On the relationship between C and N fixation and amino acid synthesis in nodulated alfalfa (*Medicago sativa* L.)

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Abstract

Legumes such as alfalfa (*Medicago sativa* L.) are vital N_2-fixing crops accounting for a global N_2 fixation of ≈35 Mt N y^{-1}. Although enzymatic and molecular mechanisms of nodule N_2 fixation are now well documented, some uncertainty remains as to N_2 fixation is strictly coupled with photosynthetic carbon fixation. That is, the metabolic origin and redistribution of carbon skeletons used to incorporate nitrogen are still rather undefined. Here, we carried out isotopic labelling with both $^{15}N_2$ and ^{13}C-depleted CO_2 on alfalfa plants grown under controlled conditions and took advantage of isotope ratio mass spectrometry to investigate the relationship between carbon and nitrogen turn-over in respired CO_2, total organic matter and amino acids. Our results indicate that CO_2 evolved by respiration had an isotopic composition similar to that in organic matter regardless of the organ considered, suggesting that the turn-over of respiratory pools strictly followed photosynthetic input. However, carbon turn-over was nearly 3 times larger than nitrogen turn-over in total organic matter, suggesting that new organic material synthesised was less N-rich than pre-existing organic material (due to progressive nitrogen elemental dilution) or that N remobilization occurred to sustain growth. This pattern was not consistent with the total commitment into free amino acids where the input of new C and N appeared to be stoichiometric. The labelling pattern in Asn was complex, with contrasted C and N commitments in different organs, suggesting that neosynthesis and redistribution of new Asn molecules required metabolic remobilization. We conclude that the production of new organic material during alfalfa growth depends on both C and N remobilization in different organs. At the plant level, this remobilization is complicated by allocation and metabolism in the different organs.

Introduction

N_2 fixation is one major flux in the biosphere, with nearly 0.15 Gt N y^{-1} fixed by terrestrial agrosystems and natural ecosystems in which N_2 fixation by legumes contribute to nearly 25%. Legumes fix atmospheric N_2 thanks to the symbiosis with bacteria (Rhizobiaceae) located in nodules. Such a nitrogen fixation is fueled by the provision of reduced carbon from the plant. Nodule respiration is essential to provide sufficient ATP and reducing power (necessary for N_2 fixation) and to synthesise carbon skeletons which act as NH_2 acceptors.. Nodule metabolism has been reported to be conditioned to carbon (sugars) provision from leaves (Hardy and Havelka 1976, Vance and Heichel 1991) and the transfer of photosynthates from leaves to nodules is very rapid (Voisin *et al.* 2003), representing up to 50% of total photosynthetic CO_2 fixation (Lluch *et al.* 2002). Other studies (Summerfield *et al.* 1979, Minchin *et al.* 1981) have suggested that 30% of C fixed by the shoot is invested in nodule metabolism, with approximately 60% of this C lost through respiration. This has been reported to represent a loss of 18% of total photosynthetically fixed C (Maxwell *et al.* 1984).

From a metabolic perspective, sucrose plays the role of the main metabolite transported to nodules where it is hydrolysed by sucrose synthase (SuSy), leading to Fru and UDP-Glc. These metabolites enter catabolism thus yielding cellular energy (required by nitrogenase activity) and organic acids (required as C-skeletons for amino acid synthesis) (Marino *et al.* 2006). Within nodules, reduced nitrogen is converted into key amino acids such as Asp and Ala, as well as amines (Asn) and ureides (Waters *et al.* 1998, Allaway *et al*. 2000, Poole & Allaway 2000, Day *et al.* 2001, White *et al.* 2007). In tropical legumes (such as soybean and cowpea), ureides are the major form of N transported (Pate and Atkins 1983) and in temperate legumes (such as alfalfa [*Medicago*] and pea [*Pisum*]) amides (Asn) are the major organic N compounds transported (Groat and Vance 1981, Ta et al. 1988, Temple et al. 1998). Unsurprisingly, amino acids, which are pivotal to nitrogen nutrition at the whole-plant level, have been shown to correlate to sugars (potential carbon source) in different environmental circumstances. Under water deficit, both the sugar and amino acid content (together with N fixation) decreased in nodules (Arrese *et al.* 1999) and under elevated [CO_2], which promotes sugar accumulation, the amino acid content increased (Matt *et al.* 2001, Fischinger and Schulze 2010). That said, the relationship between C and N input and metabolism are complicated by dynamic exchanges between plant organs. First, some amino acids exported by leaves may play a role in sustaining catabolism in nodules (Kohl *et al.* 1988, Udvardi and Day 1997). Second, amino acids such as Glu exported by leaves to nodules may serve as NH_2

donors to yield Ala and Asp through transamination of oxaloacetate and pyruvate, respectively (Lodwig *et al.* 2003, Prell and Poole 2006). In addition to a possible role in down-regulating nitrogen assimilation in nodules, this contribution of above ground organs to nodular nitrogen metabolism may cause a carbon and nitrogen cycle in which C and N atoms may shuttle back and forth between leaves and nodules and favour the remobilization of C and N reserves from leaves. Further, this might lead to a metabolic instability: nitrogen assimilation by nodules sustains leaf nitrogen nutrition and thus photosynthetic capacity and the latter stimulates nodular metabolism, causing a continuous increase in both N_2 and CO_2 fixation. On the contrary, when nitrogen assimilation is limited (during, e.g., drought or other stresses), this impedes photosynthetic capacity and leaf amino acids may sustain nodular metabolism but also down-regulate nitrogen assimilation, thus causing a continuous decrease in both N_2 and CO_2 fixation.

Despite the considerable importance of such a C/N relationship for plant growth, there is relatively little information on means by which carbon and nitrogen fluxes are interconnected. The investigation of C and N fluxes into metabolites and their partitioning among organs requires the use of isotopic tracers. To our knowledge, the simultaneous $^{15}N_2/^{13}CO_2$ labelling with the specific analysis of doubly labelled plant fractions and metabolites has not been carried out yet. Even recently, double labelling on legumes was carried out with ^{15}N-nitrate or ^{15}N-ammonia (Schmitt *et al.* 2013) but not with the natural chemical species N_2. Nuclear Magnetic Resonance (NMR) technology with ^{13}C and ^{15}N detection is typically used to study isotopic labelling of metabolites. However, NMR analyses require large amount of plant material and is not very sensitive (metabolites below 1 mmol L^{-1} are usually poorly resolved). Currently, there is thus intense efforts devoted to the combined use of chromatographic techniques with isotope ratio mass spectrometry (IRMS) as a sensitive and accurate tool to follow labelling in metabolites (see, e.g., McNeill *et al.* 1994, Godin *et al.* 2007). In a previous paper, we have developed a method based on gas chromatography coupled to a combustion interface and IRMS (GC-C-IRMS) to analyse the isotope composition (δ^{13}C and δ^{15}N values) in individual amino acids (Molero *et al.* 2011). Therefore, physiological studies focused on amino acid metabolism may benefit from a sensitive, IRMS-based technique that does not strictly require the use of heavily ^{13}C- or ^{15}N-labelled material

The aim of the present study is to investigate the relationship between carbon and nitrogen turn-over and amino acids synthesis and to elucidate the metabolic origin and the redistribution of carbon skeletons used to incorporate nitrogen in the plant., We conducted

double labelling with isotopically labelled N_2 and CO_2 on alfalfa plants (*Medicago sativa* L.) and took advantage of the GC-C-IRMS technique – in addition to classical elemental analysis (EA)-IRMS. C and N input fluxes were measured and apparent commitment of the isotopic label into respired CO_2, organic matter and amino acids was computed..

Material and methods

Plant material, growth and harvesting

Alfalfa (*Medicago sativa* L. cv Magalí) plants were grown in sand (one plant per pot) and well irrigated twice per week with a full nutrient solution (devoid of N) and once with deionised water. Three different inoculations (one per week) with a suspension of *Rhizobium meliloti* were completed. Plants were grown under controlled conditions in growth chambers (Conviron E15, Controlled Environments Ltd., Winnipeg, Canada) for 90 days (total duration). Plants were supplied with a photosynthetic photon flux density (PPFD) of about 400 µmol m^{-2} s^{-1} during a 16 hours light period. There was a relative humidity of 70-80% and a temperature regime of 20/15ºC (light/dark). Eighty days after sowing, double $^{12}CO_2/^{15}N_2$ labelling was carried out for 10 days and then all the plants were harvested. Plants were dissected into whole flowers, apical and basal shoots (including leaves and stems), primary and lateral roots, and nodules. Samples were immediately frozen in liquid nitrogen and stored at –80ºC until further use.

$^{12}CO_2$ and $^{15}N_2$ labelling

Simultaneous $^{12}CO_2$ and $^{15}N_2$ labelling was conducted at the canopy level within growth cabinets. For CO_2, we took advantage of the typical ^{13}C-depletion in industrial CO_2 as compared with ambient air and thus we conducted a ^{12}C-labelling. CO_2 mole fraction was maintained slightly above (450 µmol mol^{-1}) the ambient level (390 µmol mol^{-1}) with an IRGA analyzer connected to the growth cabinet, so as to avoid external CO_2 contaminations (i.e., retro-diffusion of external ambient CO_2 into the chamber). We mixed industrial CO_2 ($\delta^{13}C$ = –38.2‰; Carburos Metálicos S.A., Barcelona, Spain) with ambient air ($\delta^{13}C$ = –12.5‰) to yield a $\delta^{13}C$ value of –22.6‰ inside the growth cabinet. Control plants (non-labelled) were grown in a second growth cabinet maintained under ambient conditions (390 µmol mol^{-1}, –12.5‰).

For nitrogen, we used 5% $^{15}N_2$ (prepared from 99% $^{15}N_2$, Euriso-top, Saint-Aubin, France). Labelled N_2 was injected to a purpose-built chamber attached to the pots using gas syringes (SGE International Pty Ltd, Australia). Control plants were also injected with N_2 from a bottle with natural isotopic composition of nitrogen ($\delta^{15}N$ = 0‰). In order to have a constant $\delta^{15}N$ value inside the labelling chamber coinciding with the maximum activity of nitrogenase (Steunou *et al*., 2008) it was necessary to undertake two injections every day (for 10 days) at 10:00 am and at 12:00 pm, one and three hours after the onset of the light period, respectively. This lead to an average $\delta^{15}N$ value inside the labelling chamber of +112‰.

Soluble sugars determination

Lyophilized and grounded samples (50 mg) were suspended with a 1 mL of distilled water and centrifuged at 12,000 g for 5 minutes at 5ºC. The supernatant was heated at 100ºC for 3 minutes and afterwards centrifuged at 12,000 g for 5 minutes at 5ºC. After centrifugation, the supernatant containing the soluble fraction was purified with a solid phase extraction column (Oasis MCX 3cc, Waters) to separate sugars from the other soluble compounds. Suc, Glc, Fru and pinitol contents were analyzed using a Waters 600 HPLC (Waters Millipore Corp., Milford, Massachusetts, USA). Samples were eluted from the columns at 85ºC (connected in series Aminex HPX-87P and Aminex HPX-87C, 300 mm x 7.8 mm, BioRad) with water as an eluent at a flow rate of 0.6 mL min^{-1} (total run time of 45 minutes). Sugars were detected with the refraction index (detector Waters 2414). Concentrations in samples were calculated using calibration curves for each sugar checked with an external standard solution (Sigma-Aldrich).

Free amino acids extraction and purification

Frozen samples were ground to a fine powder in liquid N_2 and a sub-sample was lyophilized (ranging from 50 to 200 mg FW). Extraction of the soluble fraction was performed with trifluoracetic acid (TFA) 10% (v/v) at 4ºC using a sonicator. The homogenate was centrifuged at 6,000 g for 15 minutes at 4ºC. The supernatant was collected and purified with Ultrafree-MC 10000 NMWL (Millipore, USA) tubes. It was then centrifuged (13,000 g for 45 minutes at 4ºC). 1 mL of filtered sample was taken and L-norleucine (Sigma-Aldrich) was added as an internal (both isotopic and quantitative) standard. These fractions were vacuum-dried overnight using a Speed-Vac desiccator and stored at –20ºC until further analysis. Samples were then re-suspended in 1 mL HCl 0.1 mol L^{-1} (v/v) and passed through a chromatographic column filled with a cation exchange resin (Dowex 50W X8 H^+, 200-400 mesh size, Sigma-

Aldrich) allowing extraction of acidic, basic and neutral amino acids. Details of resin construction and the solvents gradients have been previously given (Owen *et al.* 1999).

Derivatization, amino acids determination and GC-C-IRMS analysis

The method used for derivatization and GC-C-IRMS isotopic analysis has already been described in details in Molero *et al.* (2011). Briefly, the mixture of amino acids eluted from the column was completely evaporated under heat and dry nitrogen to get crystallized amino acids. Derivatization was performed with N-methyl-N-(*tert*.-butyildimethylsilyl)-trifluoroacetamide (MTBSTFA, Sigma-Aldrich, Schnelldorf, Germany) yielding N(O)-*tert*-butyildimethylsilyl (tBDMSi) derivatives. The derivatized extract was first analysed by GC-MS in order to separate, identify and also quantify amino acids (using calibration curves) to determine amino acids composition in the sample. Then extract were analysed by GC-C-IRMS to measure the isotope composition ($\delta^{13}C$ and $\delta^{15}N$) in individual amino acids. The GC-C-IRMS equipment consisted of a GC6890 (Agilent technologies , Palo Alto, CA, USA) coupled to an IRMS Delta Plus through a combustion interface GC-C-III (Thermofinnigan, Bremen, Germany). A DB-5MS column (30 m × 0.25 mm × 0.25 μm) with He as a carrier gas was used. 1-2 μL of the extract was injected in split mode. Arginine and cysteine could not be resolved because of their degradation during derivatization. Proline and γ-aminobutyric acid (GABA) were successfully resolved in GC-MS analyses but not in GC-C-IRMS analyses and therefore, a common $\delta^{13}C$ and $\delta^{15}N$ value labelled "GABA + Pro" is given in this paper. The $\delta^{13}C$ values given by the GC-C-IRMS equipment had to be corrected to account for the addition of exogenous carbon atoms ($\delta^{13}C \approx -36.4‰$) brought by the derivatization agent. Should this correction not have been carried out, this would not have caused visible changes ($\leq 3\%$ in the proportion of "new" carbon – see below) in the results presented since here, the $\delta^{13}C$ value of the derivatization agent and that of carbon fixed into amino acids during labelling ($\delta^{13}C = -37.3‰$ on average) nearly coincided, thus cancelling out in correction equations.

$\delta^{13}C$ and $\delta^{15}N$ in total organic matter and isotopic values

A sub-sample of frozen material was oven-dried at 60ºC during 48h and was weighed in tin capsules (≈ 1 mg). Samples were analysed by EA-IRMS (Flash 1112, Carbo Erba, Milan, Italy; IRMS Delta C-Conflo III Interface, Thermo-Finnigan, Bremen, Germany). EA-IRMS $\delta^{15}N$ determination used IAEA N_1 and IAEA N_2 (ammonium sulphate) and IAEA NO_3

(potassium nitrate) as reference material to correct any offset. Isotope composition are all reported in per mil (‰) with V-PDB (Vienna Pee Dee Belemnite) and atmospheric N_2 as reference material for C and N, respectively:

$$\delta^{13}\mathrm{C} \text{ or } \delta^{15}\mathrm{N} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \qquad \text{(Eq.1)}$$

where R is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio. The carbon ($\Delta^{13}C$) and nitrogen ($\Delta^{15}N$) isotope discrimination was calculated on the total organic matter (TOM) (or amino acids) basis from the isotope compositions δ_a and δ_p (Farquhar *et al.* 1989) as:

$$\Delta^{13}\mathrm{C} \text{ or } \Delta^{15}\mathrm{N} = \frac{\delta_a - \delta_p}{\delta_p + 1} \qquad \text{(Eq.2)}$$

Where subscripts "*a*" and "*p*" refer to air and TOM/amino acids, respectively. $\Delta^{13}C$ and $\Delta^{15}N$ are used thereafter to compute the percentage in "new" C and N, respectively (see below, section *Calculations*).

$\delta^{13}C$ in respired CO_2

The isotope composition in CO_2 respired in darkness was determined after Nogués *et al.* (2004). Briefly, apical shoots, basal shoots, primary root, lateral root and nodules were placed separately in a respiration chamber connected in parallel to the sample air hose of a Li-6400 (Li-Cor, Lincoln, USA). Primary roots, lateral roots and nodules were cleaned with distilled water and immediately placed over a humid paper inside the chamber. The leaf temperature in the chamber was maintained at 25°C by a water-bath (jacket of the leaf chamber). Inlet air was passed through the chamber at a rate of 1 L min^{-1} and CO_2 respired by the plant was monitored by the Li-6400 so as to determine respiration rates. In order to accumulate CO_2 for $\delta^{13}C$ analyses, the chamber was used as a closed system. It was flushed with CO_2-free air, closed and then respired CO_2 accumulated. According to respiration rates, the time required to reach ≈300 µmol mol^{-1} CO_2 (minimal mole fraction for an isotopic analysis) was calculated. CO_2 samples were collected with a 50 mL syringe (SGE, Kingwood, Australia) and immediately injected into a 10 mL BD vacutainer, being passed through magnesium perchlorate that removed water vapour. To avoid contamination by ambient CO_2 within the syringe and the needle, both were purged with pure N_2 prior to each sampling. Vacutainers were also slightly over-pressurised with pure N_2 to avoid retro-diffusion of ambient CO_2 into

the syringe (Aranjuelo *et al.* 2009). The amount of flowers available was insufficient to accumulate enough CO_2 for isotopic analyses and therefore no CO_2 data are presented for flowers (Figs. 1 and 2).

Calculations

The proportion of 'new' carbon and nitrogen (derived from labelling), denoted here as *x* (in %), was calculated after Nogués *et al.* (2004) as:

$$x = \frac{\delta_{\text{after}} - \delta_{\text{control}}}{\delta_{\text{fixed}} - \delta_{\text{control}}} \times 100 \qquad \text{(Eq.3)}$$

where δ_{control}, δ_{fixed} and δ_{after} are the isotope composition of the fraction of interest (CO_2, TOM or amino acids) of the control (no labelling), of C or N atoms fixed during labeling and of the sample after labeling, respectively. The isotope composition of fixed C or N should account for the natural isotope fractionation and was thus calculated as:

$$\delta_{fixed} = \frac{\delta_{source} - \Delta}{1 + \Delta} \qquad \text{(Eq.4)}$$

where Δ is the fractionation (see above) and δ_{source} is the isotope composition in the source ($\delta^{13}C$ of –22.6‰ in CO_2; $\delta^{15}N$ of +112‰ in N_2). Commitment values, *r*, were calculated as isotopic recovery in the fraction of interest as:

$$r = \frac{x \times c}{\Delta t} \qquad \text{(Eq.5)}$$

where *c* is the content (in µmol g DW^{-1}) and Δt the time frame considered. The mass weighted average was calculated as $r_{mw} = \sum m_i r_i / \sum m_i$ where m_i are dry weight values. The gross carbon fixation was computed from the the sum of the whole isotopic recovery in plant organic matter and respiration loss. This matched the presumed input of carbon based on total leaf surface area (≈254 cm^2 $plant^{-1}$) and average photosynthesis (15 µmol m^{-2} s^{-1}). Similarly, the total N_2 fixation matched the presumed value from nitrogenase activity (≈42 µmol g DW^{-1} h^{-1} in nodules).

Results

Isotopic $^{12}C/^{13}C$ labelling

The isotope composition ($\delta^{13}C$) in total organic matter (TOM) and dark-respired CO_2 is shown in Fig. 1A. It should be noted that no measurement in CO_2 evolved by flowers was possible due to the low flower biomass and thus insufficient CO_2 production to allow an isotopic determination. The isotopic labelling was carried out with ^{13}C-depleted CO_2 (see *Material and methods*) and thus the incorporation of the label (^{12}C) caused a ^{13}C-depletion. The ^{13}C-depletion was very clear in all instances in both CO_2 and TOM. $\delta^{13}C$ values were used to calculate the proportion of "new" carbon atoms (i.e., derived from labelling *via* photosynthesis) (Fig. 1B). Such calculations took into account the isotope fractionation (of a few ‰) because we used industrial CO_2 at near-natural ^{13}C-abundance. The proportion of "new" carbon was always around 80%, except for basal shoots (near 30%) and apical shoots (60%). A substantial difference in C elemental content between shoots and other organs cannot account for this result since %C was always within 37-40%. Therefore, this suggests that carbon fixed by photosynthesis was mainly allocated to sink organs (flowers, roots and nodules) rather than shoots.

When all organs were plotted together, there was a relationship between the proportion of "new" carbon in respired CO_2 and that in TOM, quite close to the 1:1 line (Fig. 2A), suggesting that the metabolic pool feeding respiration followed the carbon input. When compared to glucose (in roots and nodules; Fig. 2A, arrows), the relationship was less satisfactory, with more "new" carbon in glucose than in CO_2. This suggests that glucose was not the direct respiratory substrate or that respiration was fed by a mixture of different sources made of glucose and other less labelled molecules.

Isotopic $^{14}N/^{15}N$ labelling and relationship with carbon

Nitrogen labelling with $^{15}N_2$ caused a clear ^{15}N-enrichment in TOM in all organs. The $\delta^{15}N$ value was used to compute the proportion of "new" nitrogen in TOM (Fig. 2B). This proportion was always less than 30%, with highest values in sink organs (flowers, roots and nodules), as observed above with carbon. Unsurprisingly therefore, there was a close relationship between the proportion on "new" C and N, which follows a slope of around 3.5. That is, the nitrogen turn-over appeared to be about 30% only of the carbon turn-over in all organs.

Whole-plant CO_2 assimilation, respiration and N_2 fixation were calculated from isotopic data and expressed as fluxes of C and N incorporated (µmol plant^{-1} s^{-1}) or turn-over rates (mol mol^{-1} d^{-1} or g g^{-1} d^{-1}) as shown in Table 1. Gross CO_2 assimilation was calculated from the sum of

293 the isotopic incorporation into TOM and into respiratory CO_2. The respiratory loss thus
294 represented 38% of gross carbon fixation. The turn-over rate associated with carbon was very
295 close to the relative growth rate, suggesting that growth was strictly controlled by the net
296 carbon input. By contrast, the nitrogen turn-over rate was significantly lower; accordingly, the
297 rate of the symbiotic N input was about 1.3% only of the net carbon input while the N-to-C
298 ratio of the elemental composition is 5% (i.e., 3.5 times larger). This indicates that plant
299 growth yielded less N-rich tissues or that N remobilization from pre-existing tissues occurred
300 to sustain growth of new plant tissues.

301 ***Apparent commitment of the isotopic label to plant organs***

302 C and N partitioning amongst plant organs was examined using the isotopic labelling in TOM
303 and respiratory CO_2 (Fig. 3). That is, the apparent commitment of fixed CO_2 and N_2 was
304 calculated using the total isotopic abundance and converted into fluxes (µmol $plant^{-1}$ s^{-1} and
305 nmol $plant^{-1}$ s^{-1}). The largest C and N sinks were apical shoots and primary roots. Despite
306 their high metabolic activity, nodules did not represent the major C and N sink, due to their
307 rather small biomass. That said, the apparent allocation of carbon to nodules was about 0.044
308 µmol $plant^{-1}$ s^{-1}, mostly sustaining respiration: the respiratory loss represented 0.039 µmol
309 $plant^{-1}$ s^{-1}, that is, 88% of the C input in the nodules and representing 9% of the gross CO_2
310 fixation (Fig. 3). Nodules were associated with the lowest apparent commitment of ^{15}N (Fig.
311 3) because they did not retain but rather exported fixed ^{15}N to other plant organs. In fact,
312 despite their relatively large N content (elemental N content of 5.9% on average), the nitrogen
313 turn-over rate (calculated using the flux in nmol $plant^{-1}$ s^{-1} and the biomass and the N content)
314 was 0.031 mol mol^{-1} d^{-1} and thus identical to the plant-level value (Table 1).

315 ***Metabolite distribution***

316 Sugars and amino acids were analysed and quantified by HPLC and GC-MS respectively, and
317 their distribution amongst plant organs is shown in Fig. 4 as a metabolomics heat map. A
318 hierarchical clustering was carried out so as to point out characteristic metabolic patterns.
319 This yielded two groups. The first one comprised sugars (sucrose, glucose, fructose), some
320 Asp and Glu-related metabolites (Asp, Asn, Pro and γ-aminobutyrate [GABA]) and Gly and
321 Ala. The second one comprised other amino acids (including Glu and Gln) and pinitol.
322 Several metabolites showed clear shoot/root differences: Suc was less abundant while pinitol,
323 Ser and Thr were more abundant in shoots compared with underground organs. Nodules had a
324 typical metabolic signature, with a depletion in Fru and an enrichment in Ile, Leu and Val.

Isotopic labelling in amino acids

The isotopic labelling in free amino acids was measured using isotope ratio mass spectrometry coupled to gas chromatography (see *Material and methods*). This technique requires a minimal amount of carbon and nitrogen to yield reliable $\delta^{13}C$ and $\delta^{15}N$ signals and thus, only major amino acids were analysed here. Results are shown in Fig. 5A and 5B where values are expressed in µmol g DW^{-1} d^{-1} so as to *(i)* account for the concentration of each amino acid in the organ considered (which varied substantially, Fig. 4) and *(ii)* compare with C and N input rates (Fig. 3). Ala did not represent a large flux and was not ^{15}N-labelled in basal shoots and roots. Quite clearly, the major nitrogen flux appeared to be into Asn regardless of the organ considered (Fig. 5A). Nevertheless, the amount of ^{15}N recovered in organs varied a lot, with the highest value in flowers and the lowest value in nodules. The fact that nodules themselves did not capture a large amount of ^{15}N suggest that most of the ^{15}N fixed was exported to other organs. Ser in apical shoots appeared to have a quite large ^{15}N commitment, of about two third of the value in Asn.

The apparent commitment of isotopic carbon into amino acids showed a similar trend, with Asn being the major carbon sink (Fig. 5B). Quite importantly, the pattern was not similar to that with nitrogen, since the largest commitment values were obtained in nodules, while flowers showed a very low value (as lateral roots). This discrepancy between C and N clearly shows that nitrogen fixation was divorced from the production of carbon skeletons for Asn synthesis.

At the whole-plant scale, the total isotopic N and C commitment into free amino acids was found to be 1.32±0.04 and 3.42±0.11 µmol g DW^{-1} d^{-1} (C-to-N ratio of 2.6). This represented a very small part (a few ‰) of the total N and C incorporation (Table 1). The comparison of N and C commitment in individual amino acids (apart from Asn) and GABA was explored in Fig. 5C, redrawn from data in Figs. 5A and 5B. Most metabolites were above the 1:1 line and followed a regression line (forced through origin) with a slope of 3.77. Some metabolites deviated somewhat from this line: GABA + Pro, which were always more isotopically labelled with carbon than with nitrogen (open circles), and Ser in apical shoots, which incorporated more isotopic nitrogen than carbon (arrow).

Discussion

Despite the importance of N_2-fixing activity in legumes, relatively little is known on the coordination of N and C metabolisms to yields organic material. Here, we investigated the relationship between photosynthetic CO_2 assimilation and symbiotic N_2 fixation using isotopic labelling and examine the correlations between apparent C and N allocation to total organic matter or amino acids.

C and N input fluxes

In the present study, experiments were carried out on plants that experienced defoliation (clipping) and then regrowth (shoot removal 20 days before labelling, see *Material and methods*). Under such circumstances, it is believed that remobilization of reserves may be of importance to sustain growth. Nevertheless, isotopic labelling with $^{12}CO_2$ has shown that shoot growth and organic matter production after clipping relied on photosynthetic C fixation rather than remobilization (Avice *et al.* 1996). Here, we found that the carbon turn-over rate computed from fixation of isotopic carbon was similar to relative growth rate (Table 1), showing that the photosynthetic input was the main carbon source sustaining plant growth. We nevertheless recognize that our isotopic mass balance (total isotopic C fixation) was computed from the isotope composition in organic matter and evolved CO_2 with no consideration to other compartments. However, up to 20% of carbon fixed by photosynthesis after clipping has been found to be allocated to soil microbial biomass and soil-evolved CO_2 (Schmitt *et al.* 2013). In other words, our value of gross C fixation might be slightly under-estimated. Correcting for this effect would increase our carbon turn-over rate to around 0.121 g g^{-1} d^{-1}, which is satisfactorily close to the relative growth rate. By contrast, the nitrogen input rate and turn-over were both much lower than for carbon, suggesting that the net fixation of isotopic N_2 did not match all growth requirements or that new organic matter synthesised during the experiment was much less N-rich (lower elemental N content) than whole-plant total matter. An explanation may lie in either or both of the following hypotheses: *(i)* quantitative studies established that the average %N decreases during growth due to the increase in cellulosic and lignified N-poor tissues (such as stems) (Lemaire *et al.* 1992); *(ii)* shoot growth following clipping remobilises N stored in roots (Schmitt *et al.* 2013). We also recognize that one part of fixed nitrogen may have been lost to soil microbial biomass (e.g. through exudation). Should that be the case, this would have caused an under-estimation of the N input flux in Table 1; however, the correction for this effect is unlikely to exceed 20%, giving, at most, 0.039 g g^{-1} d^{-1}.

C and N partitioning

Fixed carbon was allocated to all organs since isotopic labelling was visible in all plant parts (Fig. 1B). That said, the proportion of "new" carbon in total organic matter was strikingly different between organs, with the lowest value in basal shoots. This effect may come from the nature of the basal shoot itself, which is made of slow growing tissues (stem). Effectively, total carbon in basal shoots seemed to be divorced from metabolically active pools since CO_2 evolved by respiration was much more isotopically labelled (Fig. 2A). The apparent commitment of carbon, computed from the recovery of the isotopic label in organs, also showed a much lower amount of carbon in basal shoots compared to apical shoots (Fig. 3). The proportion of "new" carbon in apical shoots was around 60% only (Fig. 1B) probably due to the substantial loss caused by respiration (as in Avice *et al.* 1996).

Respiration in nodules represented 0.039 µmol $plant^{-1}$ s^{-1}, nearly 88% of the apparent carbon flux (Fig. 3) and about 12.3 times the rate of N_2 fixation (Table 1). In other words, there was an apparent respiratory cost of N_2 fixation of 12.3 mol CO_2 mol^{-1} N, which agrees with other studies that took advantage of gas exchange (Heytler *et al.* 1985). It should be noted that about 5% of fixed nitrogen was recovered in nodule organic matter, demonstrating that 95% was exported to other organs (Fig. 3). Generally, there was a good correlation (r^2 = 0.92) between the apparent commitment of carbon and nitrogen in plant organs (Fig. 6A, redrawn from Fig. 3) and similarly, between the proportion of "new" C and "new" N in organic matter (Fig. 2B). This shows that there was a tight coupling between carbon and nitrogen partitioning (C and N remobilization matched), regardless of the organ considered.

Amino acid metabolism

The metabolic spectrum changed visibly between organs (Fig. 4) and the dynamics of metabolism among amino acids may be anticipated upon isotopic labelling. In particular, there was a tendency for organs enriched in amino acids to be relatively depleted in sugars (Fig. 6B, redrawn from Fig. 4 source data). This was visible in nodules and flowers, which were both depleted in Glc and Fru and contained high amounts in Asn (Fig. 4). However, the isotopic labelling in flowers and nodules was opposite (Fig. 5A, 5B), with a large ^{15}N commitment into Asn in flowers and a large ^{12}C commitment into Asn in nodules. This discrepancy likely came from the different allocation patterns of C and N, which, in this case, disadvantaged ^{15}N commitment in nitrogen source organs (nodules). The export of ^{15}N from

nodules was high and thus remaining ^{15}N-Asn left behind within nodules were not abundant. Additionally flowers tend to accumulate Asn with the highest concentration amongst plant organs. This could explain the larger ^{15}N commitment into Asn in flowers in comparison with nodules. High values of C commitment into amino acids in nodules suggest elevated metabolic carbon turn-over in these organs. This is evidenced by the high isotopic labelling in respiratory CO_2 and thus presumably, in carbon skeletons sustaining nitrogen assimilation. This was also observed in primary roots and apical shoots, in which respiratory CO_2 was also the highly labelled (Fig. 2A).

Apart from Asn, there was a relationship between C and N commitment into amino acids, with a coefficient of about 3.8 between C and N (Fig. 5C); this matched the concentration-weighted C-to-N ratio in detected amino acids (excluding Asn) of 4.0. That is, the input of "new" carbon and nitrogen into amino acid production appeared to be stoichiometric. Consequently, on average, the remobilization of C for biosynthesis matched that of N, as for total organic matter (see above). That said, Ser appeared to be more ^{15}N-labelled in apical shoots (Fig. 5C, arrow) possibly due to its involvement in photorespiration (rapid incorporation of ^{15}N into photorespiratory intermediates). GABA + Pro appeared to be more ^{12}C-labelled in all organs (Fig. 5C, open symbols). It is likely that those amino acids may play a role of carbon transfer from leaves to sink organs (Molero *et al.*, 2011).

Plant-level metabolic exchanges and allocation

Photosynthates that have incorporated isotopic carbon and amino acids that have incorporated isotopic nitrogen are distributed from source to sink organs but can then be converted to other metabolites that are exchanged and may return back to source organs. In that regard, Fig. 3 is not fully representative because it does not account for metabolic nitrogen recycling from leaves to nodules or carbon recycling from nodules to leaves: for example, some amino acids synthesised in leaves may be exported to nodules (Lodwig *et al.* 2003) and amino acids have been suggested to be used as a carbon source by nodules (Udvardi *et al.* 1988, McRae *et al.* 1989, Herrada *et al.* 1989, Prell and Poole 2006). Sucrose exported from leaves is believed to be cleaved within nodules by sucrose synthase (SuSy) and to fuel respiration, thereby yielding carbon skeletons for nitrogen assimilation in nodules (see, e.g., Arrese *et al.* 1999, Gordon *et al.* 1999). Therefore, the isotopic signature in leaves resulted from both photosynthates themselves and the net exchange of metabolites with nodules. The isotopic pattern apparent in Fig. 5B thus represent the net partitioning commitment in amino acids.

The total amount of C committed to free amino acids (mass-weighted sum of bars in Fig. 5B) represented 15.0 µmol g DW^{-1} d^{-1} – a relatively low proportion of total net fixed carbon (Table 1), of around 1%. Total N committed to free amino acids (mass-weighted sum of bars in Fig. 5A) represented 6.4 µmol g DW^{-1} s^{-1} – i.e., 27% of the total N input. Of course, such values are probably slightly underestimated because minor amino acids were not analysed due to the detectability threshold associated with isotope ratio mass spectrometry. That said, the C-to-N ratio is 15/6.4 = 2.34, reasonably close to the mass-weighted C and N molar content in free amino acids (2.91). At the whole plant level, the allocation of C and N into free amino acids thus followed the C:N stoichiometry, as observed in individual organs (Fig. 5C). This was achieved concurrently with CO_2 loss by respiration (Table 1), the remaining carbon fraction being allocated to other compounds such as sugars (sucrose and starch) and proteins.

Perspectives

Taken as a whole, the present study shows that under our conditions (regrowth after clipping), there was an imbalance between N_2 and CO_2 fixation, suggesting that nitrogen remobilization occurred at the whole-plant scale to yield organic matter. This imbalance was not visible in free amino acids and therefore, the remobilization of nitrogen was probably substantial during the biosynthesis of other nitrogenous fractions such as chlorophylls, nucleic acids and proteins – or alternatively, new tissues produced were less N-rich than pre-existing tissues. Future investigations are thus warranted to disentangle nitrogen fluxes in various N-containing components. We also recognize that environmental conditions are likely to influence the labelling pattern described here. For example, strong metabolic effects have been described upon water restriction, with enhancement of malate, Pro and GABA metabolism (Aranjuelo *et al.* 2013). Tracing N and C fluxes using isotopes should provide further pieces of information, such as the involvement of N-remobilization in Pro biosynthesis.

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609 **Table 1**. Relative growth rate, turn-over rate and elemental composition. “a” and “b” stand for
610 statistical classes ($p < 0.05$).
611

Parameter	Units and value
Rates	g g^{-1} d^{-1}
Relative growth rate	0.119 ± 0.024^{a}
Carbon turn-over rate	0.097 ± 0.011^{a}
Nitrogen turn-over rate	0.031 ± 0.004^{b}
C and N input	µmol C $plant^{-1}$ s^{-1} / mmol C g DW^{-1} d^{-1}
Photosynthetic carbon input	
….gross fixation	0.427 ± 0.010 / 3.18 ± 0.07
….net fixation	0.264 ± 0.009 / 1.96 ± 0.07
	nmol N $plant^{-1}$ s^{-1} / mmol N g DW^{-1} d^{-1}
Symbiotic nitrogen input (gross fixation)	3.16 ± 0.63 / 0.024 ± 0.004
Plant-average elemental composition	%
Elemental C content	39.7 ± 0.2
Elemental N content	1.99 ± 0.21

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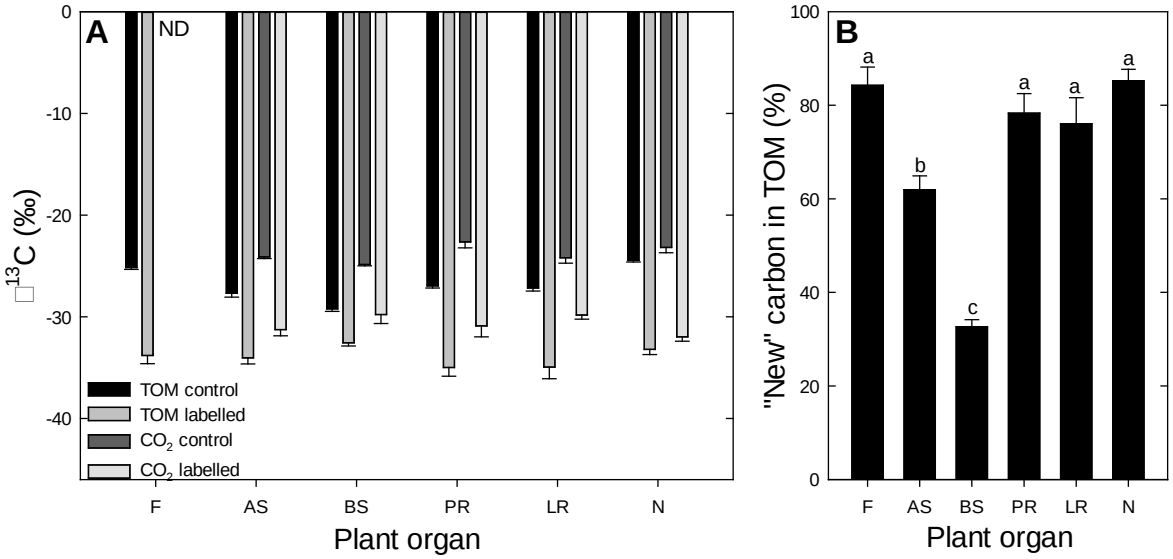

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615 **Figure 1**. ^{12}C-labelling in alfalfa: δ^{13}C values (in ‰) of total organic matter (TOM) and
616 respired CO_2 (**A**) and calculated proportion (in %) of "new" carbon (i.e., C atoms from
617 labelling) in TOM (**B**) (F, flowers; AS, apical shoot; BS, basal shoots; PR, primary roots; LR,
618 lateral roots; N, nodules). ND, not determined (not enough CO_2 evolution for isotopic
619 measurements in flowers). "a", "b" and "c" stand for statistical classes ($p < 0.05$).
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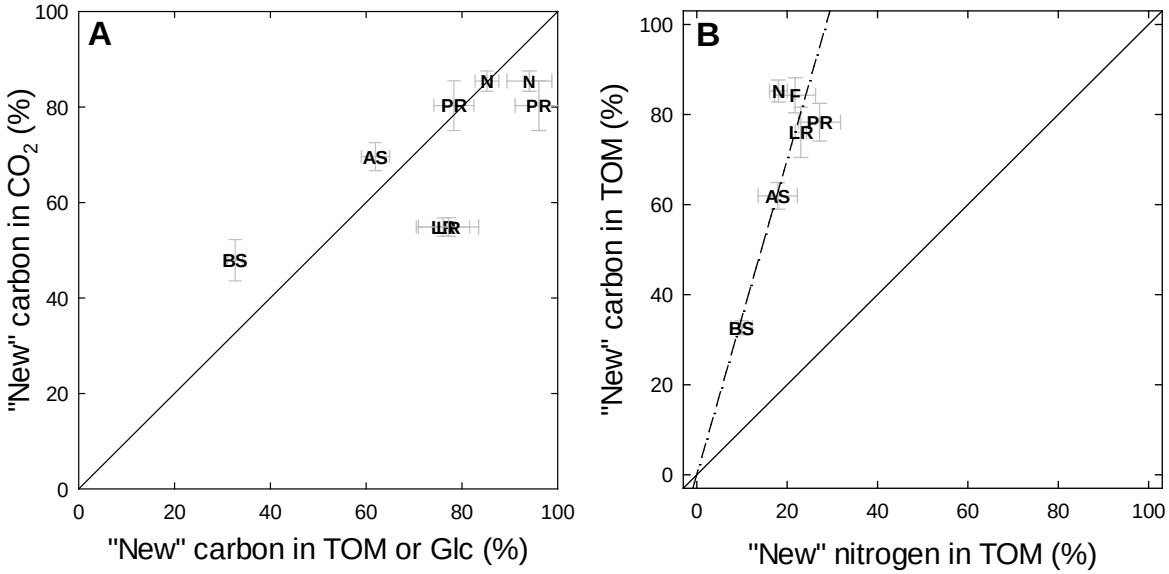

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623 **Figure 2**. Partitioning of "new" C and N atoms (i.e., from labelling) in alfalfa: **A**, relationship
624 between C allocation to total organic matter (TOM) or glucose (Glc) and respired CO_2; **B**,
625 relationship between C and N allocation to total organic matter (TOM). In both panels, the
626 continuous line stands for the 1:1 relationship. In **A**, arrows indicate data for Glc; data without
627 arrow are associated with TOM. In **B**, the broken line represents the linear fit (forced intercept
628 at 0): $y = 3.49\ x$ ($r^2 = 0.57$, regression significant, $p < 0.06$). Note that in **A**, there is no data on
629 flowers, since it was not possible to collect enough CO_2 for isotopic measurements.

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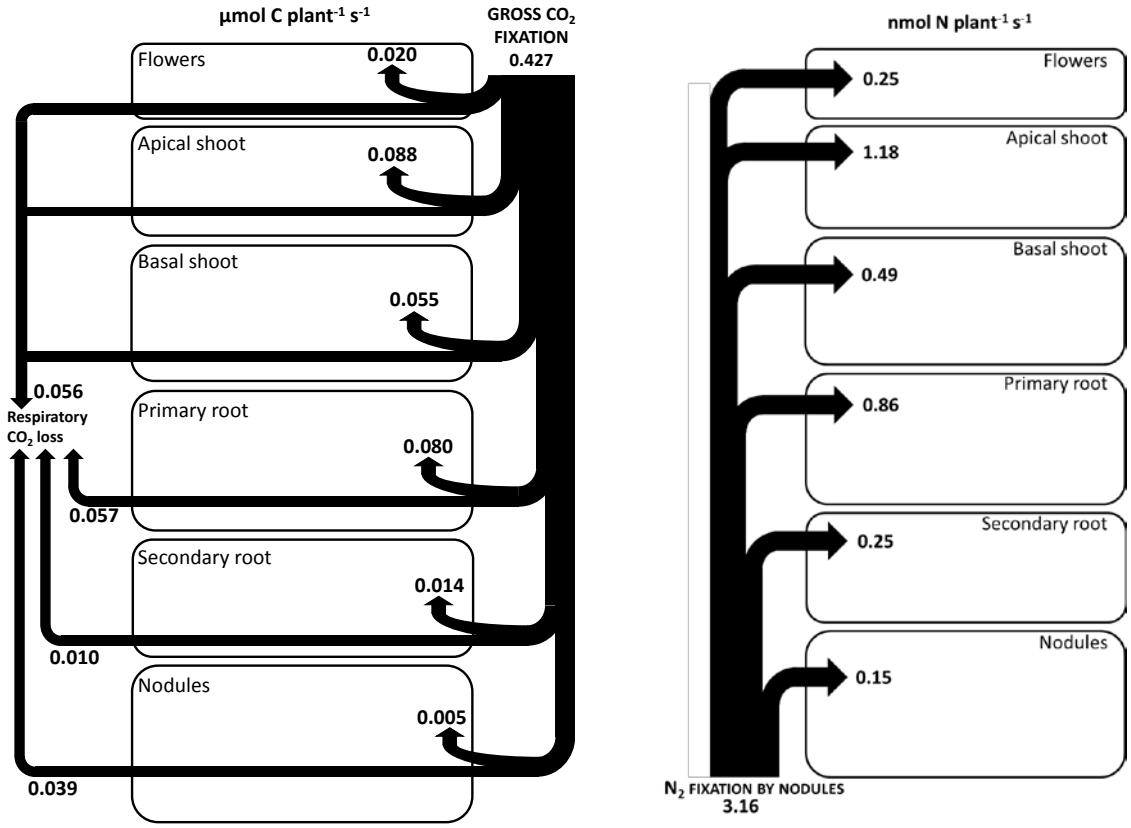

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633 **Figure 3**. Apparent commitment of fixed carbon (left) and nitrogen (right) to organic C and
634 respiratory CO_2 and total N in plant organs, obtained from ^{12}C and ^{15}N labelling data. In all
635 instances, SE are all less than 15%. This figure does not include C and N losses than cannot
636 be accounted for in the present study (volatile compounds, root exudation, etc.).
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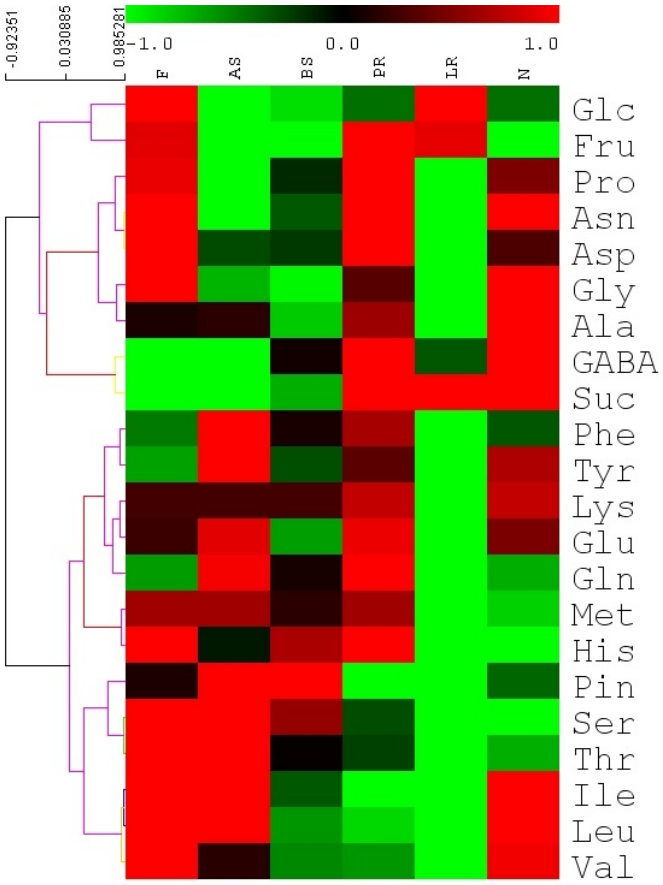

Figure 4. Metabolomic heat map of most abundant metabolites in different organs in alfalfa (F, flowers; AS, apical shoot; BS, basal shoots; PR, primary roots; LR, lateral roots; N, nodules). In each cell, the colour indicate the relative content (green, depleted; red, enriched). The hierarchical clustering is shown on the left (colours indicate robustness of branches as shown). Non-standard abbreviations: GABA, γ-aminobutyric acid; Pin, pinitol. Each column is the mean of three samples.

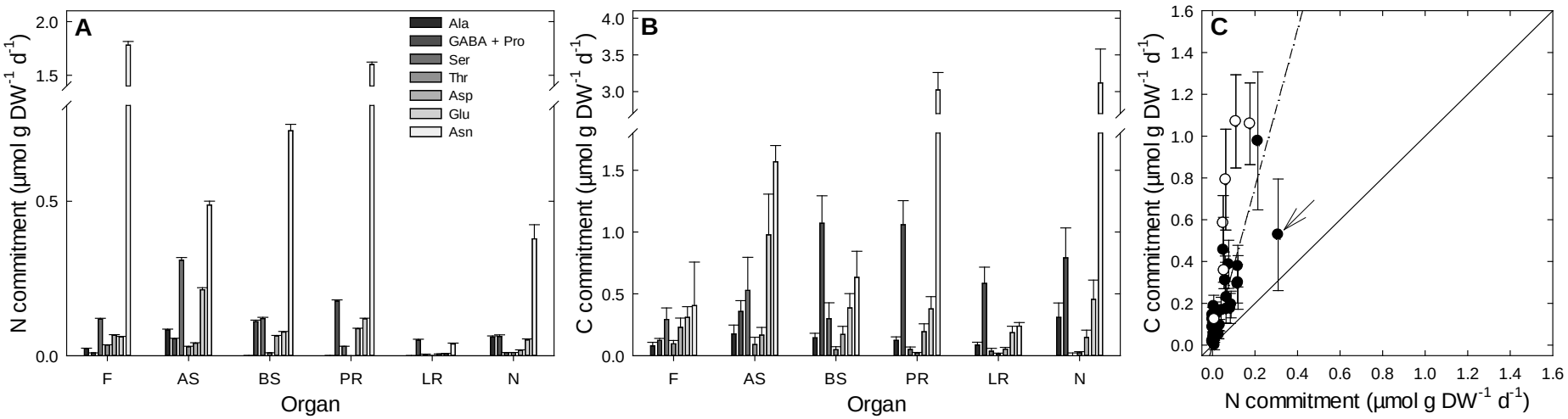

Figure 5. Isotopic labelling in major amino acids and γ-aminobutyrate (GABA) expressed as apparent commitment values (in µmol of "new" N [**A**] or N [**B**] per g DW per day) in each organ. **C**, relationship between C and N commitment (excluding Asn). Continuous line, 1:1 line. Broken line, linear regression through origin ($y = 3.77\ x$, $r^2 = 0.40$, regression significant, $p < 0.05$). Arrow: Ser in apical shoots. Open symbols, GABA + Pro; closed symbols, other amino acids. Note that GABA and Pro could not be chromatographically separated and the isotopic analysis yielded a value for the mixture of both thus here, a common value labelled "GABA + Pro" is indicated.

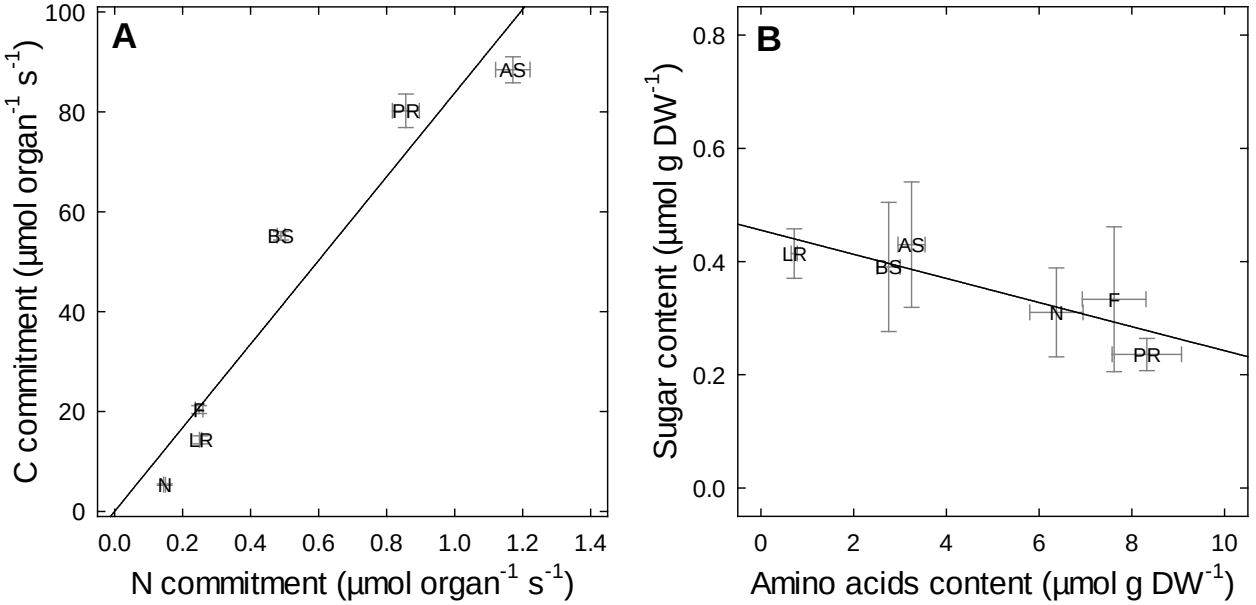

Figure 6. Relationship between isotopic C and N commitment in total organic matter in plant organs (**A**, redrawn from source data of Fig. 3) and between total sugar content and total amino acids content (**B**). Each point corresponds to an individual organ (legend as in Fig. 1). In both **A** and **B**, linear regression are significant ($p < 0.05$) with r^2 values of 0.92 and 0.77, respectively. In **A**, the regression is forced through the origin and the slope obtained was 84±6 (95% confidence interval).