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153545 - Carbon isotope fractionation during leaf and root respiration as a function of nitrogen nutrition (SNF)

Nitrate and ammonium differ in their impact on $\delta^{13}\text{C}$ of plant metabolites and respired CO_2 from tobacco leaves

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Abstract

The carbon isotopic composition ($\delta^{13}\text{C}$) of foliage is often used as proxy for plant performance. However, the effect of NO_3^- vs. NH_4^+ supply on $\delta^{13}\text{C}$ of leaf metabolites and respired CO_2 is largely unknown. We supplied tobacco plants with a gradient of NO_3^- to NH_4^+ concentration ratios and determined gas exchange variables, concentrations and $\delta^{13}\text{C}$ of TCA cycle intermediates, $\delta^{13}\text{C}$ of dark respired CO_2 , and activities of key enzymes nitrate reductase, malic enzyme and phosphoenolpyruvate carboxylase.

Net assimilation rate, dry biomass and concentrations of organic acids and starch decreased along the gradient. In contrast, respiration rates, concentrations of intercellular CO_2 , soluble sugars and amino acids increased. As NO_3^- decreased, activities of all measured enzymes decreased. $\delta^{13}\text{C}$ of CO_2 and organic acids closely co-varied and were more positive under NO_3^- supply, suggesting organic acids as potential substrates for respiration. Together with estimates of intra-molecular ^{13}C enrichment in malate, we conclude that a change in the anaplerotic reaction of TCA cycle possibly contributes to ^{13}C enrichment in organic acids and respired CO_2 under NO_3^- supply. Thus, the effect of NO_3^- vs. NH_4^+ on $\delta^{13}\text{C}$ is highly relevant, particularly if $\delta^{13}\text{C}$ of leaf metabolites or respiration is used as proxy for plant performance.

Key words: ammonium (NH_4^+), nitrate (NO_3^-), nitrate reductase (NR), phosphoenolpyruvate carboxylase (PEPC), compound specific carbon isotope analysis (CSIA), stable carbon isotopes.

Introduction

The strong link between carbon (C) and nitrogen (N) within the plant-soil system has been the subject of numerous studies [1–7]. Studying this interaction and its underlying mechanisms has been critical for understanding the biochemical cycling of these elements, particularly during respiration where C skeletons are provided for N assimilation [8]. Nitrate (NO_3^-) and ammonium (NH_4^+) are the most important inorganic N species available to plants [9], both following different uptake and assimilation patterns and resulting in fundamentally different plant metabolism. Yet, the potentially different effects of these two inorganic N species on the C isotopic composition ($\delta^{13}\text{C}$) of plant metabolites and dark respired CO_2 are largely unknown. This is particularly surprising, since increasing atmospheric CO_2 concentrations have been found to decrease NO_3^- assimilation [10]. Therefore, a shift to NH_4^+ as a preferable source of N for plants might be expected in the future [11]. Equally important, $\delta^{13}\text{C}$ values of bulk leaves and leaf metabolites are widely used to study water use efficiency (WUE), plant physiological processes and plant functional responses to climate. If nitrogen assimilation has been or will be changing over time, the proxy of $\delta^{13}\text{C}$ for WUE might be unreliable. Thus, a better understanding of how different N species availability affects plant C isotope fractionations is urgently needed.

NH_4^+ is described as a paradoxical nutrient because its redox state requires no reduction process prior to its assimilation (*via* the GS-GOGAT pathway), but most plants with NH_4^+ supply grow slower and produce less biomass compared to those supplied with NO_3^- [12,13]. Former studies have shown physiological toxicity effects caused by NH_4^+ in plants [14–16], a decrease in carbon assimilation rates [17], changes in the charge balance caused by reduced uptake of cations, such as K^+ , Mg^{2+} and Ca^{2+} , and an increased uptake of anions, such as SO_4^{2-} , PO_4^{3-} and Cl^- [12,18]. Moreover, NH_4^+ assimilation generates H^+ ions which cause a decrease in pH of the cytosol and eventually might result in growth depression [18].

Therefore, NH_4^+ assimilation takes place almost entirely in the roots [19], fuelled with C skeletons and energy delivered from the leaves [2]. In contrast to NH_4^+ supply, about 90% of the NO_3^- ions taken up by the roots are transported to the leaves for assimilation [20] together with K^+ as counter-ion [21]. NO_3^- assimilation involves reduction of NO_3^- to NH_4^+ by nitrate and nitrite reductase (NR, NiR) enzymes prior to assimilation *via* the GS-GOGAT pathway [22]. This reduction is coupled to photosynthesis [23] and to the tricarboxylic acid (TCA) cycle in the mitochondria, providing the reducing power [24,25]. In addition, the TCA cycle delivers energy and C skeletons for N uptake and assimilation [5,13,26]. N assimilation and organic acid concentrations also follow a diurnal cycle. This has been demonstrated by higher activities of NR and PEPC (i.e. the key enzymes in C and N physiology), higher accumulation of malate, NH_4^+ and glutamine during the first part of the light period and in contrast, a decrease in concentrations of citrate [26]. Given the different effects of both N species on leaf gas exchange, metabolites and enzyme activities, it is likely to expect strong and different effects of these N species on the C isotopic composition of plant material and respired CO_2 .

Within this biochemical framework, the TCA cycle intermediate malate plays a central role. Malate provides reducing equivalents for NO_3^- reduction as well as a redox storage in the vacuoles [27,28]. It has also been shown that the TCA cycle is not fully functional during the light period and consists of two loosely connected branches producing citrate and malate [8,28–30]. According to Igamberdiev and Bykova [31], during the light period malate has the potential to solely operate the TCA cycle by generating oxaloacetate (OAA) and pyruvate (supplying both substrates of the TCA cycle) via malate dehydrogenase (MDH) and malic enzyme (ME), respectively. Malate is also a key component in replenishing the loss of C skeletons from the TCA cycle during the amino acid metabolism. This replenishment happens through an anaplerotic reaction *via* phosphoenolpyruvate carboxylase (PEPC) [4]. In

addition, malate maintains the charge balance [32] and is involved in the re-transport of K^+ ions released after NO_3^- assimilation from shoots to the roots [21]. Together with citrate, malate regulates the transfer of reducing power between cell compartments. Thus, both, citrate and malate are expected to be central to unravel the underlying mechanisms if NO_3^- vs. NH_4^+ supply affects the $\delta^{13}C$ of foliage, metabolites and respired CO_2 differently.

$\delta^{13}C$ values in C_3 plants depend primarily on the $\delta^{13}C$ values of atmospheric CO_2 and photosynthetic isotope fractionation during CO_2 assimilation mainly by RuBisCO [33]. Physiological responses such as variation in assimilation rates, mesophyll and stomatal conductance can lead to changes in the ratio of leaf intercellular (C_i or more precisely chloroplast CO_2 concentration (C_c)) to atmospheric (C_a) CO_2 concentrations (C_i/C_a), which in turn changes $\delta^{13}C$ of plant organic matter and subsequently that of respired CO_2 . Consequently, $\delta^{13}C$ values of bulk plant material, metabolites and respired CO_2 vary under environmental conditions, which alter the C_i/C_a ratio such as drought and/or temperature [34–38], change over time and differ among species [39–47]. Additional “post-photosynthetic isotope fractionations” caused by reactions during plant metabolism (e.g. during dark respiration) also affect the $\delta^{13}C$ of plant material [40,48,49]. Particularly, the anaplerotic reaction of the TCA cycle, moderated *via* PEPC, results in a ^{13}C enrichment of the introduced COOH group (C-4) of OAA which often is converted to the more abundant metabolite like malate [50,51] and eventually $\delta^{13}C$ of respired CO_2 [38,52]. However, although several studies showed the influence of N source and content on C isotope discrimination during photosynthesis [53–55], to date, there is no study explicitly investigating the effects of N species supplied (changing from NO_3^- to NH_4^+) on the $\delta^{13}C$ of plant metabolites and of respired CO_2 .

Therefore, the overall aim of this study was to examine the effects of NO_3^- vs. NH_4^+ supply on C isotope fractionation of leaf metabolites and dark respired CO_2 . We hypothesized a

change in $\delta^{13}\text{C}$ of plant metabolites as well as respired CO_2 in response to changing ratios of NO_3^- and NH_4^+ . Moreover, we hypothesized that malate and the anaplerotic reaction of the TCA cycle play a central role for the fractionation during respiration of NO_3^- supplied plants. To test our hypotheses, a series of climate chamber based experiments have been conducted and relevant foliar gas exchange variables, enzymatic reactions, as well as concentrations and $\delta^{13}\text{C}$ values of selected metabolites and respired CO_2 of *Nicotiana sylvestris* leaves have been measured. Plants grew under a gradient of NO_3^- to NH_4^+ concentration ratios.

Materials and methods

Plant material

Tobacco seeds (*Nicotiana sylvestris*) were stratified for three days in water prior to sowing. The seeds were then sown in organic substrate (substrate 2, Klassmann Deilmann, Germany) and grown for two weeks in a walk-in climate chamber with average day/night temperatures of 22/20 °C and relative humidity (RH) of 50-70%. Plants were exposed to 12 hours daylight, supplemented by fluorescent lamps (Sylvania, Switzerland), with an average photosynthetic photon flux density of approximately $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the leaf surface. After two weeks, each plant was moved to a 750 mL pot filled with sterile sand (Quarzsand A 0.7-1.2 mm, Carlo Bernasconi AG, Switzerland), and perlite (Foma Sa, Switzerland) at the ratio of 5:1. Five plants were used per treatment (see below), grown in individual pots. Nutrients were supplied to plants with nutrient solutions modified according to Arnozis et al. [56] as follows: 1 mM CaCl_2 ; 1 mM MgSO_4 ; 0.25 mM KH_2PO_4 . Microelements and Fe-EDTA were provided with identical concentrations as in a Hoagland solution. In order to study the effect of different N species, 6 mM N was supplied to plants in different ratios of NO_3^- to NH_4^+ , provided in the forms of $\text{Ca}(\text{NO}_3)_2$, KNO_3 , $(\text{NH}_4)_2\text{SO}_4$, and NH_4NO_3 , while keeping the total amount of N in the solution constant. In the 100% NH_4^+ nutrient solution an additional 1 mM K_2SO_4 was added. Throughout the manuscript, when using the term ‘N

species gradient', we refer to the different ratios of the two inorganic N species supplied. Along this gradient, the following ratios were used: [%NO₃⁻-N: %NH₄⁺-N] 100:0 (NO₃⁻), 90:10 (90%), 75:25 (75%), 50:50 (50%), 25:75 (25%), 10:90 (10%), 0:100 (NH₄⁺) shown in Table 1. For simplicity, we will use the term "NO₃⁻ supply" when referring to plants fed with 100% NO₃⁻, 90% NO₃⁻, 75% NO₃⁻ and 50% NO₃⁻, and will use the term "NH₄⁺ supply" when referring to plants fed with 75% NH₄⁺, 90% NH₄⁺ and 100% NH₄⁺; sole NO₃⁻ and sole NH₄⁺ treatments represent 100% NO₃⁻ and 100% NH₄⁺ supply, respectively. Plants were treated with the NO₃⁻/NH₄⁺ solutions (pH≈7) every second day. On the days without treatment, water was provided for unlimited water supply. The pH of the drained soil solution was monitored throughout the study. At harvest time, the pH value was ca. 6.5 in NO₃⁻ supplied plants and ca. 5 in NH₄⁺ supplied plants.

This setup was repeated three times and all plants were grown in identical conditions from seeding, to ensure sufficient leaf material for prospective analyses. One set of plants was used for leaf gas exchange measurements three hours after start of light period. The same leaves were then used for ¹³C analysis of dark-respired CO₂ and for the analysis of δ¹³C and concentrations in organic matter and metabolites. The second and third sets were also harvested three hours after the start of light period and used for measurements of respiration and enzymatic analyses, respectively. All measurements and final harvest were performed after 45 days of treatment (thus plants were about 8 weeks old at harvest time).

Foliar gas exchange variables

Net assimilation rate (A_n), ratio of intercellular CO₂ to atmospheric CO₂ concentration (C_i/C_a) and stomatal conductance (g_s) were measured with a LI-6400 (LI-COR, USA) on one mature, fully expanded leaf attached to the plant. A standard photosynthesis cuvette with a leaf area of 6 cm² (6400-02B LED Light Source) was used while leaf temperature was set to 20 °C. PAR (Photosynthetically Active Radiation) was set at 400 μmol m⁻² s⁻¹ and [CO₂] (C_a) was kept at

400 $\mu\text{mol mol}^{-1}$ with a constant flow rate of 500 $\mu\text{mol s}^{-1}$. After the plants were in steady state (A_n and g_s stabilized), each gas exchange measurement took 60 seconds with the data recorded every 10 seconds and averaged afterwards. The remaining plant leaves were shock-frozen in liquid N_2 . The dry weight of the leaves was measured after freeze drying to determine plant foliar biomass. These samples were then ground to a fine powder using a ball mill (MM 200, Retsch, Germany) and stored in a desiccator for further analyses.

$\delta^{13}\text{C}$ values of leaf respired CO_2 and respiration rates

The C isotopic composition of leaf dark-respired CO_2 was measured during the day similar to previous studies [52,57,58]. After the measurement of gas exchange, the respective leaf was cut and immediately transferred into a gas tight Tedlar® bag (Keyka Ventures, USA) according to Barbour et al. [59] and Barthel et al. [60]. To avoid the light enhanced dark respiration (LEDR) period, the Tedlar bag containing the leaf was kept in the dark for 30 min before measurements started [36,61–63]. The sampling bag was flushed several times with synthetic CO_2 -free air (20% O_2 and 80% N_2 , Pangas, Switzerland) until a CO_2 free atmosphere was established, controlled by an infrared gas analyser (LI-820, LI-COR, USA). The bag with the leaf was kept in the dark for approximately one hour and the air was sampled with a gas-tight syringe (BD Plastipak, Switzerland) and transferred into a 12 mL evacuated gas-tight glass vial (“Exetainer”, Labco, England). The $\delta^{13}\text{C}$ value of dark respired CO_2 ($\delta^{13}\text{C}_R$) was measured with a modified Gasbench II as described by Zeeman et al. [64] coupled to a Delta^{plus}XP isotope ratio mass spectrometer (IRMS, ThermoFisher, Germany). In addition, the $\delta^{13}\text{C}$ value of atmospheric CO_2 in the climate chamber was determined as $-10.6 \text{ mUr} \pm 0.9 \text{ (SD)}$. All C isotope measurements in CO_2 had a typical standard deviation of less than 0.15 mUr.

For measurements of respiration rates, plants were also kept in the dark for 30 mins to avoid LEDR. A fully developed leaf was cut and put into a 445 mL glass jar covered with an opaque

171 plastic foil. The lid of the jar was equipped with a luer-stopcock fitting to allow gas sampling.
172 During a one-hour period after closing the jar, 4 samples were taken. The first sample was
173 taken right after closing the jar, and the following three samples were taken every 20 minutes.
174 CO₂ concentrations were measured with a gas chromatograph (456-GC, Scion Instruments,
175 UK). After CO₂ sampling, the leaf was scanned (Lide 20, Canoscan, Canon) and the leaf area
176 determined with the software package Fiji [65]. Leaf respiration rates were derived from the
177 linear increase in CO₂ concentrations over time. Respiration rates accounted for leaf area,
178 head space volume and temperature (ideal gas law; cf. [66]).

179 *$\delta^{13}\text{C}$ values of bulk organic matter*

180 After freeze-drying and milling, approximately 2 mg of bulk leaf material was weighed in Sn
181 capsules (5 x 9 mm, Saentis, CH) for $\delta^{13}\text{C}$ analysis of bulk leaf organic material ($\delta^{13}\text{C}_\text{B}$) with
182 a Flash EA 1112 Series elemental analyzer (ThermoFisher, Germany) coupled to a
183 Delta^{plus}XP IRMS via a ConFlo III as described by Brooks et al. [67] and Werner et al. [68].
184 Sequencing and referencing followed the recommendation by Werner and Brand [69]. The
185 measurement precision (SD) of the quality control standard (tyrosine Tyr-Z1) during the $\delta^{13}\text{C}$
186 measurements of all EA samples reported in this study was 0.16 mUr.

187 *Organic metabolites for bulk $\delta^{13}\text{C}$ analysis*

188 Extraction of organic metabolites was performed according to Lehmann et al. [38]. Briefly,
189 100 mg freeze-dried plant material were transferred to 2 mL reaction tubes. 1.5 mL 85 °C
190 deionized water was added to prevent any enzymatic activity. The samples were then
191 incubated for 30 min in a water bath at 85 °C. After centrifugation for 2 min at 10000 g, the
192 supernatant and the remainder (pellet) were separated and kept for further purifications. Ion
193 exchange chromatography was used to separate the supernatant containing the water-soluble
194 content into sugars, amino acids, and organic acids fractions. 1 mL of the supernatant was
195 added to a column filled with a cation exchanger (Dowex 50WX8, hydrogen form, 100-200

mesh, Sigma Aldrich, CH), that was directly above a second column filled with an anion exchanger (Dowex 1X8, chloride form, 100-200 mesh, Sigma Aldrich, CH). The neutral sugar fraction was eluted with 30 mL deionized water. The amino acid fraction was trapped on the Dowex 50WX8 and then eluted with 30 mL 3 M ammonia solution, while the organic acid fraction was trapped on the Dowex 1X8 column and afterwards eluted with 35 mL 1 M HCl [38,70]. All metabolic fractions were frozen, freeze dried, re-dissolved in deionized water and stored at -20 °C for further use.

Starch was enzymatically isolated from the pellet originating after the water extraction mentioned above, according to standard protocols [70,71]. In brief, the pellet was washed several times using an MCW solution (methanol/chloroform/deionized water, 12/5/3, v/v/v) and deionized water, and subsequently dried overnight. On the second day, the starch in the pellet was re-solubilized in water and gelatinized at 100 °C for 15 min and broken down to soluble sugars at 85 °C for 2 hours using the heat resistant α -amylase (EC 3.2.1.1, Sigma-Aldrich, CH). The α -amylase was separated from the soluble sugars by centrifugation filters (Vivaspin 500, Sartorius, Germany). For the respective $\delta^{13}\text{C}$ measurements, aliquots from the starch-derived sugar ($\delta^{13}\text{C}_{\text{St}}$), sugar ($\delta^{13}\text{C}_{\text{S}}$), amino acid ($\delta^{13}\text{C}_{\text{aa}}$), and organic acid ($\delta^{13}\text{C}_{\text{oa}}$) fractions were pipetted into Sn capsules, dried in an oven at 60 °C, and analysed with the EA-IRMS coupling as described above. Compound specific isotope analysis (CSIA) of individual organic acids was performed following Lehmann et al. [38] and Rinne et al. [72] and CSIA of individual amino acids was performed following Schierbeek [73] and Vlaardingerbroek et al. [74]. Detailed descriptions of these methods are provided in the supplementary information section.

Enzymatic activities

The leaf material for enzyme activity assays was frozen and immediately ground with pestle and mortar in liquid nitrogen, then stored at -80 °C until analyses. The activities of NR, PEPC

and ME were assayed following Dier et al. [75], Gibon et al. [76] and Tronconi et al. [77], respectively. Detailed descriptions of these methods are provided in the supplementary information section.

Concentration of carbohydrates

Glucose, fructose, sucrose, total sugars and total starch were extracted from the leaves according to Ruckle et al. [78]. In brief, 10 mg of freeze-dried leaf material was washed two times with 80% (v/v) ethanol and two times with 50% (v/v) ethanol to separate the soluble sugars from starch. Each ethanol extract was incubated at 80 °C for 30 min. The remaining pellet containing starch was incubated at 90 °C in 0.2 M KOH and then neutralized with 1 M acetic acid. The starch in the pellet was digested with α -amylase and amyloglucosidase (Sigma Aldrich, CH) to convert it to glucose. Starch-derived glucose, glucose, fructose and sucrose were quantified in triplicates using an enzyme-linked assay based on hexokinase, glucose-6-phosphate dehydrogenase, phosphoglucosomerase, and invertase as described by Ruckle et al. [78]. Sugar dependent conversion of NADP⁺ to NADPH (Sigma Aldrich, CH) was quantified using a plate reader (Perkin Elmer Enspire[®], USA) by measuring the absorption at 340 nm.

Statistics and calculations

To study the magnitude of post-photosynthetic C isotope fractionation we calculated the apparent respiratory isotope fractionation according to Ghashghaie et al. [79]:

$$e_{\text{dark}} = (\delta^{13}\text{C}_S - \delta^{13}\text{C}_R) / (1 + \delta^{13}\text{C}_R)$$

where $\delta^{13}\text{C}_S$ and $\delta^{13}\text{C}_R$ represent the isotopic composition of soluble sugars and the isotopic composition of respired CO₂, respectively. We used the simplified Farquhar model

$$\Delta = a + (b-a) * C_i/C_a$$

Where a is the isotope fractionation during diffusion and b during carboxylation to calculate carbon isotope discrimination [34]. We tested the effect of different N species solutions on

enzyme activities, concentrations and $\delta^{13}\text{C}$ values with one-way ANOVA using R [80]. The different solutions (NO_3^- to NH_4^+) were treated as factors. Tukey-HSD post-hoc tests were used for multiple comparisons at the 0.05 significance level. We used linear regression models to calculate the p and R^2 values given in the graphs; asterisks represent different significance levels: * for $p<0.05$, ** for $p<0.01$ and *** for $p<0.001$. All $\delta^{13}\text{C}$ values were measured on V-PDB scale.

Results

Foliar gas exchange variables

The change in N species supplied led to a shift in gas-exchange behaviour in tobacco plants (Tab. 2). We found a significant effect of the N species gradient on net assimilation rate (A_n), with approximately 50% lower A_n under NH_4^+ compared to NO_3^- supply (Fig. 1A, $p=0.012$). The ratio of intercellular to atmospheric CO_2 concentrations (C_i/C_a) was higher with increasing NH_4^+ fraction (Fig. 1B, $p=0.0014$). Although stomatal conductance (g_s) was more variable under NH_4^+ than under NO_3^- supply, no significant treatment effect was observed (Fig. 1C, $p=0.6$). In contrast, respiration rate (R_{dark}) significantly increased when NH_4^+ supply increased (Fig. 1D, $p<0.01$), whereas leaf dry biomass significantly decreased with increasing NH_4^+ (Fig. 1E, $p<0.001$).

Carbon isotopic composition of plant metabolites and respired CO_2

C isotopic composition of leaf bulk organic matter ($\delta^{13}\text{C}_B$) stayed relatively constant at about -27.4 mUr from 90% NO_3^- to 10% NO_3^- , but tended to be lower in 100% NO_3^- and 100% NH_4^+ with -28.3 mUr and -29.1 mUr, respectively (Tab. 2, Fig. 2A). Moreover, we did not find a relationship between $\delta^{13}\text{C}_B$ and N content (data not shown). $\delta^{13}\text{C}$ of leaf metabolites (e.g. sugars, starch and organic acids) and leaf respired CO_2 were clearly affected by changes in the N species ratios (Fig. 2). $\delta^{13}\text{C}$ values of sugars ($\delta^{13}\text{C}_S$) increased steadily from -27.3

271 mUr (100% NO₃⁻) to -25.8 mUr (10% NO₃⁻) but decreased sharply to -28.4 mUr under 100%
272 NH₄⁺ (Fig. 2B). Also, δ¹³C of starch (δ¹³C_{st}) increased from -27.2 mUr (100% NO₃⁻) to -25.9
273 mUr (50% NO₃⁻) before decreasing to -27.8 mUr (100% NH₄⁺). No significant differences
274 were observed for isotopic composition of amino acids (δ¹³C_{aa}: Fig. 2D, *p*=0.14). δ¹³C values
275 of organic acids (δ¹³C_{oa}) and respired CO₂ (δ¹³C_R) followed similar patterns along the N
276 species gradient from NO₃⁻ to NH₄⁺, with almost steadily decreasing δ¹³C values, which were
277 also less negative compared to those of other metabolic fractions particularly under NO₃⁻
278 supply (Figs. 2E,F). Both δ¹³C_{oa} and δ¹³C_R showed more negative values under NH₄⁺ supply
279 compared to those under NO₃⁻ supply, ranging from -24.2 mUr (δ¹³C_{oa} in 90% NO₃⁻) to -27.8
280 mUr (δ¹³C_{oa} in 100% NH₄⁺), and from -24.4 mUr (δ¹³C_R in 90% NO₃⁻) to -27.8 mUr (δ¹³C_R in
281 100% NH₄⁺), respectively.

282

283 *Compound-specific analysis of organic acids*

284 Malate and citrate were the dominant organic acids in the bulk organic acids extracted for
285 compound specific analysis. δ¹³C values of malate tended to be more negative under NO₃⁻
286 than under NH₄⁺ supply, gradually increasing from -21.6 mUr to -19 mUr, before decreasing
287 again at 100% NH₄⁺ (Tab. 2, Fig. 3A, *p*=0.07). In contrast, δ¹³C values of citrate showed a
288 clear effect of N species, with δ¹³C values decreasing from -22.9 mUr in 90% NO₃⁻ to -26.9
289 mUr in 10% NO₃⁻ treatment (Fig. 3B, *p*=0.017). Moreover, very strong effects were observed
290 in malate concentrations, which decreased with increasing NH₄⁺ from 128 μmol g DW⁻¹ in
291 100% NO₃⁻ to about 2 μmol g DW⁻¹ in 25% NO₃⁻ (Fig. 3G, *p*<0.001). Similarly, citrate
292 concentrations decreased from 47 μmol g⁻¹ in 75% NO₃⁻ to less than 1 μmol g DW⁻¹ in 10%
293 NO₃⁻ (Fig. 3H, *p*<0.001). It has to be noted that concentrations and δ¹³C values need to be
294 treated with caution since the concentrations of malate and citrate were at the detection limit
295 of HPLC-IRMS measurements for plants supplied with less than 50% NO₃⁻ and should be

interpreted with caution.

Compound specific analysis (CSIA) of amino acids

In this study, we focused on four amino acids including alanine (related to pyruvate), serine (a product of photorespiration in leaves), asparagine/aspartic acid (related to OAA) and glutamine/glutamic acid (related to 2-oxoglutarate). N species treatments significantly influenced $\delta^{13}\text{C}$ values of alanine, asparagine and serine (Fig. 3C-E, $p<0.001$ for alanine and serine, $p=0.005$ for asparagine), but not those of glutamine (Fig. 3F). While $\delta^{13}\text{C}$ of alanine was gradually decreasing with increasing NH_4^+ supply, with highest values in 100% NO_3^- (-27.9 mUr) and lowest in 100% NH_4^+ (-29.5 mUr, Fig. 3C), no clear pattern was observed along the N species gradient for asparagine (Fig. 3D). Similar to alanine, serine was more enriched in ^{13}C in plants grown under NO_3^- supply, with highest $\delta^{13}\text{C}$ value in 90% NO_3^- (-27.8 mUr), and more depleted in ^{13}C under NH_4^+ supply, with the lowest $\delta^{13}\text{C}$ value in 10% NO_3^- (-32.5 mUr, Fig. 3E). Furthermore, concentrations of all four amino acids increased from NO_3^- supply towards 10% NO_3^- , before sharply decreasing again at 100% NH_4^+ , almost reaching the same values as under NO_3^- supply (Figs. 3 I-L, $p<0.01$). The N species effect was very clear in alanine and also in serine, with the highest concentrations of alanine in 10% NO_3^- (2.8 $\mu\text{mol g DW}^{-1}$) and lowest in 100% NO_3^- (0.5 $\mu\text{mol g DW}^{-1}$) and highest concentrations of serine in 10% NO_3^- (23.7 $\mu\text{mol g DW}^{-1}$) and lowest in 90% NO_3^- (2.6 $\mu\text{mol g DW}^{-1}$). The influence of N species on the concentrations of asparagine and glutamine was not as clear, although NH_4^+ supplied plants showed higher concentrations compared to NO_3^- supplied plants.

Enzyme activity

Enzymatic activities of NR, PEPC and ME were negatively affected by the gradient of NO_3^-

to NH_4^+ supply (Fig. 4, $p<0.001$), most clearly seen in NR and PEPC. As the fraction of NH_4^+ increased, NR activity decreased from $1.9 \text{ nmol NO}_2^- \text{ g FW}^{-1} \text{ s}^{-1}$ (100% NO_3^-) to $0.7 \text{ nmol NO}_2^- \text{ g FW}^{-1} \text{ s}^{-1}$ (10% NO_3^- , Fig. 4A). Similarly, PEPC activity decreased from $9.5 \text{ nmol NAD g FW}^{-1} \text{ s}^{-1}$ (100% NO_3^-) to $4.3 \text{ nmol NAD g FW}^{-1} \text{ s}^{-1}$ (100% NH_4^+ , Fig. 4B). In contrast, ME activity was lowest under 25% NO_3^- ($269 \text{ nmol NAD g FW}^{-1} \text{ s}^{-1}$) and highest under 50% NO_3^- supply ($505 \text{ nmol NAD g FW}^{-1} \text{ s}^{-1}$, Fig. 4C). The activities of NR and ME in plants growing under 100% NH_4^+ could not be analysed due to insufficient amount of plant material.

Concentration of carbohydrates

The gradient of N species from NO_3^- to NH_4^+ , strongly influenced concentrations of both water-soluble carbohydrates and starch (Fig. 5). Concentrations of glucose, fructose, sucrose and total sugars significantly increased as the percentage of NH_4^+ in the N supply increased, except under 100% NH_4^+ (Fig. 5A-D). In contrast, starch concentrations were highest in plants supplied with higher percentage of NO_3^- , with concentrations decreasing from 359 mg g DW^{-1} in 50% NO_3^- to 95 mg g DW^{-1} under 10% NO_3^- supply (Fig. 5E).

Potential substrates for respiration and apparent isotope fractionation

As stated above, leaf respiration rates as well as $\delta^{13}\text{C}_\text{R}$ were influenced by the gradient of N species. In addition, we found a significant and positive relationship between $\delta^{13}\text{C}_\text{R}$ and $\delta^{13}\text{C}_\text{Oa}$, with a slope close to one and an intercept close to zero, representing a 1:1 relationship between these two variables (Fig. 6, $R^2=0.93$, $p<0.001$, $y = 0.97x - 0.72$). This relationship clearly showed that both organic acids and respired CO_2 became more depleted in ^{13}C along the N species gradient from NO_3^- to NH_4^+ .

Also, apparent isotope fractionation (e_dark), calculated from $\delta^{13}\text{C}_\text{S}$ and $\delta^{13}\text{C}_\text{R}$, was influenced

by the N species gradient, with negative e_{dark} values indicating higher ^{13}C enrichment in respired CO_2 compared to sugars as the first products of photosynthesis. e_{dark} in plants supplied with more than 50% NO_3^- was more negative compared to plants supplied dominantly with NH_4^+ (Fig. 7). e_{dark} values were the lowest (-2.4 mUr) in plants of the 90% NO_3^- treatment and increased along the gradient to the highest values in plants under 10% NO_3^- (0.9 mUr).

Discussion

NO_3^- to NH_4^+ gradient affects foliar gas exchange and metabolism

Our main aim was to study C isotope fractionation in response to a supply gradient of two different N species. We therefore setup an experiment with tobacco plants supplied with a N gradient from 100% NO_3^- to 100% NH_4^+ . As expected, foliar gas exchange characteristics and biomass were strongly influenced by this N supply gradient (Fig. 1). As NO_3^- decreased, net assimilation rate also decreased in our tobacco plants (Fig. 1A), similar to earlier studies with spinach [81], sugar beet [82] and maize [83]. The decreasing net assimilation rate and the simultaneously increasing respiration rate along the gradient (Fig. 1D), can explain the observed lower biomass in plants under NH_4^+ supply (Fig. 1E), most likely a sign of NH_4^+ toxicity caused by NH_4^+ supply [16, 12, 18], supported by the lower pH values (by 1.5) we observed in the drained soil solution from NH_4^+ supplied plants. Moreover, lower assimilation rate with decrease in NO_3^- supply is in line with the lower needs for reducing equivalents.

We observed strong impacts of the ratio of N species supplied on all measured metabolite concentrations such as organic acids (malate and citrate), soluble sugars, starch and amino acids (alanine, serine, asparagine and glutamine), as well as on measured enzyme activities. As suggested by the higher assimilation of plants dominantly supplied with NO_3^- , there was probably a higher supply of reducing power, in line with the observed higher NR activity

(Fig. 4A). Decreasing PEPC and ME activities (Fig. 4B,C) together with decreasing concentrations of organic acids along the gradient from NO_3^- to NH_4^+ (Fig. 3G,H) imply an important role of organic acids (e.g. malate) and the anaplerotic reaction of the TCA cycle under NO_3^- , but not under NH_4^+ supply [5,29,55]. Altogether, these findings clearly confirmed that a change in N species supplied to plants has an influence on carbon metabolism and thus on leaf respiratory metabolism.

We further observed higher concentrations of soluble sugars in the leaves of plants supplied dominantly with NH_4^+ (Fig. 5D) which aligned with higher respiration rates in these plants (Fig. 1D), resulting in a strong positive relationship between sugar concentrations and respiration ($R^2=0.75$, $p<0.05$, Fig. S1). This might be a stress response to NH_4^+ toxicity, supported by suppressed growth observed in this study as also suggested by Hachiya et al. [84]. N assimilation under high NH_4^+ supply takes place mostly in the roots [17,19] and requires C skeletons to be delivered from the leaves [85]. However, not only aboveground but also root biomass was low in NH_4^+ supplied plants (data is not shown) [86].

Moreover, the N species gradient not only influenced concentrations of organic acids and carbohydrates, but also the concentrations of individual amino acids (Fig. 3). We observed an increase in concentrations of alanine, serine, asparagine and glutamine with increasing NH_4^+ in the supply solutions (Fig. 3 I-L), supporting previous findings [2,22,87]. These observations might simply be signs of a detoxification strategy, i.e., remove free NH_4^+ ions from the cytosol by converting them into amino acids [19,22,88]. Moreover, decreasing C:N ratios are reported to relate to increasing amino acid biosynthesis [24,87], as seen for the NH_4^+ supplied plants in our study (Fig. S2 A). Consequently, we are confident that our experimental setup allowed in-depth assessment of the C isotopic composition of metabolites and respired CO_2 in relation to a N species gradient.

NO₃⁻ to NH₄⁺ gradient influences δ¹³C of amino acids

Moreover, we conducted compound specific isotope analysis of individual amino acids to investigate if changes observed in the post-photosynthetic fractionation between NO₃⁻ and NH₄⁺ supplied plants were mirrored in the δ¹³C of individual amino acids as the products of N assimilation. Analysis of amino acids showed different effects of the two N species on δ¹³C values of individual amino acids, particularly of alanine and serine (Fig. 3C,E; except for serine under 100% NH₄⁺ supply). A possible explanation for ¹³C enriched alanine in NO₃⁻ supplied plants can be the isotope fractionation caused by pyruvate dehydrogenase (PDH), resulting in an accumulation of ¹³C enriched glycolytic pyruvate that is known as a precursor for alanine biosynthesis [89,90]. Alternatively, the observed ¹³C enriched alanine in NO₃⁻ supplied plants can be explained by higher ME activity (Fig. 4C), supplying amino acid biosynthesis with ¹³C enriched pyruvate from anaplerotically synthesized malate.

δ¹³C values of serine and alanine were similar in NO₃⁻ supplied plants (around -27 mUr), but serine was by 3 mUr more ¹³C depleted in NH₄⁺ plants compared to alanine (-32.5 mUr to -29.5 mUr, respectively). Tcherkez [91] suggested a ¹³C depletion in serine molecules compared to glycine in relation to photorespiration. Our findings therefore can convey possible higher photorespiration in NH₄⁺ supplied plants, resulting in ¹³C depletion of serine. Although δ¹³C of asparagine was not influenced by the gradient of N species, it showed the highest ¹³C enrichment among all other amino acids. Sechley et al. [92] underlined the role of OAA/malate as donors of C skeleton for synthesis of asparagine. Given that malate was the most ¹³C enriched compound in our study (Fig. 3A), a contribution of this compound to the ¹³C enrichment of asparagine is likely.

NO₃⁻ to NH₄⁺ gradient influences carbon isotope fractionation

420 We observed distinct changes in $\delta^{13}\text{C}$ values of respired CO_2 and various metabolites such as
421 soluble carbohydrates, starch, organic acids and consequently leaf bulk organic matter in
422 relation to the N species gradient (Fig. 2). Despite the clear increase of C_i/C_a ratio from NO_3^-
423 to NH_4^+ supplied plants (Fig. 1B), suggesting larger ^{13}C discrimination and therefore more
424 ^{13}C depleted assimilated along the gradient, the $\delta^{13}\text{C}$ values of sugars, starch and bulk organic
425 matter showed no significant response (Fig. 2A,B). Therefore, we could not explain the
426 differences between $\delta^{13}\text{C}$ values of metabolites (sugars and starch as the first products of
427 photosynthesis) along the gradient ($R^2 < 0.54$) using the C_i/C_a ratio alone, based on the
428 simplified Farquhar model ($\Delta = a + (b-a) * C_i/C_a$, Fig. S3, [34]). This might have several
429 reasons: (1) N concentration positively influences mesophyll conductance and thus C_c/C_a ,
430 resulting in higher ^{13}C discrimination which in turn causes more ^{13}C depleted sugars
431 [43,45,93,94]. However, although we found higher N concentration in the leaves of NH_4^+
432 supplied plants (as shown in Fig. S2 B) and thus potentially increased mesophyll
433 conductance, soluble sugars in NH_4^+ supplied plants were not depleted in ^{13}C . (2) The foliar
434 sugar pool has accumulated over longer time periods than can be represented by individual
435 snap-shot C_i/C_a measurements. (3) According to Caemmerer and Evans [43], there is a
436 possible influence of PEPC activity on the expected C isotope discrimination during
437 carboxylation by RuBisCo. Considering the different PEPC activities measured in NO_3^- and
438 NH_4^+ supplied plants, such an effect of PEPC on discrimination cannot be excluded.

439 To further assess the mechanisms underlying the effects of NO_3^- vs. NH_4^+ supply on the
440 fractionation during respiration, we calculated the apparent isotope fractionation (e_{dark})
441 between the sugars $\delta^{13}\text{C}_s$ (as the first photosynthetic product) and respired CO_2 ($\delta^{13}\text{C}_R$)
442 according to Ghashghaie et al. [79]. Interestingly, e_{dark} values were much more negative in
443 plants supplied with more than 50% NO_3^- than in plants supplied predominantly with NH_4^+
444 (Fig. 7). This implies that in plants supplied with NO_3^- , substrates for the respiration were

445 more enriched in ^{13}C relative to sugars (as the first products of photosynthesis and main
446 metabolites in the respiratory pathway) which in turn resulted in the observed ^{13}C enrichment
447 of respired CO_2 . In contrast, plants with higher percentage of NH_4^+ supply showed e_{dark}
448 values which were on average close to or slightly above zero, reflecting a very minor and
449 thus negligible difference between $\delta^{13}\text{C}_\text{S}$ and $\delta^{13}\text{C}_\text{R}$ (Fig. 7). These results confirmed our
450 hypothesis that a shift from NO_3^- to NH_4^+ supply mainly influences post-photosynthetic
451 isotope fractionation.

452 The high concentrations of malate and the high PEPC activity in NO_3^- supplied plants
453 together with relatively high $\delta^{13}\text{C}$ values of malate amongst all other metabolites (Fig. 3A)
454 further support the idea that the anaplerotic reaction of the TCA cycle could play an
455 important role in the ^{13}C enrichment of CO_2 respired by the leaves of NO_3^- supplied plants. In
456 order to determine the relative contribution of the anaplerotic reaction responsible for the ^{13}C
457 enrichment in leaf-respired CO_2 , we calculated the possible ^{13}C enrichment of the C-4 atom
458 in malate [95]. According to the model proposed by Mezler and O'Leary [95], the carbon
459 introduced into malate *via* PEPC is about 6 mUr more enriched in ^{13}C than that of
460 intercellular (internal) CO_2 . After calculating the $\delta^{13}\text{C}$ of intercellular CO_2 , we adjusted the
461 Melzer and O'Leary model by assuming that in plants supplied with 100% NO_3^- , PEPC has
462 the highest activity (supported by our own results, Fig. 4) and therefore these 100% NO_3^-
463 plants have the highest enrichment of the C-4 atom in malate (i.e. an additional 6 mUr). The
464 ^{13}C enrichment of all other N treatments along the gradient were then calculated by using the
465 ratio of their PEPC activity compared to that of 100% NO_3^- supplied plants (Fig. S4). Our
466 results showed a clear decrease in the ^{13}C enrichment of the C-4 atom in malate along the N
467 species gradient. These findings indicated that a higher proportion of anaplerotically
468 introduced carboxyl group(s) in malate might be released as CO_2 during amino acid
469 biosynthesis or plant dark respiration, particularly in NO_3^- supplied plants (Fig. 8). We

therefore conclude that such a change in the PEPC related mechanism along the N species gradient is the most likely pathway to explain ^{13}C enrichments observed in $\delta^{13}\text{C}$ of organic acids and of respired CO_2 under NO_3^- supply (see Fig. 6).

Conclusion

This study aimed to understand the influence of a supply gradient in NO_3^- and NH_4^+ supply on the C isotope fractionation as seen in leaf metabolites and during leaf respiration. Several lines of evidence suggest that modifications of the N species supplied to plants, which changed foliar gas exchange, enzymatic activities and concentrations of metabolites (i.e. sugars, amino acids and organic acids), also changed the C isotopic composition of leaf metabolites and respired CO_2 , supporting our first hypothesis. With our second hypothesis, we suggested a major role of the anaplerotic reaction of the TCA cycle (*via* PEPC) and organic acids. Indeed, our results indicated a higher contribution of anaplerotically assimilated carbon in the process of dark respiration in plants supplied dominantly with NO_3^- , leading to enrichments in metabolites and respiratory CO_2 . Our findings on the impact of N species on $\delta^{13}\text{C}$ of organic acids and respired CO_2 might gain additional relevance since the N species available to vegetation is strongly affected by either management or anthropogenic pollution while $\delta^{13}\text{C}$ values of leaf metabolites or respiration are widely used in plant sciences, e.g. as a proxies for plant physiological responses to environmental change or to partition ecosystem CO_2 fluxes.

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Declaration of authorship

SG, RAW, NB, FWB, JG, MML, RTWS developed the presented idea, planned, and designed the research. SG conducted the experiments as well as data analysis and wrote the manuscript. SG, RH, RM, SS, MH performed enzyme activity assays and MER and DAC contributed in carbohydrate concentration measurements. MML carried out compound specific analysis of organic acids. All authors discussed the results and contributed to the final manuscript.

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Supplemental Material

The following supplemental materials are available.

Figure S1. Linear regression between respiration rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$) and concentration of soluble sugars (mg g DW^{-1})

Figure S2. Carbon to nitrogen ratio (C:N) and nitrogen concentration (%)

Figure S3. Linear regression between measured $\delta^{13}\text{C}$ of starch/sugar and modeled $\delta^{13}\text{C}$ values using the simplified Farquhar model

Figure S4. Modelled carbon isotopic composition of C-4 of malate in the leaves of tobacco plants

Methods of compound-specific $\delta^{13}\text{C}$ and concentration analysis of amino acids and organic acids

Enzyme activity analysis: NR, PEPC and ME

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1. Nitrogen species gradient. In total, 6 mmol/L nitrogen were provided in the form of NO_3^- or NH_4^+ as sole N sources (100%) or in different ratios (10 to 90%). Each treatment had been applied to 5 individual plants. The colour code and descriptions of this table was applied in Figures 1-7. In all Figures, NO_3^- and NH_4^+ represent the 100% NO_3^- and 100% NH_4^+ treatments, respectively, while percentages on the x axes represent the fraction of NO_3^- in the nutrient solutions, decreasing from left to right.

		NO_3^-	90%	75%	50%	25%	10%	NH_4^+
6 mmol/l N	NO_3^-	100%	90%	75%	50%	25%	10%	0%
	NH_4^+	0%	10%	25%	50%	75%	90%	100%

Table 2. Effects of the N species gradient on foliar gas exchange, metabolites and their $\delta^{13}\text{C}$ as well as enzyme activities. The effects of the NO_3^- vs. NH_4^+ gradient on net assimilation rate (A_n , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), ratio of intercellular concentration to atmospheric concentration (C_i/C_a), stomatal conductance (g_s , $\text{mol m}^{-2} \text{ s}^{-1}$), respiration rate (RR , $\mu\text{mol m}^{-2} \text{ s}^{-1}$), leaf dry biomass (Biomass, g), isotopic composition of bulk organic matter ($\delta^{13}\text{C}_B$), sugars ($\delta^{13}\text{C}_S$), starch ($\delta^{13}\text{C}_{st}$), amino acids ($\delta^{13}\text{C}_{aa}$), organic acids ($\delta^{13}\text{C}_{oa}$), respiration ($\delta^{13}\text{C}_R$), malate ($\delta^{13}\text{C}_{malate}$), citrate ($\delta^{13}\text{C}_{citrate}$), glutamine ($\delta^{13}\text{C}_{glu}$), asparagine ($\delta^{13}\text{C}_{asp}$), alanine ($\delta^{13}\text{C}_{ala}$), serine ($\delta^{13}\text{C}_{ser}$), all in (mUr), together with activities of nitrate reductase (NR, $\text{nmol NO}_2^- \text{ s}^{-1} \text{ g FW}^{-1}$), phosphoenolpyruvate carboxylase (PEPC, $\text{nmol NAD s}^{-1} \text{ g FW}^{-1}$), malic enzyme (ME, $\text{nmol NAD s}^{-1} \text{ g FW}^{-1}$), concentrations of malate, citrate, glutamine, asparagine, alanine, serine ($\mu\text{mol g DW}^{-1}$) and concentrations of glucose, fructose, sucrose and starch (mg g DW^{-1}) are presented. Values denote p values from one-way ANOVA analysis, significant values are indicated in bold.

Variables				
A_n	C_i/C_a	g_s	RR	Biomass
0.013	0.001	0.67	0.008	<0.001
$\delta^{13}\text{C}_B$	$\delta^{13}\text{C}_S$	$\delta^{13}\text{C}_{st}$	$\delta^{13}\text{C}_{aa}$	$\delta^{13}\text{C}_{oa}$
0.042	0.007	0.005	0.14	<0.001
$\delta^{13}\text{C}_R$	$\delta^{13}\text{C}_{malate}$	$\delta^{13}\text{C}_{citrate}$	$\delta^{13}\text{C}_{glu}$	$\delta^{13}\text{C}_{asp}$
0.005	0.07	0.017	0.69	0.005
$\delta^{13}\text{C}_{ala}$	$\delta^{13}\text{C}_{ser}$	NR	PEPC	ME
<0.001	<0.001	<0.001	<0.001	<0.001
[malate]	[citrate]	[glutamine]	[asparagine]	[alanine]
<0.001	<0.001	<0.001	0.001	<0.001
[serine]	[glucose]	[fructose]	[sucrose]	[starch]
0.004	<0.001	<0.001	<0.001	<0.001

Figure legends

Fig. 1 Foliar gas exchange and biomass of tobacco leaves along the N species gradient. (A) Net assimilation rate (A_n , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), (B) ratio of intercellular CO_2 concentration to atmospheric CO_2 concentration, (C) stomatal conductance (g_s , $\text{mol m}^{-2} \text{ s}^{-1}$), (D) respiration rate (R_{dark} , $\mu\text{mol m}^{-2} \text{ s}^{-1}$) and (E) leaf dry biomass per plant (g) along the N species gradient from 100% NO_3^- to 100% NH_4^+ given (see Table 1; percentages on the x axis represent the fraction of NO_3^- in the nutrient solutions, decreasing from left to right). The bar underneath the figure reflects the gradient of N species changing from NO_3^- to NH_4^+ . Different letters denote significant differences ($p < 0.05$, $n = 5$).

Fig. 2 Carbon isotopic composition ($\delta^{13}\text{C}$, mUr) in tobacco leaves along the N species gradient. $\delta^{13}\text{C}$ of (A) bulk organic matter, (B) water soluble sugars, (C) starch, (D) bulk amino acids, (E) bulk organic acids, and (F) dark respired CO_2 in the leaves of tobacco plants along the N species gradient from 100% NO_3^- to 100% NH_4^+ are given (see Table 1; percentages on the x axis represent the fraction of NO_3^- in the nutrient solutions, decreasing from left to right). The bar underneath the figure reflects the gradient of N species changing from NO_3^- to NH_4^+ . Different letters denote significant differences ($p < 0.05$, $n = 5$).

Fig. 3 Compound specific isotope analysis in tobacco leaves along the N species gradient. Carbon isotopic composition ($\delta^{13}\text{C}$, mUr) of (A) malate, (B) citrate, (C) alanine, (D) asparagine, (E) serine, (F) glutamine and (G-L) their corresponding concentrations ($\mu\text{mol g DW}^{-1}$), in the leaves of tobacco plants along the N species gradient from 100% NO_3^- to 100% NH_4^+ are given (see Table 1; percentages on the x axis represent the fraction of NO_3^- in the nutrient solutions, decreasing from left to right). The bar underneath the figure reflects the gradient of N species changing from NO_3^- to NH_4^+ . Different letters denote significant differences ($p < 0.05$, $n = 5$). Note: Values of malate and citrate in plants supplied with more than 50% NO_3^- were measured at the detection limit of HPLC-IRMS.

Fig. 4 Enzymes activity in tobacco leaves along the N species gradient. (A) Nitrate reductase activity (NR, $\text{nmol NO}_2^- \text{ g FW}^{-1} \text{ s}^{-1}$), (B) phosphoenolpyruvate carboxylase activity (PEPC, $\text{nmol NAD g FW}^{-1} \text{ s}^{-1}$), (C) malic enzyme activity (ME, $\text{nmol NAD g FW}^{-1} \text{ s}^{-1}$) in the leaves of tobacco plants along the N species gradient from 100% NO_3^- to 100% NH_4^+ are given (see Table 1; percentages on the x axis represent the fraction of NO_3^- in the nutrient solutions, decreasing from left to right). The bar underneath the figure reflects the gradient of N species changing from NO_3^- to NH_4^+ . Different letters denote significant differences ($p < 0.05$, $n = 5$). In NR and ME activity assays, 100% NH_4^+ has not been measured due to lack of adequate plant material.

Fig. 5 Carbohydrate concentrations in tobacco leaves along the N species gradient. Concentrations of (A) glucose (mg g DW^{-1}), (B) fructose (mg g DW^{-1}), (C) sucrose (mg g DW^{-1}), (D) soluble sugars (mg g DW^{-1}), (E) transitory starch (mg g DW^{-1}) in the leaves of tobacco plants along the N species gradient from 100% NO_3^- to 100% NH_4^+ are given (see Table 1; percentages on the x axis represent the fraction of NO_3^- in the nutrient solutions, decreasing from left to right). The bar underneath the figure reflects the gradient of N species changing from NO_3^- to NH_4^+ . Different letters denote significant differences ($p < 0.05$, $n = 5$).

Fig. 6 Relationship between $\delta^{13}\text{C}$ of respiration and organic acids. Linear regression showing relationship between carbon isotopic composition of bulk organic acids ($\delta^{13}\text{C}_{\text{oa}}$, mUr; see Figure 2) and of respired CO_2 ($\delta^{13}\text{C}_{\text{R}}$, mUr). *** denotes $p \leq 0.001$ along the N species gradient from 100% NO_3^- to 100% NH_4^+ (see Table 1). Data represent average values $\pm$ SE ($n = 5$). Dotted line represents the 1:1 line; the regression equation is $y = 0.97x - 0.72$.

Fig. 7 Apparent respiratory isotope fractionation. ϵ_{dark} values are shown for tobacco leaves along the N species gradient from 100% NO_3^- to 100% NH_4^+ (see Table 1; percentages on the x axis represent the fraction of NO_3^- in the nutrient solutions, decreasing from left to right). The bar

underneath the figure reflects the gradient of N species changing from NO_3^- to NH_4^+ . The dashed line indicates $e_{\text{dark}}=0$ as a reference line. Different letters denote significant differences ($p<0.05$, $n=5$).

Fig. 8 Schematic representation of leaf dark respiration as influenced by NO_3^- and NH_4^+ supply. (A) Under NO_3^- supply, the anaplerotic reaction of the TCA cycle is highly active, introducing malate to this cycle. Malate is incorporated in citrate and eventually respired, while the ^{13}C enriched C-4 atom is lost with the CO_2 . (B) Under NH_4^+ supply, sugar molecules are being respired through the catabolic TCA cycle. Lines represent dominant reactions and dashed lines represent smaller fluxes. Dotted circles show the anaplerotic reaction to the TCA cycle. Abbreviations are Pyr (pyruvate), Cit (citrate), PEP (phosphoenolpyruvate), Mal (malate), Fum (fumarate), OAA (oxaloacetate), ME (malic enzyme), MDH (malate dehydrogenase), 2-OG (2-oxoglutarate), Asp (asparagine), Cyt (cytosol), Ala (alanine), Ser (serine) and Gln (glutamine).

Figure 1.

Figure2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Supplementary Information

Figure S1. Linear regression between respiration rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$) and concentration of soluble sugars (mg g DW^{-1}) in tobacco leaves along the N species gradient from 100% NO_3^- to 100% NH_4^+ (see Table 1). Different letters denote significant differences ($p < 0.05$, $n=5$).

Figure S2. (A) Carbon to nitrogen ratio (C:N) and (B) nitrogen concentration (%) in tobacco leaves along the N species gradient from 100% NO_3^- to 100% NH_4^+ (see Table 1; percentages on the x axis represent the fraction of NO_3^- in the nutrient solutions, decreasing from left to right). The bar underneath the figure reflects the gradient of N species changing from NO_3^- to NH_4^+ . Different letters denote significant differences ($p < 0.05$, $n = 5$).

Figure S3. Linear regression between (a) measured $\delta^{13}\text{C}$ of starch and modeled $\delta^{13}\text{C}$ values using the simplified Farquhar model and between (b) measured $\delta^{13}\text{C}$ of sugar and modeled $\delta^{13}\text{C}$ values using the simplified Farquhar model along the N species gradient from 100% NO_3^- to 100% NH_4^+ (see Table 1). Data represent average values $\pm$ SE ($n=5$). (+) denotes $p \leq 0.1$.

Figure S4. Modelled carbon isotopic composition of C-4 of malate in tobacco leaves according to Melzer and O’Leary (1991) along the N species gradient from 100% NO₃⁻ to 100% NH₄⁺ (see Table 1; percentages on the x axis represent the fraction of NO₃⁻ in the nutrient solutions, decreasing from left to right). The bar underneath the figure reflects the gradient of N species changing from NO₃⁻ to NH₄⁺. Data represent average values ± SE (n=5).

Compound-specific $\delta^{13}\text{C}$ and concentration analysis of amino acids and organic acids

$\delta^{13}\text{C}$ and concentration analyses of individual organic acids in the organic acid fraction were performed with a high-performance liquid chromatograph (HPLC) coupled to a Delta V Advantage IRMS by an LC IsoLink (all ThermoFisher, Germany) as described by Lehmann *et al.* (2015). The organic acid fraction was filtered with a 0.45 μm PTFE syringe filter (Infochroma AG, Switzerland) into 1.5 mL glass vials (Macherey-Nagel GmbH and Co, Germany). The separation was performed on a 4.6 $\times$ 300 mm Allure Organic Acids HPLC column (Restek, USA) at 5-10 $^{\circ}\text{C}$. The mobile phase was a 100 mM monopotassium phosphate buffer (pH=3.0) with a flow speed of 500 $\mu\text{L min}^{-1}$. A standard mixture of commercially available malate and citrate were analyzed at different concentrations (10-180 ng C μL^{-1}) between the samples to allow correction of the $\delta^{13}\text{C}$ offset, relative to measured $\delta^{13}\text{C}$ values with the EA coupling and used to determine the concentration of both organic acids (Rinne *et al.*, 2012; Lehmann *et al.*, 2015). The LC IsoLink measurement had a typical standard deviation of 0.2‰ for malate and 0.3‰ for citrate.

The amino acid fraction from the Dowex extraction was freeze-dried and re-dissolved in 400 μL deionized water. An aliquot of 200 μL was transferred to a tube, followed by an addition of 20 μL internal standard containing 0.25 $\mu\text{g } \mu\text{L}^{-1}$ norvaline and 0.25 $\mu\text{g } \mu\text{L}^{-1}$ norleucine in 0.1 M HCl. To derivatize the amino acids, 140 μL of ethanol:pyridine (4:1) and 20 μL ethyl-chloroformate were added to the solution. After 5 min incubation at room temperature, the samples were extracted by liquid-liquid extraction. 400 μL hexane:dichloromethane:ethyl-chloroformate (50:50:1, v/v/v) was added to the samples and an aliquot of 200 μL was transferred to a glass vial after a vortex mixing and centrifugation step (1 min, 2000 g). This step of extraction was repeated once, and 400 μL of the supernatant was combined with the first aliquot. The combined fractions were dried under a gentle N_2 flow and the residuals were re-dissolved in 50 μL ethylacetate according to Vlaardingerbroek *et al.* (2014) and Schierbeek (2017). Isotopic composition of amino acids and concentration measurements were performed with a Delta-XP isotope ratio mass spectrometer coupled online with a trace gas chromatograph and a combustion interface type 3 (GC-C-IRMS, ThermoFisher, Germany). Aliquots were injected into the gas chromatogram GC system by a PAL auto sampler (CTC, Switzerland). We corrected for the exogenous carbon added during the derivatization by analyzing a standard curve with known ^{13}C enrichments for each of the amino acids of interest. The slope and intercept of the curves are used to calculate the corrected $\delta^{13}\text{C}$ values of the samples. The GC-C-IRMS measurement had a typical standard deviation of about 1.1% for each amino acid.

Enzymatic activities

Nitrate reductase activity (NR, EC 1.7.1.1) was measured with an *in vitro* assay as described by Dier *et al.* (2017), with only minor modifications. Briefly, 100 mg of leaf material was mixed with four volumes of extraction buffer at 4 °C (100 mM HEPES-KOH (pH=7.5), 5 mM Mg(Ac)₂, 1 mM EDTA, 10% (v/v) glycerol, 0.5% (w/v) bovine serum albumin (BSA), 0.1% Triton X-100, 1% polyvinylpolypyrrolidone (PVPP), 5 µM NaMoO₄, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM DTT, 20 µM FAD and 25 µM leupeptin). To start the enzymatic reaction for measuring the total NR activity, one volume of this leaf extract was added to five volumes of an assay buffer (100 mM HEPES-KOH (pH=7.5), 6 mM KNO₃, 6 mM EDTA, 0.6 mM NADH, 20 µM leupeptin, 12 µM FAD, 0.3 mM DTT and 6 µM NaMoO₄) preheated to 25.5 °C. After 5, 10 and 15 min, the reaction was stopped by adding 300 µL of this assay mixture to a new reaction tube containing 25 µL 600 mM Zn(OAc)₂. Unreacted NADH was removed by adding 75 µL of 0.25 mM phenazine methosulphate followed by a 15 min dark incubation. Produced NO₂⁻ was then converted by diazotization with N-(1-naphthyl) ethylenediamine (NED) and sulfanilamide (SA) to a red dye that was determined colorimetrically as described by Scheible *et al.* (1997b) at 540 nm. Two technical replicates were carried out for each sample and afterwards averaged.

The activity of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) was determined as described by Gibon *et al.* (2004), with minor modifications. In brief, 150 mg of leaf plant material was shock frozen and ground in liquid nitrogen. This tissue was mixed with 1 mL of protein extraction buffer (50 mM HEPES, 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA and 0.25% w/v BSA, pH=7.5). Additives containing 10% glycerol, 5 mM DTT and 1 tablet of protease inhibitor (Roche complete, Sigma Aldrich, CH) were added to this mixture, followed by a short vortex mixing and then samples were thawed on ice. After 10 min of centrifugation (10000 g at 4 °C), 20 µL protein extract was transferred to an assay volume containing 100 mM tricine (pH=8), 20 mM MgCl₂, 10 mM NaHCO₃, 0.05% Triton X-100, 0.5 mM NADH, 0.2 U malate dehydrogenase (MDH), 1 mM PEP, pH 8.0. The reaction was assessed by monitoring the consumption of NADH spectrophotometrically at 340 nm over 2 hours with a plate reader (Infinite M1000, TECAN, Switzerland). Enzyme activity was calculated using the time window (approximately 30 min) when the reaction production rate of NAD⁺ was constant.

NAD-malic enzyme (ME, EC 1.1.1.39) extraction was carried out by adding 200 μ L of extraction buffer (50 mM 3-N-morpholino-propanesulfonic acid (MOPS)-NaOH, pH=7.0, 5 mM MnCl_2 , 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.05% (v/v) triton X-100, 20% (v/v) glycerol, and 1 mM PMSF, pH 7.0) to 100 mg shock frozen and ground plant material. After 15 min of centrifugation (10000 g at 4°C), the supernatant was transferred to a new tube. 10 μ L of the supernatant was directly pipetted into a 96 well plate containing 186 μ L assay buffer (50 mM MOPS-NaOH, 4 mM NAD^+ , 5 mM DTT, 10 mM MnCl_2 and 10 U MDH (pig heart, Roche, Switzerland, pH=7.0)). The enzyme assay was started by adding 4 μ L L-malate to a final concentration of 20 mM into each well. Production of NADH was then monitored spectrophotometrically at 340 nm for 20 min with a plate reader (Synergy HT Platereader, BioTek, USA).