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Metabolic origin of $\delta^{15}N$ in nitrogenous compounds from *Brassica napus* L. leaves

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Abbreviations: GABA, γ-aminobutyric acid; GC-C-IRMS, gas chromatography coupled to isotope ratio mass spectrometry; SF, deproteinated soluble fraction;

Abstract

Nitrogen isotope composition ($\delta^{15}N$) in plant organic matter is currently used as a natural tracer of nitrogen acquisition efficiency. However, the $\delta^{15}N$ of whole leaf material does not properly reflect the way by which N is assimilated by metabolism because isotope fractionation along metabolic reactions may cause substantial difference among leaf compounds. In other words, any change in metabolic composition or allocation pattern may cause undesirable variability in leaf $\delta^{15}N$. Here, we investigated the $\delta^{15}N$ of different leaf fractions and individual metabolites from rapeseed (*Brassica napus*) leaves. We show that there were substantial differences between nitrogenous compounds (up to 30‰) and the content in (^{15}N-enriched) nitrate had a neat influence on leaf $\delta^{15}N$. Using a simple steady-state model of day metabolism, we suggest that $\delta^{15}N$ in major aminoacids was mostly explained by isotope fractionation associated with enzymes of primary nitrogen metabolism. $\delta^{15}N$ values were further influenced by light vs. dark conditions and the probable occurrence of alternative biosynthetic pathways. We conclude that biochemical pathways which fractionate between isotopes and nitrogen sources used for aminoacid production should be considered to interpret the $\delta^{15}N$ of leaf material.

Introduction

It is now more than fifty years that the first isotope fractionation between ^{14}N and ^{15}N isotopes associated with a biological reaction (N_2 fixation by *Azotobacter*) has been measured (Hoering & Ford, 1960). Since then, considerable advance has been done on nitrogen isotope composition ($\delta^{15}N$) in plants, with clear isotopic patterns along nitrogen assimilation or symbiotic N_2 fixation. As a matter of fact, $\delta^{15}N$ values are considered as useful tools to investigate the physiology of plant nitrogen assimilation and nitrogen use efficiency. It is now well recognized that $^{14}N/^{15}N$ fractionation occurs during nitrate absorption and assimilation so that plant organic matter is on average 2-3‰ ^{15}N-depleted compared to inorganic soil nitrogen (for a review see Evans, 2001; Tcherkez & Hodges, 2008). Such a ^{15}N-depletion is nevertheless variable since it depends upon soil N availability (Mariotti *et al.*, 1982; Evans, 2001) and correlates to transpiration efficiency of net N uptake (Cernusak *et al.*, 2009).

Still, the $\delta^{15}N$ value in total plant organic matter cloaks disparities among plant metabolites. For example, it has been shown that leaf nitrates are ^{15}N-enriched (Yoneyama & Tanaka, 1999) while secondary metabolites such as alkaloids are ^{15}N-depleted (Weilacher *et al.*, 1996). This isotopic difference stems from isotope effects along metabolism (for a review, see Werner & Schmidt (2002). For example, Gln synthetase, that fixes ammonia onto Glu to evolve Gln, fractionates against ^{15}N by 16‰ (Yoneyama *et al.*, 1993) and nitrate reductase fractionates against ^{15}N by 15‰ (Ledgard *et al.*, 1985; Tcherkez & Farquhar, 2006) thereby enriching in ^{15}N nitrate molecules left behind and depleting the primary aminoacids Glu and Gln. More generally, most enzymes associated with primary nitrogen metabolism (transaminases, Glu synthase, Asn synthetase, etc.) fractionate between nitrogen isotopes (Werner & Schmidt, 2002). However, the isotope composition in metabolites is not only influenced by enzymatic isotope effects but also by metabolic fluxes and commitments (Schmidt & Kexel, 1997; Tcherkez *et al.*, 2011). Typically, metabolic reactions that run to

79 completion do not fractionate between isotopes simply because all substrate molecules are
80 consumed; by contrast, limited reactions can fractionate between isotopes. Using a flux-
81 modelling approach, we have recently shown that the $\delta^{15}N$ in leaf aminoacids does depend on
82 source nitrate $\delta^{15}N$ and isotope effects but also on photorespiration and N-reduction input
83 rates (Tcherkez, 2011). Experimental $\delta^{15}N$ measurements in aminoacids show a ^{15}N-depletion
84 in Gly and Ser (relative to glutamate) and a ^{15}N-enrichment in others (Hayes, 2001),
85 suggesting indeed the ^{15}N-depleting effect of photorespiration and the ^{15}N-enriching effect of
86 other reactions (such as transaminases). However, experimental data with simultaneous
87 isotopic analysis of several aminoacids, nitrate and secondary metabolites are scarce and
88 therefore, the means by which isotopic fractionations and metabolic fluxes are integrated into
89 metabolite $\delta^{15}N$ are still uncertain. Furthermore, most studies on $\delta^{15}N$ in aminoacids use
90 protein hydrolysates and not free aminoacids (Werner & Schmidt (2002) but see Hofmann *et*
91 *al.* (1997) and Bol *et al.* (2002)).

92 As an aid in clarifying ^{15}N distribution among plant compounds, we took advantage of
93 isotope ratio mass spectrometry (IRMS) techniques to measure the $\delta^{15}N$ in free aminoacids
94 and several metabolic fractions from rapeseed leaves (*Brassica napus* L.) and explored the
95 relationships between them. We show that the nitrate content had a major influence on $\delta^{15}N$
96 in leaf soluble fraction. Known metabolic pathways and fluxes satisfactorily explained the
97 $\delta^{15}N$ in metabolites on a steady-state basis in the light. We further show that although Glu
98 and Gln content remained the same in the light and in the dark, their $\delta^{15}N$ value was
99 dissimilar, likely because of changes in source N. $\delta^{15}N$ in other compounds mostly reflects
100 the influence of precursors and biosynthetic/consumption reactions.

101

Material and methods

Plant material

Seeds of canola (*Brassica napus* var *oleifera* cv Darmor) germinated in Petri dishes on wet Whatman paper. After 72 h, seedlings were transferred to 500 mL pots filled with potting mix. Plants were grown in the glasshouse under 22/18°C, 60/55% relative humidity, 16/8h photoperiod (day/night) as described by (Vartanian *et al.*, 1987). Plants were automatically watered 3 times a day with nutritive solution (Hydrokani C2) in which ammonium nitrate had a $\delta^{15}N$ value of +2.69±0.61. Carbon dioxide in air was at natural ^{13}C-abundance ($\delta^{13}C$ = –8.92±0.55‰, where $\delta^{13}C$ is the carbon isotope composition with respect to V-PDB). The sampling was done on 6-weeks old plants: mature leaves (rank 5 or 6 from the apex) were cut and instant-frozen with liquid nitrogen. Samples were collected either in the dark or in the light. Sampled leaves were freeze-dried (lyophilized) and ground in fine powder.

Soluble, protein and chlorophyll fractions

Soluble fraction and proteins were extracted as described in Nogués *et al.* (2004). 100 mg of leaf powder were resuspended in 2 mL of distilled water. After centrifugation (5 min, 10,000 g, 5°C), the aqueous supernatant was transferred in another tube and the pellet was conserved at –80°C for chlorophyll extraction. The supernatant was heated at 100°C for 5 min for protein precipitation. After centrifugation (5 min, 10,000 g, 5°C), the protein precipitate was frozen and lyophilized and the supernatant was used in the following as the deproteinated soluble fraction (SF, which contains sugars, organic and aminoacids and nitrates). The SF was lyophilized and 1.4 mg was weighted in tin capsules for isotopic analysis. The solvent extraction of chlorophylls was carried out on the pellet obtained above, using ethanol 96% v/v. After 10 min agitation at ambient temperature and centrifugation (5 min, 10,000 g, 5°C),

the solvent phase was transferred in a tube. 200 µL were poured in thick tin capsules adapted for solvents and ethanol was oven-evaporated at 35°C.

Nitrate content and purification

The nitrate content was measured in the soluble fraction SF using a nitrate-selective electrode (CI-6735, PASCO Scientific, Roseville CA, USA) calibrated with standard nitrate solutions of known concentrations at ambient temperature. 1 mL of SF was first diluted 50 times with distilled water and then measured under continuous agitation with a magnetic stirrer. For isotopic analyses, nitrates were purified from aqueous leaf extracts by collecting the HCl loading fraction through a cation-exchange column (see below, *Aminoacids extraction*).

Isotopic analyses

Dried SF, chlorophyll, proteins, nitrate and DNA were analysed by elemental-analysis-isotope-ratio-mass spectrometry (EA-IRMS) using a Flash-EA (ThermoFisher Scientific) and Optima (Elementar, Villeurbanne, France). EA-IRMS settings were adjusted (split ratio and trap current) so as to have a sufficient mass-44 signal (1.0 10^{-9} A) in all instances including for small samples (proteins). Any possible $\delta^{15}N$ offset was corrected using reference material of known isotope composition (glutamic acid USGS40, –4.5±0.1‰ and caffeine IAEA600, +1±0.2‰, International Atomic Energy Agency, Vienna, Switzerland) included in each sample trials. Similarly, $\delta^{13}C$ values were corrected for any offset using standard glutamic acid (USGS40, –26.4±0.04‰). In this paper, all $\delta^{15}N$ and $\delta^{13}C$ values are given with respect to atmospheric N_2 and V-PDB as the standard reference, respectively.

Aminoacids extraction, derivatization and isotopic analysis

The extraction and analysis of amino acids was carried out after Molero *et al.* (2011). Briefly, 250 µL of L-norleucine (1 mmol L^{-1}) were added to 2 mL of SF as an internal reference ($\delta^{15}N$

= 17.0±0.4‰). Samples were then spin-dried and kept at –80°C. The samples were resuspended in 1 mL HCl 0.1 mol L^{-1} and purified through a cation-exchange column (Dowex 50W X8 H+, 200-400 mesh size, Sigma-Aldrich, Saint-Quentin Fallavier, France). The aminoacid-enriched fraction was obtained by elution with NH_4OH and dried with an infrared lamp under a non-oxidative atmosphere (N_2 flow). Samples were then derivatized with 50 µL of N-methyl-N-(ter-butyldimethylsilyl)trifluoroacetamide and 50 µL pyridine and incubated at 70°C for 1 hour. The isotopic analysis of amino-acids was carried out by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). The GC-C-IRMS device was made of a GC6890 gas Chromatograph (Agilent Technologies, Palo Alto CA, USA) coupled to a Delta-Plus spectrometer through the GC-C-III combustion interface (ThermoFisher-Scientific, Courtaboeuf, France). Chromatography was carried out with a PTE-5 column (30 m x 0.32 mm x 1 µm; Supelco, Schelldorf, Germany). Helium was used as the carrier gas for separation. A volume of 1 µL was injected in splitless mode at an injector temperature of 270°C. The temperature program used was: 90°C for 1 min, ramping at 8°C min^{-1} to 140°C for 5 min, then ramping at 3°C min^{-1} to 220°C and finally ramping at 12°C min^{-1} to 285°C, holding for 12.5 min. Water was trapped using a Nafion® membrane. CO_2 was trapped with liquid N_2. The chromatographic sequence of aminoacid derivatives was checked by injecting the same samples in a GC8060 gas chromatograph (Fisons, Manchester, UK) coupled to a MD800 mass spectrometer (ThermoFinigan, Bremen, Germany) using helium as the carrier gas. Amino acids were separated on a DB-5MS column (30 m x 0.25 mm x 0.25 µm; Agilent Technologies, Santa Clara, CA, USA). Aminoacid derivatives were identified by their mass spectra (Mass Spectral Library: NIST 05: NIST, Gaithersburg, MD, USA). The absolute concentration of aminoacids was obtained from GC-MS signals with calibration curves using a standard aminoacids mix (AAS18, Sigma-Aldrich in which norleucine, Gln, Asn and GABA were added), after correction for the recovery of norleucine.

173 The $\delta^{15}N$ value of aminoacids was corrected for any offset using norleucine as an internal
174 isotopic standard.

175 *DNA purification*

176 1 g of leaf fresh material was extracted in a mortar at ambient temperature with 20 mL of
177 extraction buffer (Tris-HCl 200 mmol L^{-1}, NaCl 250 mmol L^{-1}, EDTA 25 mmol L^{-1}, SDS
178 0,5%, pH 7.5). After centrifugation (15 min, 12,000 g, 5°C), the supernatant was mixed with
179 phenol/chloroform/3-methylbutanol (25/24/1 v/v/v) and agitated to separate the different
180 phases. The aqueous phase was collected and mixed with isopropanol to induce DNA
181 flocculation. After centrifugation (15 min, 12,000 g, 4°C), the supernatant was discarded and
182 the DNA pellet was frozen with liquid nitrogen and lyophilized.

183 *Modeling*

184 The $\delta^{15}N$ in aminoacids was computed using the model developed by Tcherkez (2011)
185 Briefly, the model is based on steady-state (mass-balance) iso-fluxes through nitrogenous
186 compounds, taking into account known isotope effects. With the 'standard' model, the steady
187 $^{15}N/^{14}N$ isotope ratio in Glu in the light can be rearranged to:

188 $$R_{\mathrm{Glu}} = \frac{iR^*/\alpha_5}{\alpha_1^N \alpha_2^N \tau_{\mathrm{Gln-N}} \left(t + \gamma/\alpha_1^N\right) \left(\frac{\tau_{\mathrm{Glu}}}{\gamma(\gamma - e)} - \frac{1}{\alpha_1^G \alpha_2^G \tau_{\mathrm{Gln-G}}} \right) - \frac{v_o - \mu_g}{2\alpha_6^N \tau_{\mathrm{Gly}}}} \qquad (1)$$

189 Where α_1^N (1.016), α_1^G (1.000), α_2^N (1.022), α_2^G (1.000), α_5 (1.015), α_6^N (0.995) are the
190 isotope effects associated with glutamine synthetase (subscript 1), glutamate synthase
191 (subscript 2), nitrate reductase (subscript 5) and glycine decarboxylase (subscript 6). For
192 glutamate synthase and glutamine synthetase, isotope effects at the amido and amino N-atom
193 level are distinguished with superscripts N and G, respectively. *e* is the rate of Gln

accumulation/export in the light (within the 0.05-0.2 μmol m^{-2} s^{-1} range), v_o is the Rubisco-catalysed oxygenation rate (typically 5 μmol m^{-2} s^{-1}), μ_g the rate of Gly escape from photorespiratory recycling to Ser (0.018 μmol m^{-2} s^{-1}), i the reduced-N input rate (within the 0.05-0.35 μmol m^{-2} s^{-1} range), t the rate of NH_3 escape from the leaf (0.0004 μmol m^{-2} s^{-1}) and γ is equal to $i+(v_o-\mu_g)/2-t$. R^* is the isotope ratio in utilized leaf nitrate. Parameters denoted as τ are homogeneous to consumption iso-fluxes (linear combination of leaving fluxes and inverse isotope effects, $1/\alpha$). In this model, the rate of Asp production from Glu by transamination is fixed by mass-balance on the Glu content.

However, the Asp content decreased in the light (Figure 3A), suggesting that Asp could have been consumed rather than produced by transamination from Glu. Under the assumption that Asp is a nitrogen source (with a fixed isotope ratio R_{Asp}), we have:

$$R_{Glu} = \frac{iR^*/\alpha_5 + r(\gamma - e)\left(t\frac{\alpha_1^N}{\gamma} + 1\right)\alpha_2^N \tau_{Gln-N}\alpha_8 R_{Asp}}{\alpha_1^N \alpha_2^N \tau_{Gln-N}\left(t + \gamma/\alpha_1^N\right)\left(\frac{\tau_{Glu}}{\gamma(\gamma - e)} - \frac{1}{\alpha_1^G \alpha_2^G \tau_{Gln-G}}\right) - \frac{v_o - \mu_g}{2\alpha_6^N \tau_{Gly}}} \quad (2)$$

Where r is the rate of Asp consumption and α_8 the equilibrium isotope effect associated with Asp transaminase in the direction of Asp synthesis (0.9985).

In the above equations (1) and (2), it should be noted that the influence of α_1^N on the isotope ratio in Glu is extremely small because t is negligible (when $t = 0$, α_1^N disappears in the equations). That is, NH_3 is fully committed to recycling when $t = 0$ such that the isotope effect associated with glutamine synthetase is of negligible importance (furthermore, note that $\alpha_1^G = 1$). In its simplified form ($\alpha_1^G = \alpha_2^G = 1$, $t = 0$), Eqn 1 gives:

213 $$R_{\mathrm{Glu}} = \frac{iR^{*}/\alpha_5}{\alpha_2^N \tau_{\mathrm{Gln-N}}\left(\frac{\tau_{\mathrm{Glu}}}{(\gamma - e)} - \frac{\gamma}{\tau_{\mathrm{Gln-G}}}\right) - \frac{v_o - \mu_g}{2\alpha_6^N \tau_{\mathrm{Gly}}}} \qquad (3)$$

214 And Eqn (2) gives:

215 $$R_{\mathrm{Glu}} = \frac{iR^{*}/\alpha_5 + r(\gamma - e)\alpha_2^N \tau_{\mathrm{Gln-N}} \alpha_8 R_{\mathrm{Asp}}}{\alpha_2^N \tau_{\mathrm{Gln-N}}\left(\frac{\tau_{\mathrm{Glu}}}{(\gamma - e)} - \frac{\gamma}{\tau_{\mathrm{Gln-G}}}\right) - \frac{v_o - \mu_g}{2\alpha_6^N \tau_{\mathrm{Gly}}}} \qquad (4)$$

216 In the present paper, *r* can be roughly estimated with the dark-to-light decrease of Asp
217 content (Figure 3), which gives 0.22 µmol m^{-2} s^{-1}. The minimal *i* value can be obtained from
218 the N content (excluding nitrates) in leaf organic matter, that is, 0.025 µmol m^{-2} s^{-1}. This
219 value is under-estimated however, simply because leaf-assimilated N is redistributed in other
220 organs. The nitrate reduction rate in rapeseed shoots under ammonium nitrate growth
221 conditions has been shown to be 0.29 µmol m^{-2} s^{-1} (Leleu & Vuylsteker, 2004). In the two
222 scenarios investigated here (Asp production, Asp consumption), the total aminoacid
223 accumulation (sum of Gly, Ser, Glu, Gln, Asp if applicable) matched the total input (nitrate
224 reduction + Asp utilization). To facilitate comparisons, the two scenarios considered here are
225 symmetrical for Asp metabolism: when Asp is assumed to accumulate, the associated rate is
226 fixed at 0.22 µmol m^{-2} s^{-1}, that is, identical to the rate of Asp remobilization when Asp is
227 assumed to be consumed (see above). Under both scenarios, input and output iso-fluxes of the
228 whole metabolic system were similar (steady-state condition), at 2.9 (Asp production
229 scenario) and 5.2 (Asp consumption scenario) ‰ µmol m^{-2} s^{-1}. Note that the iso-flux with
230 Asp consumption was a bit ^{15}N-enriched because Asp was more enriched ($\delta^{15}N$ = +12.5‰)
231 than nitrogen evolved by nitrate reduction (+9.8‰).

232

Results

Comparison of $\delta^{15}N$ in leaf fractions

The average $\delta^{15}N$ value in total organic matter and leaf fraction is reported in Table 1. Leaf nitrates appeared to be substantially ^{15}N-enriched. Soluble proteins were isotopically very close to total organic matter while amino-acids (weighted average) were ^{15}N-enriched; so was chlorophyll. By contrast, DNA was ^{15}N-depleted (nearly 30‰ compared to nitrates). The apparent isotope fractionation between leaf source nitrate and organics was thus comprised between 15 and 30‰.

The natural carbon and nitrogen isotope composition in leaf fractions is represented in Figure 1, as a deviation from total organic matter. Quite clearly, leaf fractions are more widely distributed along the $\delta^{15}N$ axis (with a difference of nearly 25‰ between extreme values) than along the $\delta^{13}C$ axis (10‰ difference between extreme values). Proteins appeared to be close to total organic matter though slightly ^{13}C-enriched by 0.8‰ on average. Chlorophyll and DNA showed substantial scattering along both $\delta^{15}N$ and $\delta^{13}C$ axes; nevertheless, chlorophyll was on average ^{13}C-depleted by 3‰ compared to total organic matter and DNA was ^{13}C-enriched by 1‰. As a result, there was limited overlapping between compounds in the $\{\delta^{13}C, \delta^{15}N\}$ space. Such a pattern likely reflected contrasted metabolic precursors or biosynthetic pathways: chlorophylls were ^{13}C-depleted due to their phytol group, DNA was ^{15}N-depleted due to isotope fractionation associated with bases biosynthesis (see Discussion below).

Relationship between nitrates and leaf soluble fraction

As visible in Figure 1, the leaf soluble fraction is always ^{15}N-enriched but the nitrogen isotope composition is quite variable, with $\delta^{15}N$ values between +2‰ and +15‰. Due to the

256 substantial enrichment in leaf nitrates (Table 1), the leaf soluble fraction is certainly
257 influenced by the nitrate content. In fact, there was a positive relationship between $\delta^{15}N$ and
258 leaf nitrate content (Figure 2A). Furthermore, when expressed on a nitrogen mole fraction
259 basis (fraction of leaf N represented by nitrates), there was a linear relationship between $\delta^{15}N$
260 and nitrate content (Figure 2B), with a regression coefficient of 0.7. In other words, leaf
261 nitrates content explained 70% of the $\delta^{15}N$-variation in the soluble fraction. However, the
262 linear relationship did not coincide with the predicted mixing-line between the two major
263 soluble N compounds: nitrates (+25.7%) and aminoacids (average $\delta^{15}N$ of +7.5‰). That is,
264 there was a systematic depletion of 5 to 8‰ in the soluble fraction, likely explained by the
265 contribution of other compounds, such as free aminoacids not analysed here, free nucleotides,
266 NAD, etc. $\delta^{15}N$ in proteins was also related to that in the soluble fraction (Figure 2C),
267 although with a lower regression coefficient and a slope of nearly 0.6. It remains possible that
268 nitrates still present in the protein fraction adulterated the $\delta^{15}N$ value in proteins. However,
269 using the nitrate concentration in sample, such a contribution of nitrates was certainly less
270 than 15%, that is, much less than the slope between nitrate content and proteins ($0.7 \times 0.6 =$
271 0.42). It is likely therefore that the N-source effect of nitrate $\delta^{15}N$ on aminoacids had in turn
272 an influence on proteins synthesized therefrom.

273 *$\delta^{15}N$ in free aminoacids*

274 Individual free aminoacids were extracted and analysed by gas chromatography coupled to
275 isotope ratio mass spectrometry (Figure 3). There were clear differences between aminoacids,
276 with Thr and Asp being the most enriched of them and Gly and Asn the most depleted
277 (Figure 3B). In addition, there were day/night differences, Glu, Gln and most aminoacids
278 being more ^{15}N-enriched in the light than in the dark. Such a ^{15}N-enrichment was statistically
279 significant in Gln only. Except for Asp that decreased in the light, there was little change in

280 leaf aminoacid content in the light compared to the dark (Figure 3A). Ala, Asn, Gly and Val
281 were aminoacids of minor importance. The ^{15}N-enrichment in most aminoacids in the light
282 may have come from the ^{15}N-enrichment in source nitrogen used in biosyntheses or changes
283 in (fractionating) metabolic fluxes causing a net ^{15}N-enriching effect.

284 It should be noted that the δ^{15}N values reported in Figure 3 do not distinguish between amino
285 and amido groups of Gln and Asn, although the amino-N atom has been shown to be
286 substantially ^{15}N-depleted compared to the amido-N atom (Sacks & Brenna, 2005). The δ^{15}N
287 value in Asn was very different (more than 14‰-depleted) from that in Asp, suggesting a
288 very large isotope effect associated with Asn synthesis from Asp and Gln as an amido-donor.
289 This isotope effect would over-compensate for the natural ^{15}N-enrichment in Gln amido-N
290 atom. Since amino-N in Asp was probably similar to that in Asn (small or no isotope effect at
291 the amino-site) and the Asn molecular average was at ca. 10‰, the δ^{15}N in Asn amido-N was
292 presumably at –18‰. If Gln amido-N was 18‰-enriched compared to the molecular average
293 (Tcherkez, 2011), the apparent isotope fractionation associated with Asn synthetase might
294 have been of 36‰.

295 *Comparison of modeled vs observed values*

296 The δ^{15}N value in aminoacids has been computed using the steady-state model of Tcherkez
297 2011, modified to account for changes in parametrization (see Material and Methods) and the
298 scenario of Asp consumption in the light suggested by Asp content (Figure 3). The
299 comparison of observed values and modeled values is shown in Figure 4, in which the two
300 scenarios have been represented (Asp production, Asp consumption). There was a rather
301 good agreement between actual and computed δ^{15}N values in Asp, Glu, Gly and Ser but
302 modeled values were less satisfactory in Gln and GABA. In the latter case, this may have
303 come from the fact that GABA and Pro coeluted in GC-C-IRMS profiles, such that the

304 observed $\delta^{15}N$ also integrated the contribution of Pro – not accounted for in the model. In
305 addition, the direct precursor of both GABA and Pro is Glu, which was a bit underestimated
306 indeed.

307 The computation of $\delta^{15}N$ in the dark was not carried out. In fact, dark metabolism does not
308 appear to involve Asn accumulation nor Glu degradation in the dark (Figure 3A) and
309 furthermore, the typical ^{15}N-enrichment in Gln caused by its consumption (that fractionates
310 against ^{15}N) to sustain Asn synthesis in the dark was not observed (Figure 3B). It is rather
311 clear therefore that the dark aminoacid conversion did not involve Asn metabolism under our
312 conditions. However, both Glu and Gln appeared to be ^{15}N-depleted in the dark, suggesting
313 the involvement of depleting reactions. For example, Asp was synthesized in the dark while
314 Asp aminotransferase fractionates against ^{14}N for Asp production (equilibrium isotope effect)
315 (Rishavy *et al.*, 2000) thereby depleting Glu in ^{15}N.

316

317

Discussion

The use of $\delta^{15}N$ to gain information on N metabolism may be complicated by the multiplicity of fractionating reactions and the complexity of metabolic flux patterns (Kolb & Evans, 2003). In an effort to better understand mechanisms that explain leaf $\delta^{15}N$ and ^{15}N-distribution among plant organs and compounds, we carried out isotopic analyses on fractions and individual compounds extracted from rapeseed leaves. We took advantage of GC-C-IRMS for the analysis of aminoacids (Evans, 2001; Molero *et al.*, 2011) and integrated compound-specific $\delta^{15}N$ values into primary nitrogen metabolism with a simple steady-state model.

Nitrate has a major influence on leaf $\delta^{15}N$

Nitrate purified from leaves has been shown to be ^{15}N-enriched compared to organic matter or other compounds (Yoneyama & Tanaka, 1999) and in fact, nitrate was on average enriched by 24‰ compared to total organic matter under our conditions. Such an enrichment is believed to come from the nitrate allocation pattern (for a review, see Tcherkez & Hodges, 2008): while nitrate absorption per se may fractionate against ^{15}N by a few per mil (Mariotti *et al.*, 1982), nitrate molecules left behind after reduction by root metabolism are partly exported to shoots. As nitrate reduction fractionates against ^{15}N, shoot nitrates are naturally ^{15}N-enriched. Furthermore, leaf nitrates are used as the nitrogen source for assimilation and reduction by leaf metabolism and are thus eventually enriched (isotopic Rayleigh effect). At the scale of the leaf life span, it is well possible that the nitrate pool is not in the isotopic steady-state since the input from roots on the one hand and the leaf metabolic demand on the other hand certainly vary with time. Still, the apparent isotope fractionation between nitrate and Glu (or Gln) was roughly of 25.7 (Table 1) – 9.9 (Figure 3) = 15.8‰, that matched the isotope fractionation associated with nitrate reduction (Ledgard *et al.*, 1985). Nitrate also

represented a significant part of leaf soluble nitrogen and the variation of leaf nitrate content is responsible of 70% of $\delta^{15}N$ in the soluble fraction (Figure 2). In other words, nitrate had a visible influence on leaf organic matter and under our conditions, it represented nearly 15% of total leaf nitrogen, that is, had an isotopic impact on $\delta^{15}N$ of total organic matter of $0.15 \times 25.7 = +3.4‰$. This contribution may be of importance in the field since many plants have leaf-accumulated nitrate (Gebauer *et al.*, 1988).

Day and night patterns

There was a neat effect of light/dark conditions on the $\delta^{15}N$ in aminoacids, with a general ^{15}N-enrichment in most of them in the light. As nitrogen assimilation is believed to occur mainly in the light (Reed & Canvin, 1982; Pilgrim *et al.*, 1993; Delhon *et al.*, 1995; Stitt *et al.*, 2002), the ^{15}N-enrichment plausibly reflects that in nitrate. In other words, the input of ^{15}N atoms in the light stemmed from naturally ^{15}N-enriched nitrate while ^{15}N atoms were isotopically diluted by another nitrogen source in the dark, such as the recycling of proteins or other nitrogenous compounds. Protein recycling is of importance for metabolism under stressful conditions (Araújo *et al.*, 2011) and has also been suggested to occur in ordinary metabolism (Bouma *et al.*, 1994; Zerihun *et al.*, 1998). In fact, there is a systematic ^{15}N-depletion (of 3 to 10‰) in proteins compared to aminoacids (Table 1 and Figure 2) and nitrates (soluble fraction) contribute to 60% or less of the isotopic signal in proteins (Figure 2C).

$\delta^{15}N$ in aminoacids reflects leaf iso-fluxes

Here, we have analysed free aminoacids rather than aminoacids obtained from protein hydrolysis. We thus assume that the $\delta^{15}N$ values obtained here reflected dynamics of nitrogen metabolism in leaves. In the light, most aminoacids have a $\delta^{15}N$ value roughly around 8‰,

365 with significant ^{15}N-depletion in Gly, Ser and Asn. In Ser and Gly, this is likely caused by *(i)*
366 the fractionation against ^{15}N associated with NH_2-transfer from Glu to produce Gly
367 (Tcherkez, 2011) and *(ii)* the loss of ^{15}N-enriched ammonia during conversion of Gly into ser
368 by the Gly dehydrogenase complex (for a rather similar reaction Rodriguez *et al.*, 1993)
369 found a fractionation against ^{14}N of 5‰) (Figure 5). Our calculations based on steady-state
370 equations applied to day metabolism and parameterized with such enzymatic isotope effects
371 satisfactorily predict $\delta^{15}N$ in Gly and Ser (Figure 4).

372 Glu and Gln are predicted to be near 7.5 and 16‰, respectively, while observed
373 values are 9.9 and 11.6‰, respectively. Simultaneous underestimation of $\delta^{15}N$ in Glu and
374 overestimation of $\delta^{15}N$ in Gln suggest that the accumulation/export rate of Gln may have
375 been slightly overestimated in the model, thereby 'trapping' ^{15}N-enriched glutamine
376 subsequently not consumed by glutamate synthase. We nevertheless recognize that
377 accumulation/export fluxes are partly uncertain since they cannot be properly obtained from
378 aminoacid content. That is, some data on phloem composition would be necessary to
379 determine the contribution of export to aminoacid metabolism. For example, the lack of
380 change in Glu and Gln content in the light compared to the dark (Figure 3) may cloaks a
381 simultaneous increase of production and export, with no net effect on leaf pools.

382 There was a large isotopic difference between Asp and Asn (Figure 3), suggesting that
383 the apparent isotope effect associated with Asn synthesis from Asp was of ca. 36‰ (see
384 Results). This value is very high and much larger than the experimental one obtained with the
385 enzyme Asn synthetase, that is, 22‰ (Stoker *et al.*, 1996). The present value may be caused
386 by: *(i)* an unusually large isotope fractionation of the rapeseed enzyme compared to other
387 species and *(ii)* the involvement of other reactions that contribute to decrease $\delta^{15}N$ in Asn.
388 While hypothesis *(i)* cannot be ruled out, hypothesis *(ii)* may involve asparaginase (that

389 hydrolyses Asn to Asp plus ammonia) or cyanoalanine hydratase that converts cyanoalanine
390 to Asn). The isotope effects of such enzymes are not well documented but presumably, they
391 both favour ^{14}N (irreversible reactions) and so the action of asparaginase would enrich Asn in
392 ^{15}N. It is therefore more likely that the alternative pathway of Asn production from
393 cyanoalanine was involved and in fact, associated enzymes (cyanoalanine nitrilase) have been
394 found in the close species *Brassica rapa* (Ishikawa *et al.*, 2007). It should be noted that in
395 rapeseed mature leaves, Asn is a minor aminoacid representing less than 5% of total
396 aminoacid content (Tilsner *et al.*, 2005). In addition, transgenic rapeseed plants with bacterial
397 Asn synthetase do not produce more Asn (Seiffert *et al.*, 2004), suggesting that Asn synthesis
398 may involve other enzymes than Asn synthetase in this species.

399 *$\delta^{15}N$ in other metabolites reflects both precursor $\delta^{15}N$ and $^{14}N/^{15}N$ fractionations*

400 The $\delta^{15}N$ value in chlorophyll was relatively close to that in aminoacids (average value) or to
401 the day/night average of Glu. Glu is the precursor of the tetrapyrrolic nucleus via δ-
402 aminolevulinate (Figure 5). Cyclization of two molecules of δ-aminolevulinate into
403 porphobilinogen likely fractionates against ^{15}N and so does porphobilinogen deaminase, that
404 condenses four porphobilinogen molecules to hydroxymethylbilane and four NH_3. To our
405 knowledge, the value of such isotope effects is not known, but the effective fractionation
406 against ^{15}N appeared to be small, probably because intermediates of chlorophyll synthesis did
407 not accumulate. That is, δ-aminolevulinate production would have been a committed step for
408 chlorophyll biosynthesis. DNA showed a clear ^{15}N-depletion compared to aminoacids, and
409 this is consistent with isotope effects associated with bases synthesis (Figure 5). Pyrimidines
410 are formed from Asp and carbamyl-phosphate, the production of which fractionates against
411 ^{15}N by 22‰ (Rishavy *et al.*, 2000). Asp and carbamyl-phosphate form carbamyl-Asp with an
412 isotope fractionation of 1.4‰ (Waldrop *et al.*, 1992). The conversion of carbamyl-Asp to

413 pyrimidine involves several enzymatic isotope fractionations that deplete evolved nucleotides
414 in ^{15}N: dihydro-orotase (11‰, Anderson *et al.*, 2006) and orotate-phosphoribosyltransferase
415 (probably near 25‰, Zhang *et al.*, 2009). Purines are formed via a complex succession of
416 reactions that use Glu, Gln, Asp and Gly as nitrogen sources. Little data is currently available
417 on isotope effects associated with their synthesis. Nevertheless, the combination of enriched
418 (e.g. Gln) and depleted (e.g. Gly) nitrogen sources is so that purines are probably only
419 slightly depleted compared to total organic matter. Taken as a whole, DNA is expected to be
420 ^{15}N-depleted but the magnitude of such a depletion is not easily predictable.

421 Thr is one of the major aminoacids in rapeseed leaves, and it appeared quite ^{15}N-
422 enriched ($\delta^{15}N$ near 17‰ in the light). Thr derives from Asp via Asp-semialdehyde without
423 N-atom transfer or modifications of C-N bonds. Little isotope effect is therefore expected
424 during Thr synthesis. By contrast, Thr is an intermediate of Ile synthesis which involves
425 deamination by Thr dehydratase. The isotope effect associated with this enzyme is not known
426 but it is probably similar to ammonia-lyases that fractionate by 14‰ (Hermes *et al.*, 1985).
427 As a result, Thr is expected to be more ^{15}N-enriched than its precursor Asp.

428 *Perspectives*

429 There are clear $\delta^{15}N$-differences among leaf compounds (see also Werner & Schmidt, 2002)
430 and here, we argue that key metabolic pathways are involved. Among them is
431 photorespiration which is associated with several isotope fractionations (aminotransferases,
432 NH_3 production by glycine dehydrogenase). In addition to depleting Gly and Ser in ^{15}N,
433 photorespiration also enriches ammonia. In the framework of our model, the predicted $\delta^{15}N$
434 in NH_3 is 15 to 19‰, that is, considerably enriched compared to total organic matter.
435 Therefore, ammonia liberated by canopies (for rapeseed canopies, see Nemitz *et al.*, 2000)
436 certainly contributes to enrich atmospheric NH_3 in ^{15}N.

The isotopic difference between compounds is also of considerable importance for isotopic mass-balance. Since Asn has a rather particular (low) $\delta^{15}N$ value and is believed to be involved in phloem transfer (Gaufichon *et al.*, 2010), Asn export from source leaves may contribute to cause a $\delta^{15}N$ difference between source and sink organs. This will be addressed in a subsequent study in which phloem aminoacid composition and $\delta^{15}N$ will be investigated.

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Figure legends

Figure 1. Bidimensional isotopic distribution ($\delta^{13}C$, $\delta^{15}N$) of leaf fractions in *B. napus*: deproteinated soluble fraction (closed discs), heat-precipitated proteins (open discs), DNA (triangles) and chlorophylls (stars). Isotope composition is expressed relative to total organic matter (TOM) so as to account for plant-to-plant isotopic variations, that is, $\Delta\delta^{13}C_{fraction} = \delta^{13}C_{fraction}-\delta^{13}C_{TOM}$ (and the same for ^{15}N). Continuous lines represent envelope curves.

Figure 2. Relationship between the $\delta^{15}N$ of the leaf soluble fraction and the nitrate content expressed as mmol NO_3^- per g dry weight (**A**) or as nitrogen mole fraction denoted as *n* (dimensionless, mol NO_3^- per mol total N) (**B**), and relationship between soluble fraction and proteins (**C**) in *B. napus* leaves. In **A**, the continuous line stands for the hyperbolic trend of the plot. In **B**, the thick line represents the mixing line between average aminoacid fraction (day/night average, +7.45‰) and nitrate (+25.7‰). The thin line is the linear regression (R^2 = 0.70, $P<0.05$) that gives $y = +1.93 + 15.12\,x$. In **C**, the thick line is the 1:1 axis and the thin line represents the linear regression ($R^2 = 0.59$, $P<0.05$) that gives $y = -3.15 + 0.59\,x$.

Figure 3. Content (**A**) and $\delta^{15}N$ values (**B**) in free aminoacids in *B. napus* leaves in the light (open bars) and in the dark (closed bars). Observed values are mean±SD (*n*=5). The nitrogen isotope composition in aminoacids was obtained by GC-C-IRMS. The $\delta^{15}N$ value and the content indicated for Pro is associated with Pro and GABA taken together since they co-eluted under our chromatographic conditions.

Figure 4. Comparison of $\delta^{15}N$ values in main aminoacids (that is, aminoacids for which isotope effects associated with biosynthesis are documented) obtained experimentally in the light (dark grey bars) or from the steady-state model: with Asp production in the light (dark bars, Eqn 1) or Asp consumption (the $\delta^{15}N$ of which is that of Figure 3) in the light (light grey bars, Eqn 2). For further modeling details, see the text (Material and Methods). In this figure, the isotope composition in GABA is assumed to represent that of Pro+GABA (see legend of Figure 3).

Figure 5. Biochemical scheme depicting the metabolic fractionation involved in $\delta^{15}N$ of leaf compounds. The present figure is simplified in that it does not include all metabolic interactions and simply represents the origin of N atoms. Question marks stand for uncertain fractionation values (see the text). The sign of fractionation values is positive when against ^{15}N and negative otherwise. δ-AL, δ-aminolevulinate; Carb-P, carbamyl-phosphate; Carb-Asp, carbamyl-Asp. The present numerical values were previously reviewed in Tcherkez (2011).

611 **Table 1.** The nitrogen isotope composition ($\delta^{15}N$) of leaf fractions in *B. napus* (mean±SD,
612 *n*=18). The large standard deviation values come from substantial $\delta^{15}N$ variations between
613 plants. The $\delta^{15}N$ of ammonium nitrate used in the nutritive solution during growth was
614 +2.7±0.6‰. The weighted average of aminoacids (see Figure 3) in the light and in the dark is
615 also indicated. There is no significant day/night $\delta^{15}N$-difference in other fractions.
616

Leaf fraction	**$\delta^{15}N$ (‰)**
Total organic matter	+1.4±1.6
Soluble fraction	+8.6±3.9
Heat-precipitated proteins	+1.6±2.9
DNA	–4.8±2.0
Chlorophylls	+6.3±3.4
Leaf nitrates	+25.7±1.4
Aminoacids (day/night)	+9.1±2.4/+5.8±2.4

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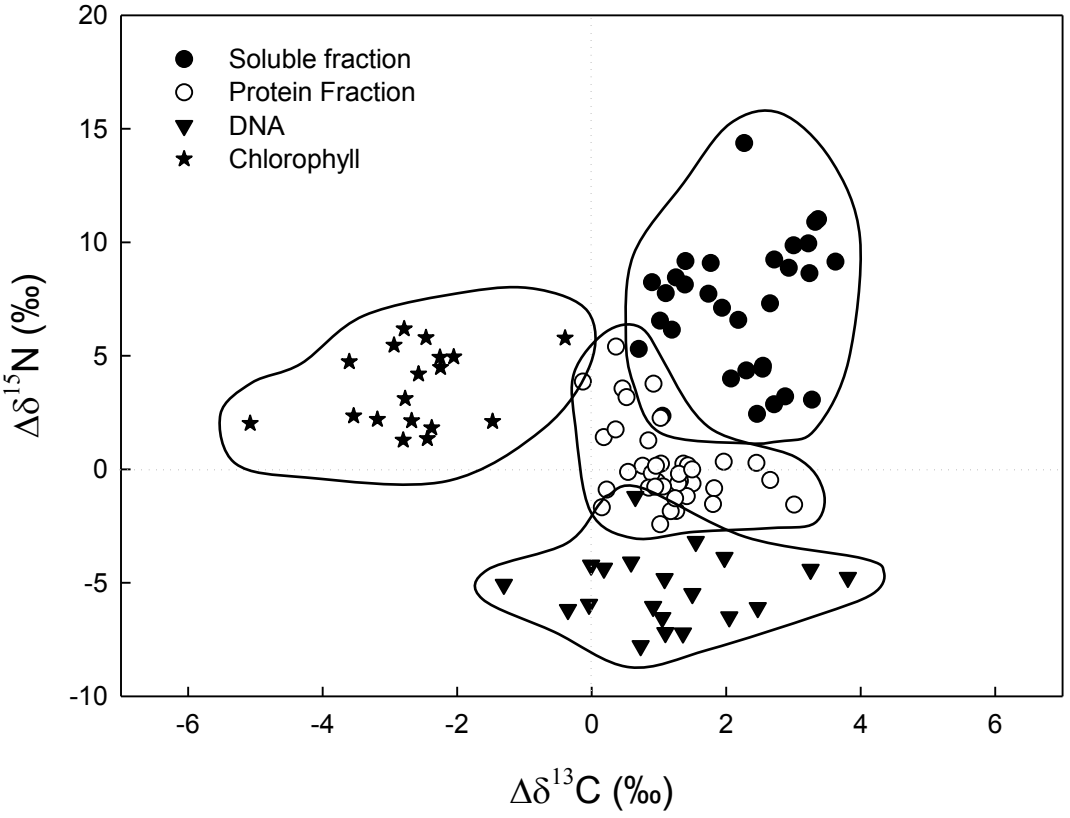

620

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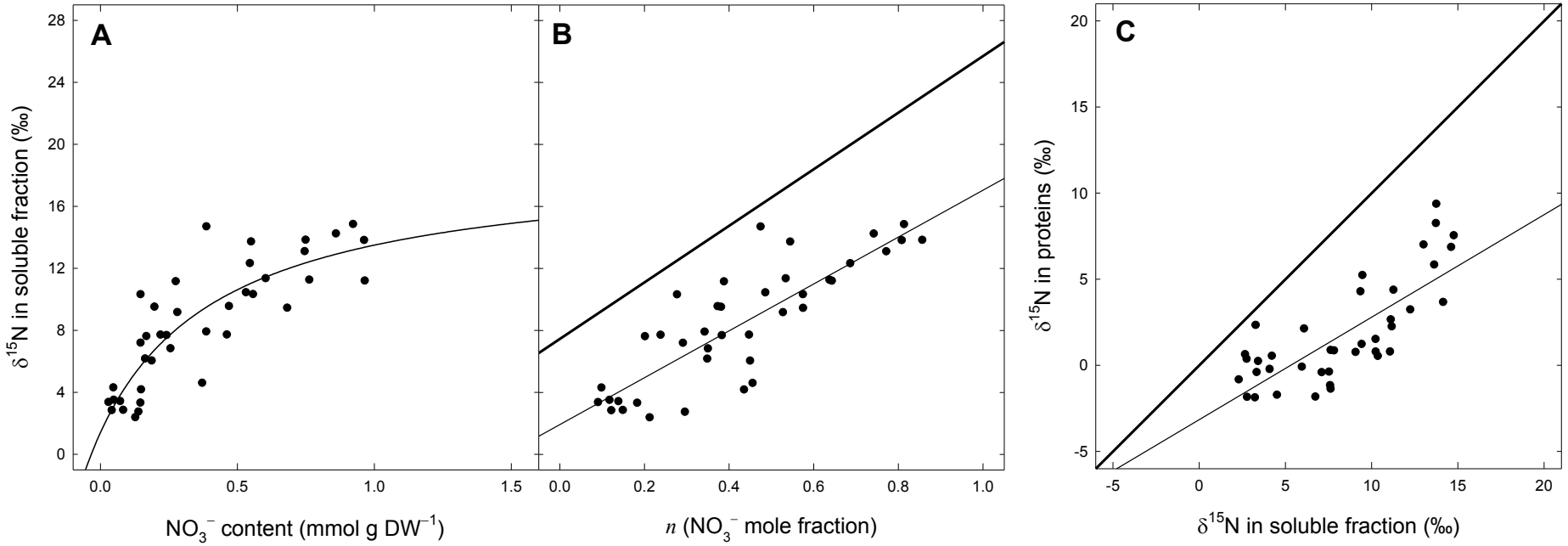

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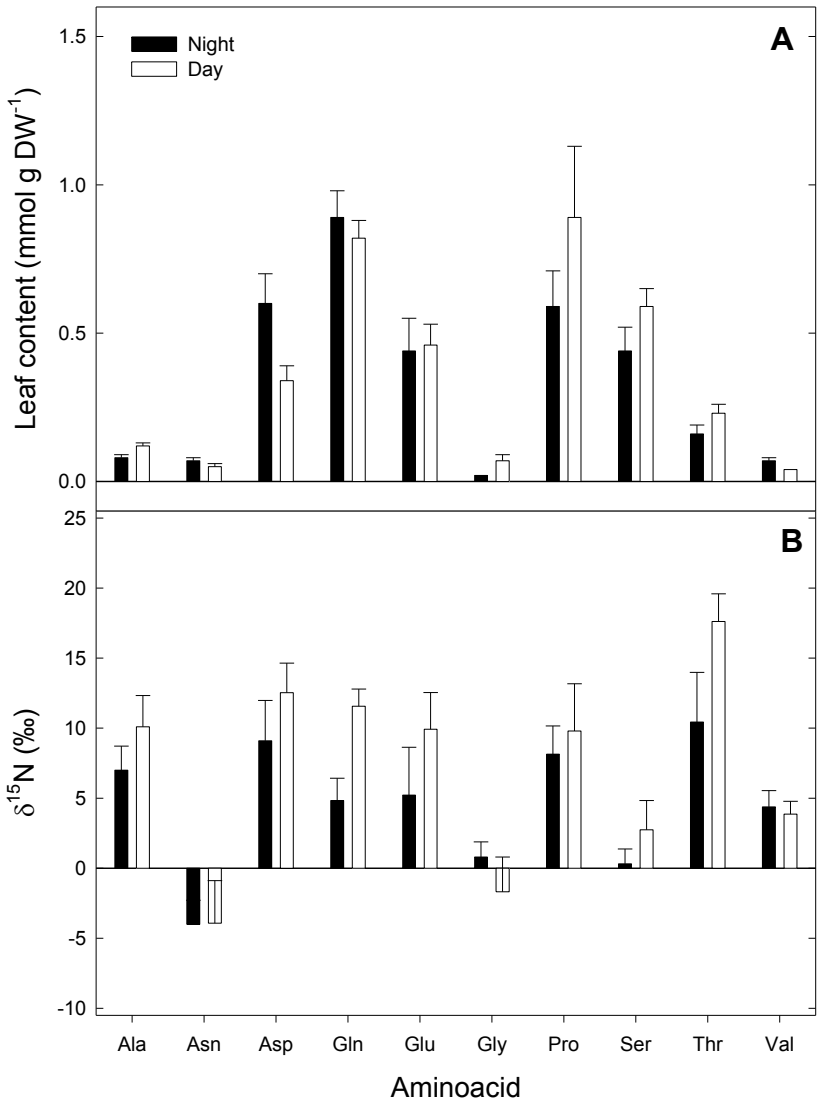

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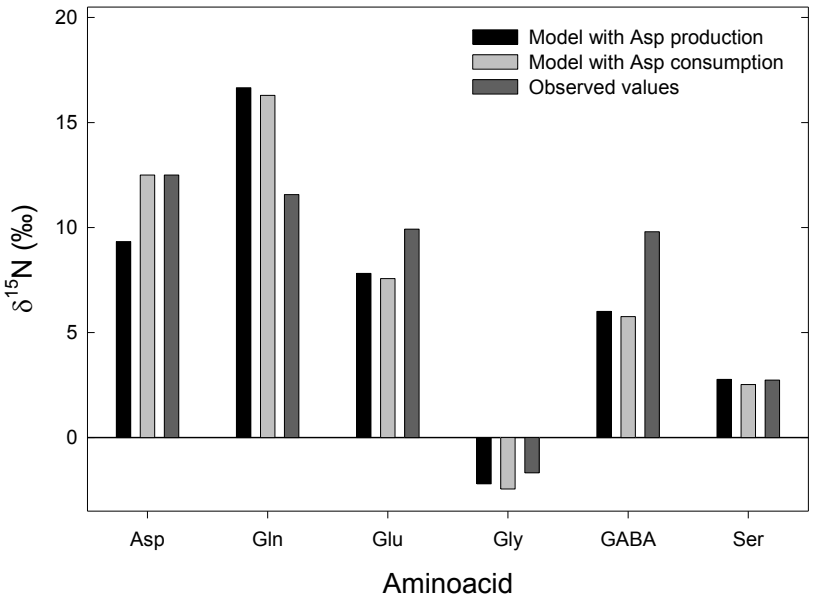

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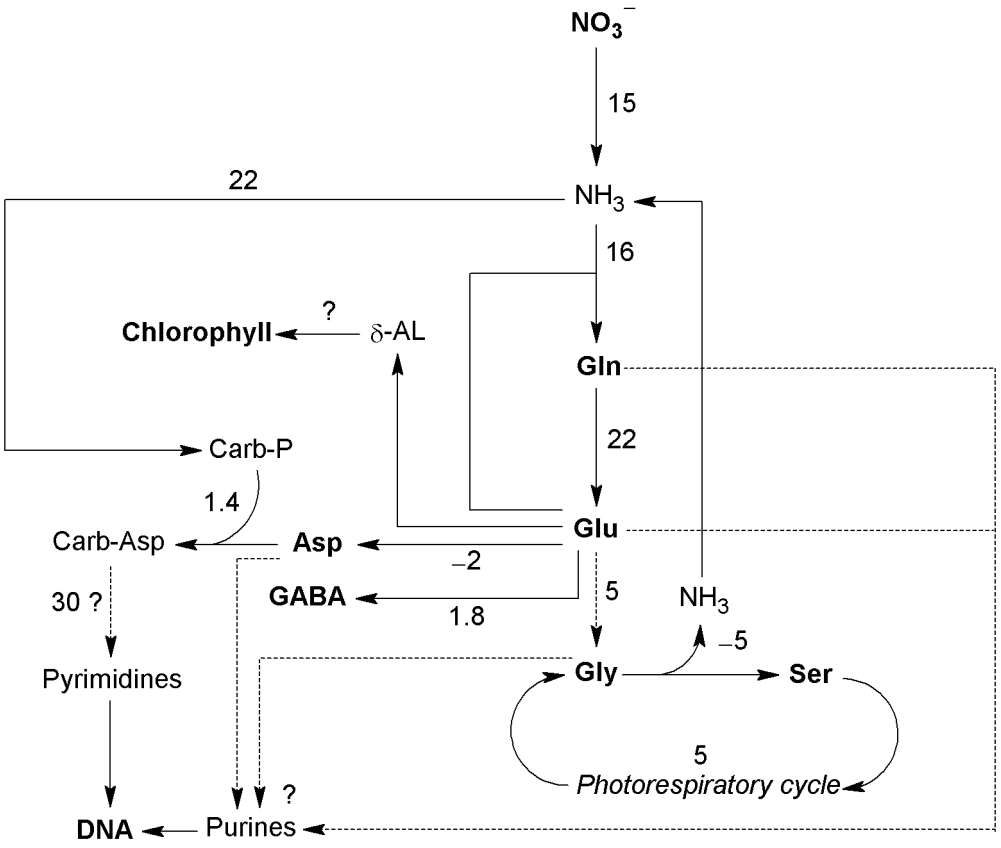

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