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

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

As an aid in clarifying ^{15}N distribution among plant compounds, we took advantage of isotope ratio mass spectrometry (IRMS) techniques to measure the δ^{15}N in free aminoacids and several metabolic fractions from rapeseed leaves (Brassica napus L.) and explored the relationships between them. We show that the nitrate content had a major influence on δ^{15}N in leaf soluble fraction. Known metabolic pathways and fluxes satisfactorily explained the δ^{15}N in metabolites on a steady-state basis in the light. We further show that although Glu and Gln content remained the same in the light and in the dark, their δ^{15}N value was dissimilar, likely because of changes in source N. δ^{15}N in other compounds mostly reflects the influence of precursors and biosynthetic/consumption reactions.
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 $+2.69\pm0.61$. Carbon dioxide in air was at natural $^{13}C$-abundance ($\delta^{13}C = -8.92\pm0.55\%$, 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 Noguès *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, $-4.5\pm0.1\%$ and caffeine IAEA600, $+1\pm0.2\%$, 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, $-26.4\pm0.04\%$). 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
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 NH₄OH and dried with an infrared lamp under a non-oxidative atmosphere (N₂ 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. CO₂ was trapped with liquid N₂. 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.
The $\delta^{15}$N value of aminoacids was corrected for any offset using norleucine as an internal isotopic standard.

**DNA purification**

1 g of leaf fresh material was extracted in a mortar at ambient temperature with 20 mL of extraction buffer (Tris-HCl 200 mmol L$^{-1}$, NaCl 250 mmol L$^{-1}$, EDTA 25 mmol L$^{-1}$, SDS 0.5%, pH 7.5). After centrifugation (15 min, 12,000 g, 5°C), the supernatant was mixed with phenol/chloroform/3-methylbutanol (25/24/1 v/v/v) and agitated to separate the different phases. The aqueous phase was collected and mixed with isopropanol to induce DNA flocculation. After centrifugation (15 min, 12,000 g, 4°C), the supernatant was discarded and the DNA pellet was frozen with liquid nitrogen and lyophilized.

**Modeling**

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

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

Where $\alpha_1^N$ (1.016), $\alpha_1^G$ (1.000), $\alpha_2^N$ (1.022), $\alpha_2^G$ (1.000), $\alpha_6$ (1.015), $\alpha_6^N$ (0.995) are the isotope effects associated with glutamine synthetase (subscript 1), glutamate synthase (subscript 2), nitrate reductase (subscript 5) and glycine decarboxylase (subscript 6). For glutamate synthase and glutamine synthetase, isotope effects at the amido and amino N-atom 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$^{-1}$), $\mu_g$ 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 $\gamma$ is equal to $i+(v_o-\mu_g)/2-t$. $R^*$ is the isotope ratio in utilized leaf nitrate. Parameters denoted as $\tau$ 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 $R_{\text{Asp}}$), we have:

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

Where $r$ is the rate of Asp consumption and $\alpha_8$ 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 $\alpha_1^N$ on the isotope ratio in Glu is extremely small because $t$ is negligible (when $t = 0$, $\alpha_1^N$ 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:
\[ R_{\text{Glu}} = \frac{iR^* / \alpha_z}{\alpha_2^N \tau_{\text{Gln-N}} \left( \frac{\tau_{\text{Glu}}}{\gamma - e} - \frac{\gamma}{\tau_{\text{Gln-G}}} \right) - \frac{v_o - \mu_g}{2 \alpha_6^N \tau_{\text{Gly}}} } \] (3)

And Eqn (2) gives:

\[ R_{\text{Glu}} = \frac{iR^* / \alpha_z + r(\gamma - e)\alpha_2^N \tau_{\text{Gln-N}} \alpha_4 R_{\text{Asp}}}{\alpha_2^N \tau_{\text{Gln-N}} \left( \frac{\tau_{\text{Glu}}}{\gamma - e} - \frac{\gamma}{\tau_{\text{Gln-G}}} \right) - \frac{v_o - \mu_g}{2 \alpha_6^N \tau_{\text{Gly}}} } \] (4)

In the present paper, \( r \) can be roughly estimated with the dark-to-light decrease of Asp content (Figure 3), which gives 0.22 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). The minimal \( i \) value can be obtained from the N content (excluding nitrates) in leaf organic matter, that is, 0.025 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). This value is under-estimated however, simply because leaf-assimilated N is redistributed in other organs. The nitrate reduction rate in rapeseed shoots under ammonium nitrate growth conditions has been shown to be 0.29 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Leleu & Vuylsteker, 2004). In the two scenarios investigated here (Asp production, Asp consumption), the total aminoacid accumulation (sum of Gly, Ser, Glu, Gln, Asp if applicable) matched the total input (nitrate reduction + Asp utilization). To facilitate comparisons, the two scenarios considered here are symmetrical for Asp metabolism: when Asp is assumed to accumulate, the associated rate is fixed at 0.22 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), that is, identical to the rate of Asp remobilization when Asp is assumed to be consumed (see above). Under both scenarios, input and output iso-fluxes of the whole metabolic system were similar (steady-state condition), at 2.9 (Asp production scenario) and 5.2 (Asp consumption scenario) \% \( \mu \text{mol m}^{-2} \text{s}^{-1} \). Note that the iso-flux with Asp consumption was a bit \( ^{15}\text{N} \)-enriched because Asp was more enriched (\( \delta^{15}\text{N} = +12.5\%) \) than nitrogen evolved by nitrate reduction (+9.8\%).
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
substantial enrichment in leaf nitrates (Table 1), the leaf soluble fraction is certainly
influenced by the nitrate content. In fact, there was a positive relationship between $\delta^{15}$N and
leaf nitrate content (Figure 2A). Furthermore, when expressed on a nitrogen mole fraction
basis (fraction of leaf N represented by nitrates), there was a linear relationship between $\delta^{15}$N
and nitrate content (Figure 2B), with a regression coefficient of 0.7. In other words, leaf
nitrates content explained 70% of the $\delta^{15}$N-variation in the soluble fraction. However, the
linear relationship did not coincide with the predicted mixing-line between the two major
soluble N compounds: nitrates (+25.7%) and aminoacids (average $\delta^{15}$N of +7.5‰). That is,
there was a systematic depletion of 5 to 8‰ in the soluble fraction, likely explained by the
contribution of other compounds, such as free aminoacids not analysed here, free nucleotides,
NAD, etc. $\delta^{15}$N in proteins was also related to that in the soluble fraction (Figure 2C),
although with a lower regression coefficient and a slope of nearly 0.6. It remains possible that
nitrates still present in the protein fraction adulterated the $\delta^{15}$N value in proteins. However,
using the nitrate concentration in sample, such a contribution of nitrates was certainly less
than 15%, that is, much less than the slope between nitrate content and proteins ($0.7 \times 0.6 =
0.42$). It is likely therefore that the N-source effect of nitrate $\delta^{15}$N on aminoacids had in turn
an influence on proteins synthesized therefrom.

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

Individual free aminoacids were extracted and analysed by gas chromatography coupled to
isotope ratio mass spectrometry (Figure 3). There were clear differences between aminoacids,
with Thr and Asp being the most enriched of them and Gly and Asn the most depleted
(Figure 3B). In addition, there were day/night differences, Glu, Gln and most aminoacids
being more $^{15}$N-enriched in the light than in the dark. Such a $^{15}$N-enrichment was statistically
significant in Gln only. Except for Asp that decreased in the light, there was little change in
leaf aminoacid content in the light compared to the dark (Figure 3A). Ala, Asn, Gly and Val were aminoacids of minor importance. The $^{15}$N-enrichment in most aminoacids in the light may have come from the $^{15}$N-enrichment in source nitrogen used in biosyntheses or changes in (fractionating) metabolic fluxes causing a net $^{15}$N-enriching effect.

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

**Comparison of modeled vs observed values**

The $\delta^{15}$N value in aminoacids has been computed using the steady-state model of Tcherkez 2011, modified to account for changes in parametrization (see Material and Methods) and the scenario of Asp consumption in the light suggested by Asp content (Figure 3). The comparison of observed values and modeled values is shown in Figure 4, in which the two scenarios have been represented (Asp production, Asp consumption). There was a rather good agreement between actual and computed $\delta^{15}$N values in Asp, Glu, Gly and Ser but modeled values were less satisfactory in Gln and GABA. In the latter case, this may have come from the fact that GABA and Pro coeluted in GC-C-IRMS profiles, such that the
observed $\delta^{15}N$ also integrated the contribution of Pro – not accounted for in the model. In addition, the direct precursor of both GABA and Pro is Glu, which was a bit underestimated indeed.

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

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with significant $^{15}$N-depletion in Gly, Ser and Asn. In Ser and Gly, this is likely caused by (i) the fractionation against $^{15}$N associated with NH$_2$-transfer from Glu to produce Gly (Tcherkez, 2011) and (ii) the loss of $^{15}$N-enriched ammonia during conversion of Gly into ser by the Gly dehydrogenase complex (for a rather similar reaction Rodriguez et al., 1993) found a fractionation against $^{14}$N of 5‰ (Figure 5). Our calculations based on steady-state equations applied to day metabolism and parameterized with such enzymatic isotope effects satisfactorily predict $\delta^{15}$N in Gly and Ser (Figure 4).

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

There was a large isotopic difference between Asp and Asn (Figure 3), suggesting that the apparent isotope effect associated with Asn synthesis from Asp was of ca. 36‰ (see Results). This value is very high and much larger than the experimental one obtained with the enzyme Asn synthetase, that is, 22‰ (Stoker et al., 1996). The present value may be caused by: (i) an unusually large isotope fractionation of the rapeseed enzyme compared to other species and (ii) the involvement of other reactions that contribute to decrease $\delta^{15}$N in Asn. While hypothesis (i) cannot be ruled out, hypothesis (ii) may involve asparaginase (that
hydrolyses Asn to Asp plus ammonia) or cyanoalanine hydratase that converts cyanoalanine to Asn. The isotope effects of such enzymes are not well documented but presumably, they both favour $^{14}$N (irreversible reactions) and so the action of asparaginase would enrich Asn in $^{15}$N. It is therefore more likely that the alternative pathway of Asn production from cyanoalanine was involved and in fact, associated enzymes (cyanoalanine nitrilase) have been found in the close species Brassica rapa (Ishikawa et al., 2007). It should be noted that in rapeseed mature leaves, Asn is a minor aminoacid representing less than 5% of total aminoacid content (Tilsner et al., 2005). In addition, transgenic rapeseed plants with bacterial Asn synthetase do not produce more Asn (Seiffert et al., 2004), suggesting that Asn synthesis may involve other enzymes than Asn synthetase in this species.

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

The $\delta^{15}$N value in chlorophyll was relatively close to that in aminoacids (average value) or to the day/night average of Glu. Glu is the precursor of the tetrapyrrolic nucleus via $\delta$-aminolevulinate (Figure 5). Cyclization of two molecules of $\delta$-aminolevulinate into porphobilinogen likely fractionates against $^{15}$N and so does porphobilinogen deaminase, that condenses four porphobilinogen molecules to hydroxymethylbilane and four NH$_3$. To our knowledge, the value of such isotope effects is not known, but the effective fractionation against $^{15}$N appeared to be small, probably because intermediates of chlorophyll synthesis did not accumulate. That is, $\delta$-aminolevulinate production would have been a committed step for chlorophyll biosynthesis. DNA showed a clear $^{15}$N-depletion compared to aminoacids, and this is consistent with isotope effects associated with bases synthesis (Figure 5). Pyrimidines are formed from Asp and carbamyl-phosphate, the production of which fractionates against $^{15}$N by 22‰ (Rishavy et al., 2000). Asp and carbamyl-phosphate form carbamyl-Asp with an isotope fractionation of 1.4‰ (Waldrop et al., 1992). The conversion of carbamyl-Asp to
pyrimidine involves several enzymatic isotope fractionations that deplete evolved nucleotides in $^{15}\text{N}$: dihydro- orotate (11%, Anderson et al., 2006) and orotate-phosphoribosyltransferase (probably near 25‰, Zhang et al., 2009). Purines are formed via a complex succession of reactions that use Glu, Gln, Asp and Gly as nitrogen sources. Little data is currently available on isotope effects associated with their synthesis. Nevertheless, the combination of enriched (e.g. Gln) and depleted (e.g. Gly) nitrogen sources is so that purines are probably only slightly depleted compared to total organic matter. Taken as a whole, DNA is expected to be $^{15}\text{N}$-depleted but the magnitude of such a depletion is not easily predictable.

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

**Perspectives**

There are clear $\delta^{15}\text{N}$-differences among leaf compounds (see also Werner & Schmidt, 2002) and here, we argue that key metabolic pathways are involved. Among them is photorespiration which is associated with several isotope fractionations (aminotransferases, NH$_3$ production by glycine dehydrogenase). In addition to depleting Gly and Ser in $^{15}\text{N}$, photorespiration also enriches ammonia. In the framework of our model, the predicted $\delta^{15}\text{N}$ in NH$_3$ is 15 to 19‰, that is, considerably enriched compared to total organic matter. Therefore, ammonia liberated by canopies (for rapeseed canopies, see Nemitz et al., 2000) certainly contributes to enrich atmospheric NH$_3$ in $^{15}\text{N}$. 
The isotopic difference between compounds is also of considerable importance for isotopic mass-balance. Since Asn has a rather particular (low) δ¹⁵N value and is believed to be involved in phloem transfer (Gaufichon et al., 2010), Asn export from source leaves may contribute to cause a δ¹⁵N difference between source and sink organs. This will be addressed in a subsequent study in which phloem aminoacid composition and δ¹⁵N will be investigated.

Acknowledgment

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Bibliographie


**Figure legends**

**Figure 1.** Bidimensional isotopic distribution (δ^{13}C, δ^{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, \( \Delta \delta^{13}C_{\text{fraction}} = \delta^{13}C_{\text{fraction}} - \delta^{13}C_{\text{TOM}} \) (and the same for \(^{15}\)N). Continuous lines represent envelope curves.

**Figure 2.** Relationship between the δ^{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 \( y = +1.93 + 15.12x \). 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 \( y = -3.15 + 0.59x \).

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

<table>
<thead>
<tr>
<th>Leaf fraction</th>
<th>$\delta^{15}$N (%o)</th>
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<tr>
<td>Total organic matter</td>
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<tr>
<td>Soluble fraction</td>
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<tr>
<td>Leaf nitrites</td>
<td>+25.7±1.4</td>
</tr>
<tr>
<td>Aminoacids (day/night)</td>
<td>+9.1±2.4/+5.8±2.4</td>
</tr>
</tbody>
</table>
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.
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Figure 3. Content (A) and δ¹⁵N 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*=4). The nitrogen isotope composition in aminoacids was obtained by GC-C-IRMS. The δ¹⁵N value and the content indicated for Pro is associated with Pro and GABA taken together since they co-eluted under our chromatographic conditions.
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