	310 ± 60 b	0.001
Chlorogenic acid (µg/g DW)	1600 ± 500 a	960 ± 1100 a	390 ± 80 a	590 ± 50 a	740 ± 320 a	0.114
Neochlorogenic acid (µg/g DW)	31.8 ± 7.8 a	16.0 ± 8.9 a	19.2 ± 14.9 a	13.2 ± 3.2 a	13.7 ± 6.0 a	0.144
Cynarin (µg/g DW)	14.0 ± 1.5 a	9.8 ± 3.2 a	10.6 ± 5.8 a	13.4 ± 2.4 a	11.5 ± 1.6 a	0.461
Isochlorogenic acid A (µg/g DW)	113 ± 31 a	96 ± 88 a	74 ± 64 a	59 ± 4 a	65 ± 25 a	0.532
Isochlorogenic acid B (µg/g DW)	23.7 ± 4.3 a	6.9 ± 3.0 b	11.4 ± 8.0 ab	4.2 ± 3.1 b	7.2 ± 7.5 b	0.018
Isochlorogenic acid C (µg/g DW)	2.85 ± 0.71 a	2.78 ± 1.51 a	5.13 ± 2.39 a	5.13 ± 0.96 a	5.59 ± 5.48 a	0.500
unknown phenolic acids (µg chlorogenic acid equivalents/g DW)	41.5 ± 3.9 ab	72.2 ± 37.0 a	25.0 ± 4.7 ab	15.3 ± 9.1 b	21.0 ± 9.2 b	0.025
total phenolic acids (mg chlorogenic acid equivalents/g DW)	13.8 ± 1.5 a	6.9 ± 3.4 b	6.9 ± 3.4 b	3.5 ± 0.8 b	3.3 ± 1.7 b	0.002
Rutin (µg/g DW)	1100 ± 30 a	350 ± 210 b	250 ± 110 b	140 ± 20 b	160 ± 70 b	0.000

unknown flavonols (µg rutin equivalents/g DW)	390 ± 80 a	178 ± 61 b	75 ± 28 b	50 ± 20 b	53 ± 12 b	0.000
total flavonols (µg rutin equivalents/g DW)	1490 ± 92 a	528 ± 274 b	325 ± 93 b	195 ± 11 b	212 ± 77 b	0.000
total phenolic compounds [b] (mg /g DW)	15.3 ± 1.5 a	7.4 ± 3.7 b	7.2 ± 3.4 b	3.8 ± 0.8 b	4.2 ±1.8 b	0.001

[a] Values are the mean ± SD of three plants for each N level (n=3). In a row, different letters (a, b and c) indicate significant differences at $P < 0.05$ among nitrogen levels.
[b] Sum of mg of authentic compounds or their equivalents

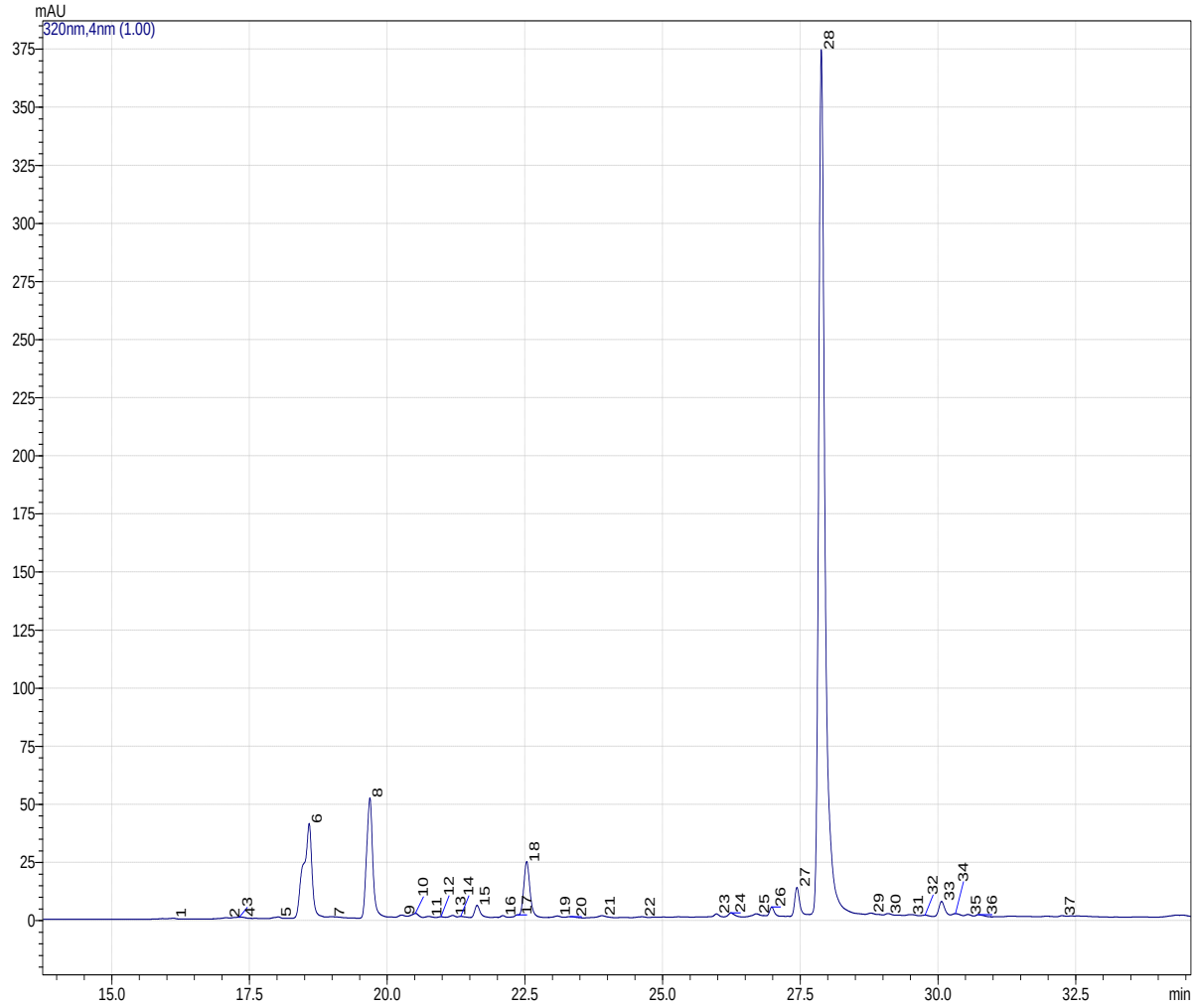


Figure 1. HPLC Chromatogram of phenolic compounds in lettuce

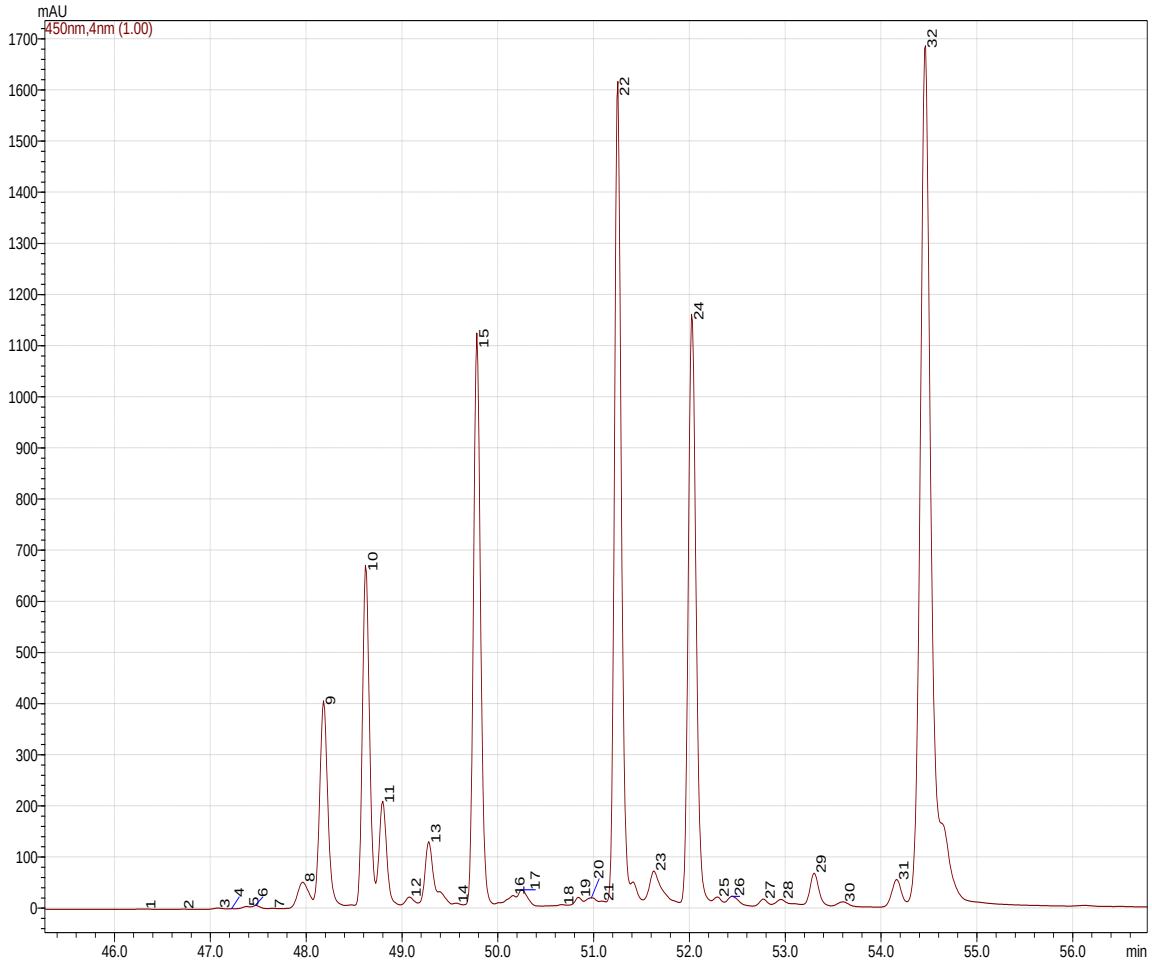


Figure 2. HPLC Chromatogram of carotenoids and chlorophylls in lettuce.

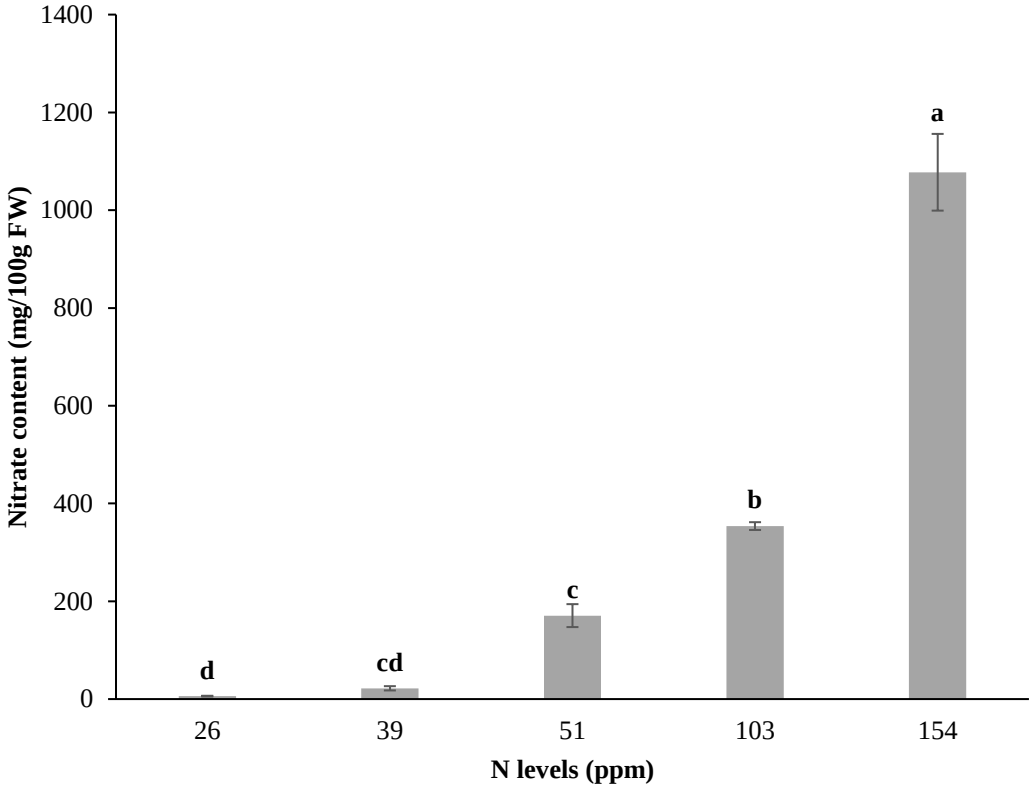
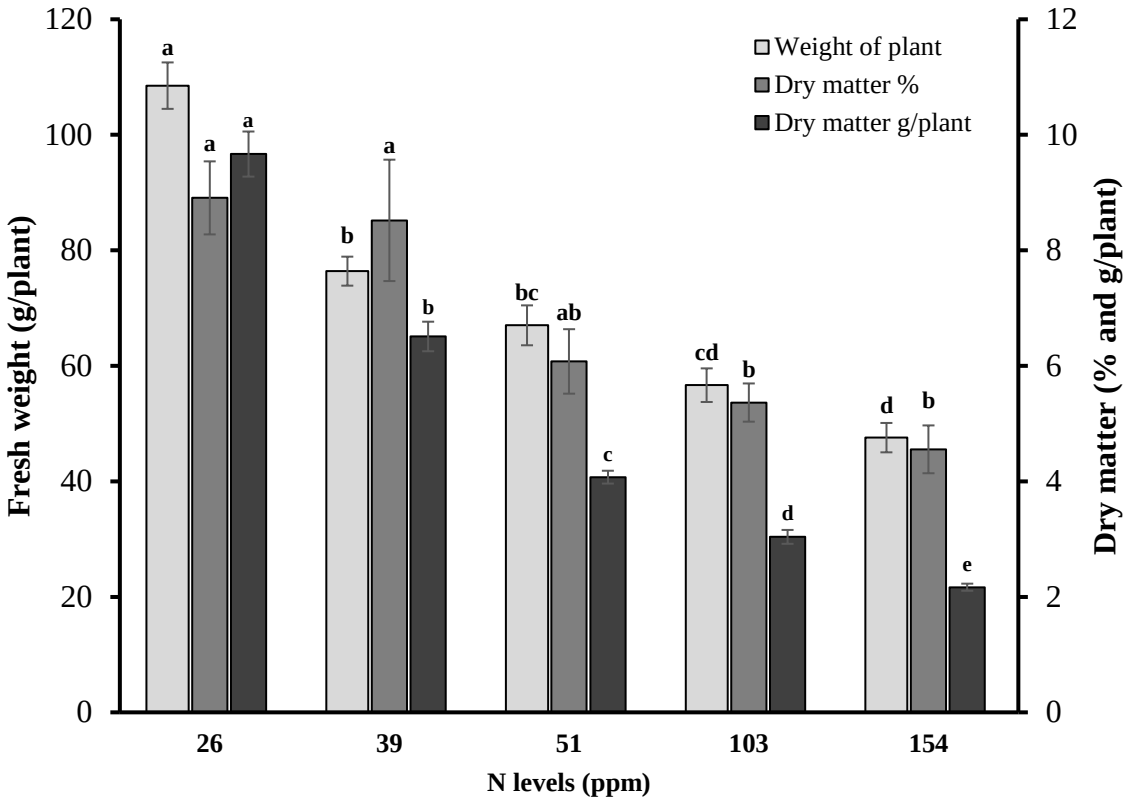


Figure 3. Effect of nitrogen levels on nitrate content (FW) in Butterhead lettuce.



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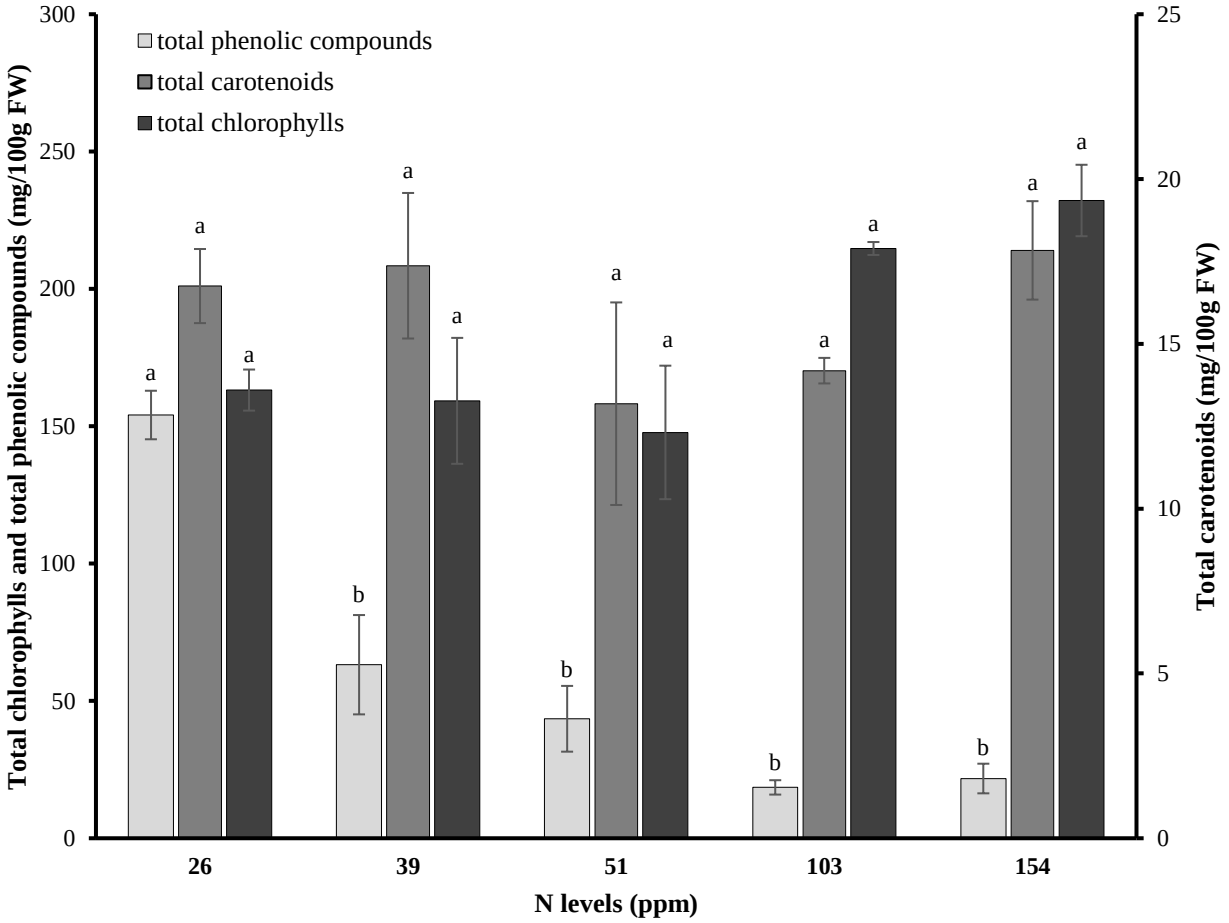

Figure 5. Effect of nitrogen levels on total phenolic compounds, total carotenoids and total chlorophylls contents (FW) in Butterhead lettuce.

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