



## Article

# For a Better Understanding of the Effect of N Form on Growth and Chemical Composition of C<sub>3</sub> Vascular Plants under Elevated CO<sub>2</sub>—A Case Study with the Leafy Vegetable *Eruca sativa*

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**Abstract:** Plant responses to elevated atmospheric CO<sub>2</sub> (eCO<sub>2</sub>) are well studied, but the interactions of the carbon and nitrogen metabolism in the process are still not fully revealed. This is especially true for the role of nitrogen forms and their assimilation by plants under eCO<sub>2</sub>. This study investigated the interacting metabolic processes of atmospheric CO<sub>2</sub> levels and N form in the short-term crop arugula. The effects on physiological processes and their consequences for crop growth, yield and nutritional value were elucidated. Two varieties of arugula were grown in climate cabinets under 400 or 800 ppm CO<sub>2</sub>, respectively. The plants were fertilized with either pure nitrate or ammonium-dominated-N. Photosynthetic CO<sub>2</sub> assimilation increased in response to eCO<sub>2</sub> regardless of the N form. This did not affect the assimilation of nitrate and consequently had no impact on the biomass of the plants. The extra photosynthates were not invested into the antioxidative compounds but were probably diverted towards the leaf structural compounds, thereby increasing dry mass and “diluting” several mineral elements. The fertilization of arugula with ammonium-dominated N had little benefits in terms of crop yield and nutritional quality. It is therefore not recommended to use ammonium-dominated N for arugula production under future elevated CO<sub>2</sub> levels.

**Keywords:** ammonium; climate change; food quality; photosynthesis; nitrogen source; nitrate; vegetable

## 1. Introduction

The atmospheric CO<sub>2</sub> concentration is predicted to rise from 400 ppm up to 1142 ppm until the end of the 21st century [1]. Elevated CO<sub>2</sub> concentrations (eCO<sub>2</sub>) promote growth for many plants as the water use efficiency is ameliorated and the carbon gain by photosynthetic activities is increased, at least in the short term, compared to ambient CO<sub>2</sub> concentrations (aCO<sub>2</sub>) [2]. The beneficial effect of eCO<sub>2</sub> in higher plants, however, is linked to certain conditions, such as (e.g., [3]): i. the plant's type of photosynthesis (e.g., C<sub>3</sub> or C<sub>4</sub> pathway), ii. the plant's growth period and therefore effect duration (short-, medium- or long-term), iii. the presence of other photosynthesis limiting factors (e.g., light, nutrients), and iv. the balance between source activity (net export of photo-assimilates by mature leaves) and sink activity (growth, storage). This complexity highlights that the finely-tuned mechanism of carbon assimilation is the prerequisite for better plant production under future increased carbon dioxide supply. From an agricultural perspective, the physiological processes involved that can be influenced by crop management are of particular interest. The supply of nitrogen and its assimilation in plants represents one such adjusting screw. Nitrogen (N) is incorporated in many organic compounds comprising all amino acids, thus being highly relevant for the biosynthesis of enzymes which are essential for metabolic

activities. Plants expend about one quarter of their energy to convert inorganic into organic N [4]. N deficiency of plants induces a shift in the metabolic processes with a reduction of N-based products, such as proteins, resulting in reduced growth [5]. Due to the high vulnerability of crop development to N deficiency, the relationship between eCO<sub>2</sub> and nitrogen assimilation deserves special attention.

Nitrogen assimilation in plants is coupled to photosynthesis primarily through Rubisco, the enzyme catalyzing C<sub>3</sub> carbon fixation. Rubisco activity determines the availability of electrons for the reduction of inorganic nitrogen, NO<sub>3</sub><sup>−</sup> via NH<sub>4</sub><sup>+</sup>, to organic compounds [6]. The reductants originate from the oxygenation reaction of Rubisco, better known as photorespiration. The carboxylation reaction and photorespiration compete for reductants and the balance between these processes depends largely on the relative concentrations of CO<sub>2</sub> and O<sub>2</sub>, as well on the high specificity of Rubisco for CO<sub>2</sub> [7]. At eCO<sub>2</sub>, photorespiration is initially reduced in favour of stimulated net CO<sub>2</sub> assimilation (e.g., [8]). Limited photorespiratory activity provides less reductants for the nitrate reduction in photosynthetic tissue (e.g., [4,9]), which limits the nitrate assimilation under eCO<sub>2</sub> conditions [4]. Bloom [4] concludes that the nitrogen form supplied to plants matters for the efficiency of converting inorganic N into organic N and consequently, for the photosynthates- and protein-driven growth response under eCO<sub>2</sub>. Thus, a reduced assimilation of inorganic N under eCO<sub>2</sub> can be attenuated by using other N forms as fertilizers for crops, such as ammonium (NH<sub>4</sub><sup>+</sup>) [6,10]. In fact, NH<sub>4</sub><sup>+</sup> can be incorporated into organic N compounds independently of photorespiration (see details in [11]). Growing plants with ammonium as the sole N source, however, may exert toxic effects [12]. For ammonium-sensitive plant families, such as the economically relevant Brassicaceae [13], it is therefore recommended to use mixtures of ammonium-N and nitrate-N [14,15] in order to improve plant productivity under eCO<sub>2</sub> levels [16].

The effects of eCO<sub>2</sub> on nitrate assimilation in the context of N forms and their consequences for plant growth are highly controversial in the literature [4,6,11,17]. The two main criticisms are the CO<sub>2</sub> acclimation [18–20] and the site of nitrate assimilation [17]. CO<sub>2</sub> acclimation is characterized by short-term stimulated net CO<sub>2</sub> assimilation, which stabilizes at rates close to those of plants at aCO<sub>2</sub> in long-term exposure to eCO<sub>2</sub> [3]. This may result from an accumulation of photosynthates that are generated by the initially increased photosynthesis. The imbalance in the source-sink ratio then down-regulates the photosynthetic activity [3]. Under these conditions, photorespiration might not be inhibited to the extent that is necessary to lower nitrate photoreduction. This is supported by observations of increased growth of C<sub>3</sub> species under eCO<sub>2</sub> with the supply of nitrate as the sole N source [21–23]. In addition, nitrate assimilation of C<sub>3</sub> plants proceeds at several sites, in leaves as well as in roots. Shifts in the partitioning of NO<sub>3</sub><sup>−</sup> assimilation from leaves to roots under eCO<sub>2</sub> have been observed [17,24] and were explained by the increased transport of photosynthates to the root to provide essential reductants [17].

The impacts of eCO<sub>2</sub> on the mechanisms of N assimilation in C<sub>3</sub> plants are thus not fully understood, especially for short-term crops. Among them, leafy vegetables which play an important role in the human diet have to comply with thresholds of their foliar nitrate content. This is related to the high demand for fertilizer, such as nitrogen (e.g., as a component of chlorophyll), to ensure good yields and qualities. Reducing the N supply is therefore not an option, but reaching high yields and low contents of nitrate in the foliage can be achieved by complete or partial fertilization with other N forms [25–29].

For the model plant *Arabidopsis thaliana* and several vegetable crops of the Brassicaceae family, the supply of mixtures of ammonium- and nitrate-N was shown to increase the concentrations of secondary compounds [6,25,30], thus further improving the nutritional value and the defensive capacity of the plants [31]. This might become relevant in the future as recent publications suggest an increased occurrence of fungal infections in leafy vegetable crops [32,33] due to altered temperature and humidity in larger and denser canopies developing under eCO<sub>2</sub> [34]. However, several studies suggest that plants grown under eCO<sub>2</sub> will have increased contents of C-based secondary compounds [35–37], which

might offer protection against pathogen and pest attack and have beneficial effects for human nutrition at the same time. Nevertheless, the negative effects of eCO<sub>2</sub> on the nutritional value of crops have also been observed: for example, larger biomass potentially reduces the concentration of macro- and micronutrients [35,38–40].

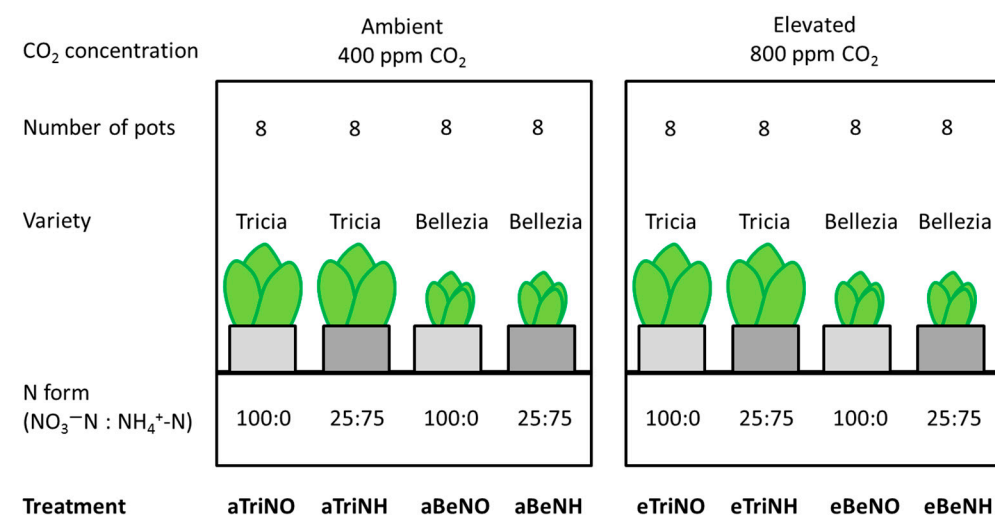
This is particularly relevant for arugula, a leafy vegetable of growing economic importance. Besides the characteristic aroma and taste, low caloric value, and high content of minerals and vitamin C, arugula offers additional benefits to human health as it contains many phytochemicals, such as carotenoids, phenolics and glucosinolates [41,42]. Arugula plants are often affected by downy mildew and thus the resistance to this biotic stressor is an important target for breeding activities on arugula. A higher defensive capacity can be counterbalanced by yield decreases as the plants are continuously faced by the dilemma to invest their resources into growth or defence-related processes [43], especially under altered environmental conditions.

Against the background of complex and partly unknown interactions between CO<sub>2</sub> concentrations and N form, we aimed at elucidating the effects of nitrate-N and ammonium-N nutrition under elevated CO<sub>2</sub> on the physiology, morphology and chemical composition of the short-term crop *Eruca sativa*, differentiated between two varieties.

## 2. Materials and Methods

### 2.1. Plant Cultivation

The experiment was conducted in a split-plot design in climatic chambers (HGC 0714, Weiss Technik UK Ltd., Königswinter, Germany). The atmospheric CO<sub>2</sub> concentration was the main factor with the arugula variety and N form being the subfactors. Each combination of the factors was repeated 8 times, realized by 8 pots with 30 plants each per factor sub-combination (Figure 1) and climatic chamber. Thus, 1 climatic chamber contained 32 pots in total.



**Figure 1.** Design of one experimental set. One rectangle represents one climatic chamber. This set-up was repeated three times.

Three sets of experiments (Figure 1) were run ( $n = 3$ ). The first and second sets were grown in parallel (late August until mid-October 2016), having two climatic chambers each set to the same CO<sub>2</sub> conditions. The third set was run after the first two (late October until early December 2016), consisting of one climatic chamber set to 400 ppm CO<sub>2</sub> and one climatic chamber set to 800 ppm CO<sub>2</sub>.

The *Eruca sativa* varieties ‘Tricia’ and ‘Bellezia’ (both Enza Zaden, Warmenhuizen, The Netherlands) were chosen for the experiment as they showed the highest and lowest incidences of downy mildew in a field trial, respectively [44]. The plants were grown in pots (diameter 10 cm) in clay substrate (325 mL·pot<sup>-1</sup>; Nullerde fein; Alpenflor Er-

denwerke GmbH & Co. KG, Weilheim, Germany) with 30 seeds each. The substrate contained 41.6–49.8 mg total N·L<sup>-1</sup>. Sowing dates were 29 August 2016 for the first and second set of experiments and 24 October 2016 for the third set. The pots were covered with Styrofoam sheets and placed in trays into the climatic chambers set to the following conditions: 20/18 °C (day/night), no light, 400 ppm CO<sub>2</sub>. After 4 days, the Styrofoam cover was removed. Another 3 days later, when the cotyledons were unfolded, the pots were arranged to their final position (distances of 11.5 cm × 14 cm). The photosynthetic photon flux density (metal halide lamps) was 1000 µmol·m<sup>-2</sup>·s<sup>-1</sup> at the height of the plants during a photoperiod of 11 h (with dusk and dawn phases of one hour each). The light intensity at plant height was recorded by a quantum sensor (LI190R, LI-COR Environmental, Lincoln, NE, USA) coupled to a data logger (LI1400, LI-COR Environmental) in each climatic chamber separately. The relative humidity was set to 50% and the temperature to 20 °C/18 °C (day/night). Water was applied according to the demands of the single pots in order to fill up to a 90% water holding capacity per pot. The pots were thus weighed every 2 days at the beginning and daily later-on, and water was supplied when the water holding capacity fell below 80%.

Fertilizer was applied 4 times during the cultivation period, separately for N and P + K, yielding 300 mg N, 185 mg P<sub>2</sub>O<sub>5</sub> and 500 mg K<sub>2</sub>O per pot. N was given on days 6, 20, 27 and 33 after sowing as calcium nitrate (N + P + K = 15.5 + 0 + 0; 100% NO<sub>3</sub><sup>-</sup>) in the pure nitrate treatment. On the same days, the ammonium-dominated N treatment received a mixture of 50% ammonium sulphate (N + P + K 21 + 0 + 0) and 50% ammonium nitrate (N + P + K 34.8 + 0 + 0), yielding 75% NH<sub>4</sub><sup>+</sup> and 25% NO<sub>3</sub><sup>-</sup>. P and K was applied on days 13, 25, 29 and 35 after sowing ("Ferty Basisdünger 1" (N + P + K 0 + 14 + 38; Planta Düngemittel GmbH, Regenstauf, Germany). The fertilizer was added into the saucers in portions of 50–75 mL per pot. The plants were cultivated for 44 to 45 days. Harvests were conducted on 11 and 12 October 2016 (first and second set) and on 6 December 2016 (third set), respectively.

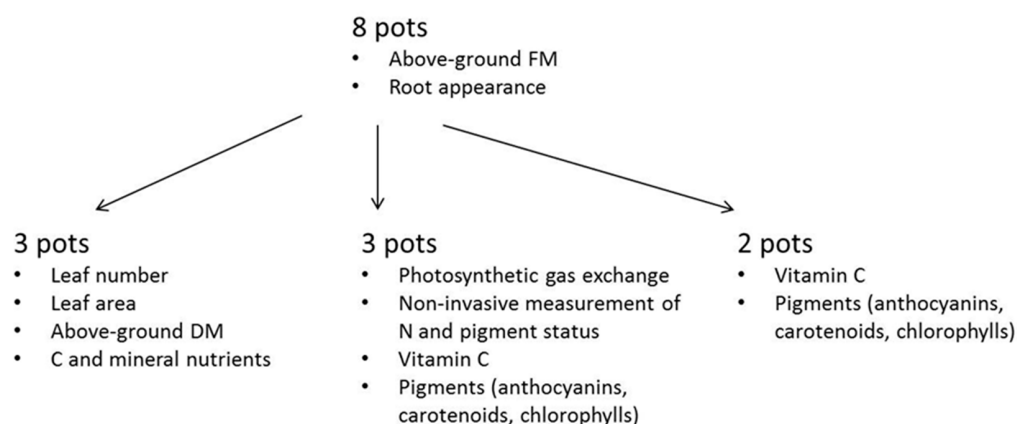
## 2.2. Biomass and Physiological Measurements

At harvest date, the number of inflorescences with at least 1 open flower and the total above-ground fresh mass were assessed from all 8 pots per treatment and climatic chamber (Figure 2). Three pots per treatment and climatic chamber were chosen for the recording of leaf numbers per pot, leaf area (LI 3100, LI-COR Biosciences, Lincoln, NE, USA), and the dry mass of the above-ground plant parts. Three other pots per treatment and climatic chamber were used for the measurement of photosynthetic gas exchange and non-invasive measurements of the N status and pigment indices. The above-ground biomass was dried at 60 °C for 24 h (when dry mass of the leaf material was constant) and then used for elemental analyses. The plants of 3 other pots per treatment and climatic chamber were used for the measurement of photosynthetic gas exchange. The above-ground biomass of all plants of these 3 pots per treatment and climatic chamber was sampled and frozen for later chemical analyses of plant pigments and vitamin C. The above-ground fresh mass in the remaining 2 pots per treatment and climatic chamber were sampled as well for the analysis of vitamin C and pigment content.

The gas exchange was measured with the GFS-3000 device (Heinz Walz GmbH, Effeltrich, Germany) on a single leaf per pot. The following cuvette conditions were used: flow 750 µmol, impeller 5, PAR 1100 µmol·m<sup>-2</sup>·s<sup>-1</sup>, relative humidity 50%, measured leaf area 8 cm<sup>2</sup>, temperature 20 °C. The CO<sub>2</sub> supply was either set to 400 ppm or 800 ppm, depending on the respective growing conditions of the plants. Measuring points were recorded when the rates of transpiration and photosynthesis were stable. After 4 measurements, the gas analyzer was calibrated by taking measurements of an empty cuvette.

After the measurements, the leaves were cut at the edge of the cuvette and the edge was subtracted by cutting along a model made of a piece of paper. The remaining leaf part, which represented the actual measured leaf area, was photographed against a piece of paper of a defined size. The measured leaf area was calculated by colour segmentation

with the software Fiji [45]. The data on photosynthetic gas exchange were then corrected for the actual measured leaf area.



**Figure 2.** Parameters assessed at harvest of the arugula plants. Each pot contained 30 plants. FM = fresh mass, DM = dry mass.

On the same leaves as used for photosynthetic measurements, indices for the N status and pigments were assessed with the Dualex device (Force-A, Orsay Cedex, France) and the N-Tester (based on the SPAD device, YARA GmbH & Co. KG, Dülmen, Germany).

### 2.3. Chemical Analyses

For ascorbic acid analyses, the frozen leaf material was homogenized in oxalic acid and an aliquot was taken for quantification by titration with iodide and iodate (TitroLine alpha plus with automatic dispenser TITRONIC universal and sample changer TW alpha plus, all SCHOTT Instruments GmbH, Mainz, Germany). Chlorophylls and carotenoids were extracted from frozen and ground leaves in 100% acetone buffered with  $\text{NaHCO}_3$  and quantified after photometric readings (modified procedure of [46]). Anthocyanins were analyzed according to [47]. Briefly, the anthocyanins were extracted from 0.5 g of frozen and ground leaf material using 80% methanol, which was acidified with 1% acetic acid. The extracts were measured photometrically at 530 nm and 657 nm and the concentrations of anthocyanins were calculated as equivalents to cyanidine-3-glycoside.

For elemental analysis, dried and ground plant materials were wet-digested in a microwave digester (MLS 1200 mega; MLS GmbH, Leutkirch, Germany). All micro- and macro-nutrients (excluding N & C) were analyzed using inductively coupled plasma optical emission spectrometry (Perkin-Elmer Optima 3000 ICP-OES; Perkin-Elmer Corp., Norwalk, CT, USA). Total nitrogen was extracted using the Kjeldahl method and measured in a continuous flow apparatus (FIAstar™ 5000 Analyser; FOSS Analytical A/S, Hilleroed, Denmark). The organic C content in the dry material was measured by a colometric assay (Lambda 25 UV/VIS Spectrophotometer; PerkinElmer, Inc., Shelton, CT, USA) after wet oxidation of the organic matter using potassium dichromate [48].

The substrate of the 8 pots of the same treatment and the same climate chamber was combined and mixed before taking an aliquot as a single subsample. In this sample, the pH was recorded (inoLab pH 7310; Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany). Additionally, the contents of the total N,  $\text{NO}_3^-$ -N and  $\text{NH}_4^+$ -N were determined after Kjeldahl distillation (Vapodest; C. Gerhardt GmbH & Co. KG, Königswinter, Germany) and titration with 0.02 M HCl.

### 2.4. Statistical Analyses

The averages of the plants per climatic chamber and treatment were used for statistical analyses, yielding  $n = 3$ . The data assessed in a split plot design ( $\text{CO}_2$  as the main factor with variety and N form as subfactors) were analyzed using the R software package [49]. The following model for a nested ANOVA was chosen:  $\text{CO}_2 \times \text{N form} \times \text{variety} + \text{error (climatic)}$



chamber/(N form\*variety)). Following this, the interactions of the 3 experimental factors CO<sub>2</sub> concentration, N form and arugula variety were assessed. When significant impacts were observed, they were further dissected by *post hoc* F-tests at  $\alpha = 0.05$ .

### 3. Results

#### 3.1. Substrate Composition

The total N remaining in the substrate after 44–45 days of arugula cultivation was neither impacted by CO<sub>2</sub> supply, nor by N form, nor by arugula variety (Table 1). The concentrations of ammonium-N and nitrate-N did not vary between the treatments. As well, the pH was not altered by the CO<sub>2</sub> supply (Table 1). However, the substrate of the ‘Bellezia’ plants had a slightly higher pH than those of the ‘Tricia’ plants. The N form also impacted the pH: with pure nitrate, the pH was higher when compared to ammonium-dominated N.

**Table 1.** Chemical parameters of the growth substrate at harvest (44–45 DAS). n = 3.

Treatment	Total N (mg·L <sup>-1</sup> )	NH <sub>4</sub> <sup>+</sup> -N (mg·L <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> -N (mg·L <sup>-1</sup> )	pH
aBeNO	65.6 a	30.1 a	35.5 a	7.0 a
aTriNO	62.6 a	15.6 a	47.0 a	7.0 a
aBeNH	83.1 a	28.7 a	54.4 a	6.1 b
aTriNH	63.4 a	27.7 a	35.7 a	5.8 c
eBeNO	60.1 a	23.4 a	36.7 a	7.1 a
eTriNO	67.7 a	20.1 a	47.5 a	7.0 a
eBeNH	84.0 a	31.4 a	52.6 a	6.1 b
eTriNH	56.1 a	24.7 a	31.5 a	5.6 c

Different letters show differences between the experimental treatments at  $\alpha = 0.05$ . a = ambient CO<sub>2</sub>, e = elevated CO<sub>2</sub>, Be = ‘Bellezia’, Tri = ‘Tricia’, NO = pure nitrate, NH = ammonium-dominated N.

#### 3.2. Plant Growth and Yield Parameters

The number of leaves per pot (=30 plants) was not significantly affected by the three experimental factors, albeit tendencies for more leaves in ‘Bellezia’ were obvious ( $p = 0.1$ ). However, differences in the leaf area were detected, which consequently influenced the total leaf area per pot: The CO<sub>2</sub> concentration had no significant effect on the leaf area, albeit ‘Bellezia’ with ammonium-dominated N supply had a lower leaf area under elevated CO<sub>2</sub> as compared to ambient CO<sub>2</sub> concentrations. Thus, the interactions of N form, variety and CO<sub>2</sub> supply were apparent ( $p = 0.048$ ). In general, plants with ammonium-dominated N supply, as well as those of the variety ‘Bellezia’, had lower leaf areas. The same pattern was found for the total above-ground fresh mass. While the CO<sub>2</sub> supply did not affect the fresh mass, there was an increase in the dry mass under elevated CO<sub>2</sub>. The plants with pure nitrate had a higher dry mass. Concerning the arugula varieties, there were tendencies ( $p = 0.053$ ) for larger above-ground dry mass in the ‘Tricia’ plants.

Inflorescences with at least one open flower showed up at day 39 after sowing (DAS), independently of CO<sub>2</sub> supply, N form and arugula variety (Figure S1). On 43 DAS, significant interactions of the three experimental factors’ CO<sub>2</sub> supply, N form and arugula variety were observed ( $p = 0.035$ ).

When fertilized with ammonium-dominated N, ‘Bellezia’ had less inflorescences than ‘Tricia’ under 400 ppm CO<sub>2</sub>. This difference between the arugula varieties was not found at 800 ppm CO<sub>2</sub> when both ‘Tricia’ and ‘Bellezia’ with ammonium-dominated N supply had significantly more inflorescences than under ambient CO<sub>2</sub> concentrations. With pure nitrate, the number of inflorescences was not altered by the CO<sub>2</sub> supply in both arugula varieties. In general, no significant effects due to CO<sub>2</sub> were observed ( $p > 0.1$ , Table 2).

The N form exhibited significant effects on the number of inflorescences. At elevated CO<sub>2</sub> conditions, both arugula varieties had more inflorescences when grown with pure nitrate as compared to those with ammonium-dominated N.

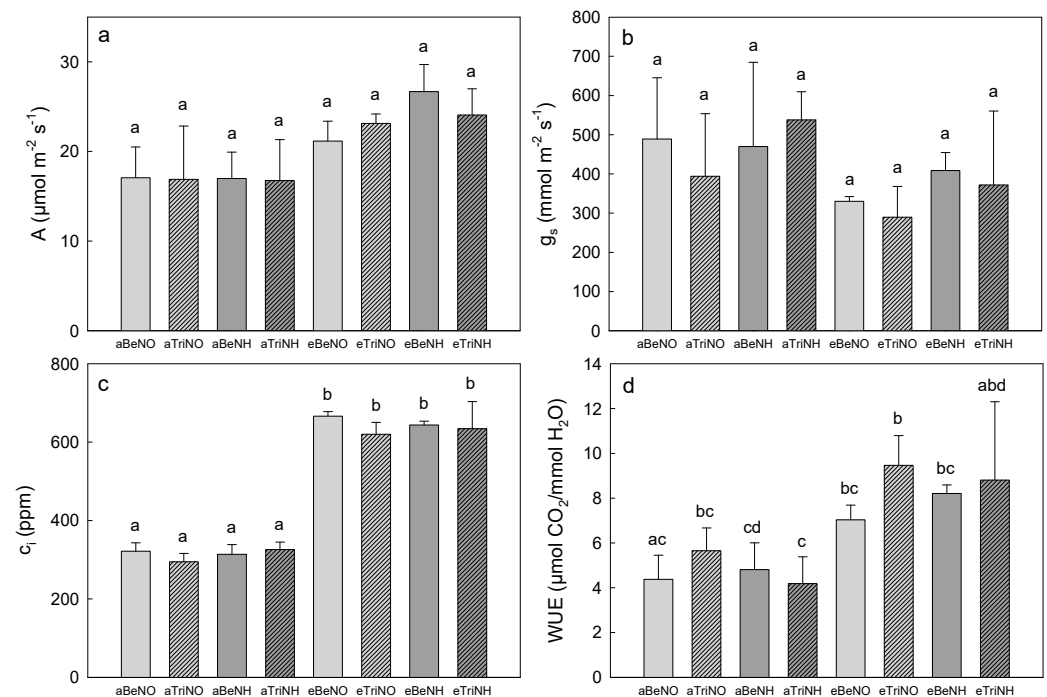
**Table 2.** Above-ground biomass parameters at harvest. Data are averages of n = 3.

Treatment	Fresh Mass (g·plant <sup>-1</sup> )	Dry Mass (g·plant <sup>-1</sup> )	Leaf Number (plant <sup>-1</sup> )	Total Leaf Area (cm <sup>2</sup> ·plant <sup>-1</sup> )	Inflorescences (Number pot <sup>-1</sup> )
aBeNO	1.53 a	0.27 ac	8.11 a	25.48 ab	4.7 c
aTriNO	1.64 a	0.28 ad	7.81 a	30.10 c	4.1 c
aBeNH	1.11 b	0.18 b	8.09 a	21.25 de	2.6 d
aTriNH	1.28 ce	0.22 ab	7.70 a	25.84 ab	3.7 c
eBeNO	1.59 ad	0.32 cd	8.52 a	25.66 ab	6.5 ac
eTriNO	1.66 a	0.32 cd	8.20 a	28.30 ac	7.4 ac
eBeNH	1.14 bc	0.23 ab	8.46 a	19.04 d	5.8 b
eTriNH	1.30 e	0.26 a	8.19 a	24.03 be	5.9 b

Different letters indicate significant differences between the treatments at  $\alpha = 0.05$ . a = ambient CO<sub>2</sub>, e = elevated CO<sub>2</sub>, Be = 'Bellezia', Tri = 'Tricia', NO = pure nitrate, NH = ammonium-dominated N.

### 3.3. Photosynthetic Gas Exchange

The photosynthetic CO<sub>2</sub> assimilation was not affected by N form and arugula variety but increased at eCO<sub>2</sub> (Figure 3a). However, eCO<sub>2</sub> significantly reduced the stomatal conductance (Figure 3b), thereby decreasing transpiration (data not shown), while increasing the leaf-internal CO<sub>2</sub> concentration (Figure 3c). The photosynthetic water use efficiency (WUE = assimilated CO<sub>2</sub> per water lost by transpiration) was 1.7 to 2.4 times higher at 800 ppm compared to 400 ppm CO<sub>2</sub> (Figure 3d).



**Figure 3.** Photosynthetic CO<sub>2</sub> assimilation (a), stomatal conductance (b), leaf-internal CO<sub>2</sub> concentration (c) and photosynthetic water use efficiency (d) at harvest (45–46 days after sowing). a = ambient CO<sub>2</sub>, e = elevated CO<sub>2</sub>, Be = 'Bellezia', Tri = 'Tricia', NO = pure nitrate, NH = ammonium-dominated N. Data are averages of n = 3. Different letters indicate significant differences between the treatments at  $\alpha = 0.05$ .

### 3.4. Plant Composition

The eight experimental treatments did not differ in terms of the chlorophyll content of the leaves, as confirmed by non-invasive measurements (Figure S2) and by chemical analyses (Table 3).

**Table 3.** Pigment and ascorbic acid contents of leaves of two arugula varieties grown with two different N forms and two atmospheric CO<sub>2</sub> concentrations. n = 3.

Treatment	Chlorophylls (mg 100 g <sup>-1</sup> FM)	Carotenoids (mg 100 g <sup>-1</sup> FM)	Anthocyanins (mg 100 g <sup>-1</sup> FM)	Ascorbic Acid (mg 100 g <sup>-1</sup> FM)
aBeNO	30.5 a	0.25 a	8.3 a	24.5 ab
aTriNO	29.8 a	0.16 a	8.1 a	18.1 a
aBeNH	37.5 a	0.18 a	9.4 a	18.1 a
aTriNH	29.1 a	0.15 a	6.8 a	21.5 ab
eBeNO	29.7 a	0.49 a	5.5 a	24.8 ab
eTriNO	25.5 a	0.40 a	6.2 a	21.8 ab
eBeNH	30.3 a	0.25 a	5.7 a	26.6 ab
eTriNH	30.1 a	0.32 a	6.4 a	35.2 b

Different letters indicate significant differences between the treatments at  $\alpha = 0.05$ . a = ambient CO<sub>2</sub>, e = elevated CO<sub>2</sub>, Be = 'Bellezia', Tri = 'Tricia', NO = pure nitrate, NH = ammonium-dominated N.

The concentrations of total carotenoids, anthocyanins and ascorbic acid were not impacted by the experimental factors CO<sub>2</sub> concentration, N form and variety (Table 3). Ascorbic acid concentrations showed significant interactions of N form and variety (Table 3). The concentration of ascorbic acid of 'Tricia' increased by almost two-fold when grown with ammonium-dominated N at eCO<sub>2</sub> as compared to pure nitrate under aCO<sub>2</sub>.

The N content of the leaves did not differ between the eight experimental treatments (Table 4). The elevated CO<sub>2</sub> concentrations tended to increase the C content of the arugula leaves ( $p = 0.09$ ). Consequently the C/N ratio was significantly increased exclusively by 100% nitrate N, and in the variety 'Tricia' (data not shown).

**Table 4.** Concentration of macro- and micronutrients in above-ground dry mass of arugula. n = 3.

Element	Unit	aBeNO	aTriNO	aBeNH	aTriNH	eBeNO	eTriNO	eBeNH	eTriNH
C	%	39.1 a	37.8 a	38.1 a	38.6 a	39.2 a	39.5 a	39.9 a	39.6 a
N	%	2.19 a	2.16 a	2.97 a	2.47 a	1.92 a	1.85 a	2.61 a	2.51 a
S	%	0.96 ab	1.12 abc	1.31 b	1.20 ab	0.81 c	0.90 c	1.02 abc	0.94 ac
K	%	2.91 bc	3.18 bc	3.17 bc	3.30 b	2.68 ac	2.73 ac	2.94 bc	3.01 bc
P	%	0.39 a	0.36 a	0.71 b	0.61 bc	0.33 a	0.33 a	0.63 bc	0.57 c
Ca	%	1.87 a	1.97 a	1.65 a	1.37 a	1.73 a	1.74 a	1.38 a	1.33 a
Mg	%	0.22 a	0.24 a	0.22 a	0.22 a	0.21 a	0.22 a	0.21 a	0.21 a
Fe	ppm	41.4 a	42.7 a	60.1 a	71.5 a	39.4 a	39.1 a	71.9 a	63.6 a
Zn	ppm	20.3 a	21.0 abd	28.3 bc	30.9 c	20.5 ab	20.9 abd	31.1 c	28.3 cd
Mn	ppm	11.4 a	10.7 a	12.5 a	18.4 a	11.0 a	9.1 a	13.7 a	16.7 a
Cu	ppm	6.44 a	7.04 a	8.09 a	6.87 a	7.34 a	6.47 a	7.19 a	6.84 a
Na	ppm	0.16 ab	0.15 ab	0.22 a	0.15 ab	0.13 ab	0.13 b	0.15 ab	0.13 ab

Different letters indicate significant differences between the treatments at  $\alpha = 0.05$ . a = ambient CO<sub>2</sub>, e = elevated CO<sub>2</sub>, Be = 'Bellezia', Tri = 'Tricia', NO = pure nitrate, NH = ammonium-dominated N.

The concentrations of the mineral elements Ca, Mg, Fe, Mn and Cu did not differ between the eight experimental treatments. The concentration of S was not altered by eCO<sub>2</sub>, N form or arugula variety, with the exception of a decrease due to eCO<sub>2</sub> for 'Bellezia' fertilized with pure nitrate-N. Potassium concentrations were not altered by N form, variety and CO<sub>2</sub> concentration. However, 'Tricia' grown with ammonium-dominated N under aCO<sub>2</sub> had significantly larger K concentrations in the leaves as compared to 'Tricia' grown under eCO<sub>2</sub> with pure nitrate-N. The foliar concentrations of P were neither impacted by CO<sub>2</sub> concentration nor arugula variety. When supplied with ammonium-dominated N, the P concentrations increased in the leaves regardless of the CO<sub>2</sub> concentration. No effects of variety or CO<sub>2</sub> concentration on the Zn content of leaves were observed, except for increased contents in "eTriNH". For Na, no significant differences between the treatments were observed, albeit 'Bellezia' grown with ammonium-dominated N under aCO<sub>2</sub> (aBeNH) had larger Na concentrations than 'Tricia' grown under eCO<sub>2</sub> with pure nitrate-N (eTriNO; Table 4).



#### 4. Discussion

This study is a contribution to the highly controversial debate of whether  $C_3$  plants benefit from ammonium as a nitrogen source under elevated atmospheric  $CO_2$  concentrations (e $CO_2$ ). This assumption is based on [50], who stated that nitrate assimilation in plants is reduced under non- $CO_2$ -limited conditions, under which photorespiration is reduced in favour of carboxylation. Ammonium-N nutrition, compared to nitrate, would then be expected to increase the efficiency of carbon fixation and N assimilation, both leading to higher net production and product quality. However, Andrews et al. [17] argue against that theory with underestimated nitrate assimilation by roots under e $CO_2$  conditions. Besides the reduced leaf nitrate assimilation, the phenomenon of  $CO_2$  acclimation, occurring potentially a few days or weeks after e $CO_2$  exposure [51], may mitigate the changes in the N metabolism and thus, the effect of the ammonium-nutrition of plants. Against this controversial background, we tested the effect of N form under e $CO_2$  on plant performance and leaf chemical composition using the short-term leafy vegetable arugula (*Eruca sativa*), which as a Brassicaceae highly contributes to human health.

When grown under 800 ppm  $CO_2$ , arugula plants showed a clear  $CO_2$  fertilization effect as  $c_i$  (Figure 3c), photosynthetic  $CO_2$  assimilation (Figure 3a) and dry mass (Table 2) increased in response to e $CO_2$ , independently of the N form. These effects were recently confirmed for a close relative of arugula, *Arabidopsis thaliana* [52]. Characteristically for the  $CO_2$  fertilization effect, the water use efficiency of arugula plants was improved (Figure 3d), as shown for several other  $C_3$  species at e $CO_2$  [2].

The photosynthetic activity was enhanced, as there were no limitations by light, water supply, N supply or  $CO_2$ . The same is true for other mineral elements, such as Mg. As there were neither effects of the N form nor the  $CO_2$  level on the Mg contents in the leaves (Table 4), we conclude that the carboxylation activity of Rubisco was favoured at the expense of the oxygenation of RuBP (see [4]).

This initially enhanced  $CO_2$  assimilation was often shown to decrease under long-term e $CO_2$  exposure due to feedback inhibition by the accumulated carbohydrates in the leaves [53]. The so-called  $CO_2$  acclimation effect was not observed for the short-term crop arugula (Figure 3a). Consequently, lower photorespiratory activities in plants under e $CO_2$  might be assumed compared to a $CO_2$ , which is the prerequisite for limited nitrate assimilation in leaves [50].

However, our study cannot confirm Bloom's theory [50]. As nitrate assimilation was not quantified we used other methods, such as nitrate depletion from the growth medium which was considered to indicate altered nitrate assimilation in  $C_3$  plants [4]. In fact, the lack of differences in soil nitrate-N and ammonium-N concentrations between all treatments at the end of the experiment suggests that nitrate depletion from the medium was unaffected by the  $CO_2$  concentration (Table 1). Moreover, leaf N contents were similar in all N form\* $CO_2$  treated plants (Table 4), which would not be expected with reduced leaf nitrate assimilation under e $CO_2$  levels. This further yielded similar fresh mass of the plants grown with ammonium-dominated N compared to pure nitrate-N under e $CO_2$  (Table 2). If nitrate assimilation in leaves was nevertheless reduced—and in fact there are indicators of reduced photorespiration and thus, limited nitrate-photoreduction—it must have been compensated by increased assimilation in other plant organs [17,54]. For *Arabidopsis thaliana*, it was suggested that root N assimilation is favoured in plants under e $CO_2$  conditions in order to “offset the decline in nitrogen metabolism in the leaves” [54]. A shift in the partitioning of nitrate assimilation consequently results in little alterations of the plant's N status under e $CO_2$  conditions, which we thus can confirm for arugula.

For future fertilization under elevated  $CO_2$ , we do not recognize any advantage of ammonium-N nutrition for arugula in context of biomass and N accumulation. In general, using ammonium-dominated N is rather detrimental in arugula production as leaf area and fresh and dry mass may be decreased, regardless of the  $CO_2$  level (Table 2). Moreover, smaller diameters and less visible roots [55] indicate overall restricted growth processes in response to ammonium-dominated N, pointing to mild toxicity symptoms [12].

This is especially true for the variety ‘Bellezia’ which probably is more sensitive towards ammonium-N supply than the variety ‘Tricia’.

These negative aspects of ammonium-N nutrition can be counterbalanced by lower numbers of inflorescences at harvest (Table 2), especially under eCO<sub>2</sub> conditions, resulting in more marketable plants than with nitrate-N. This implies that senescence processes are not enhanced in response to the mild stress imposed by ammonium. Moreover, there was no increased exposure of plants to oxidative stress due to the lower photorespiratory activity under eCO<sub>2</sub> [4], as suggested by several antioxidants in the leaves (carotenoids, anthocyanins, ascorbic acid) that were not altered by CO<sub>2</sub> level and N form (Table 3). This is similar to results of other studies [50], but contrasts with reported increases in the contents of ascorbic acid and other antioxidants and their precursors in vegetables under eCO<sub>2</sub> [35,36,56–58].

Thus, the proposed carbon surplus generated by the increased photosynthetic activity under eCO<sub>2</sub> conditions was likely not utilized for increased synthesis of secondary metabolites with antioxidative properties [59]. In addition, C surplus was not increasingly metabolized to soluble carbohydrates in the leaves (as the C content was not significantly affected, Table 4), and thus did not cause feedback limitation of photosynthesis in the long-term (Figure 3a). The option of transporting extra carbon to the root system [59] can be excluded since the visible root growth was not promoted (see [55]). We therefore suggest that the surplus carbon was used for modifications of the leaves’ morphological and anatomical structures, as proposed by Gamage et al. [2]. This hypothesis is fostered by the significantly higher dry mass of the plants under eCO<sub>2</sub> levels (Table 2).

This higher dry mass resulted in the “dilution” of the macronutrients P, K and S (Table 4). The phenomenon of reduced concentrations of nitrogen and other mineral elements in plants grown under elevated CO<sub>2</sub> concentrations [35,39,40,57,60] is a consequence of accumulated photosynthates and reduced root uptake due to increased CO<sub>2</sub> assimilation and decreased transpiration, respectively [61]. In our study, the lower concentrations of relevant macronutrients in the above-ground biomass were apparently not limiting the plant growth processes, but they might lower the nutritional quality of the vegetables in the future [35,40].

This is especially true for the micronutrient Zn, as pointed out by Dong et al. [36] and Soares et al. [39]. However, foliar Zn levels were not affected by the CO<sub>2</sub> level in our study but were lower when grown with nitrate as the sole N source (Table 4). This can be regarded as a dilution effect by the larger biomass under pure nitrate fertilization.

To overcome these low contents of Zn in the leaves of arugula, the fertilization with ammonium-dominated N may be beneficial under ambient and elevated CO<sub>2</sub> (Table 4). However, this conclusion cannot be drawn for several other minerals (Ca, Mg, Na, Mn, Fe, Cu) for which no significant differences between the CO<sub>2</sub>\*N form treatments were observed (Table 4). The same applies to N (Table 4), which is closely linked to the leaf chlorophyll contents (Table 3). Several studies reported that the total chlorophyll content of leaves is not altered even at very high CO<sub>2</sub> levels [32,36,52,57,62], which enables the high photosynthetic capacities and the increased water use efficiency under non-limiting conditions, regardless of N form and arugula variety.

## 5. Conclusions

A CO<sub>2</sub> acclimation does not occur in the short-term leafy vegetable arugula. This crop benefits from elevated CO<sub>2</sub> levels in terms of photosynthetic activities and biomass development. The supply of ammonium-dominated N, in contrast to the hypothesis of Bloom [50], does not provide further advantages as the biomass development of arugula is rather impeded by this N form, regardless of the atmospheric CO<sub>2</sub> level. The same is true for the nutritional quality of arugula leaves, as pure nitrate supply and ammonium-dominated N fertilization have very similar impacts. It is thus not recommended to use ammonium-dominated N for the production of arugula under future elevated CO<sub>2</sub> levels.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/horticulturae7080251/s1>, Figure S1: Number of inflorescences with at least one open flower observed from days 18 to 39 after the start of the experiments, Figure S2: Indices for the chlorophyll content of the leaves as observed by non-invasive measurements.

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