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Original Article

Experimental evidence for diel $\delta^{15}N$-patterns in different tissues, xylem and phloem saps of castor bean (*Ricinus communis* L.)

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ABSTRACT

Nitrogen isotope signatures in plants might give insights in the metabolism and allocation of nitrogen. To obtain a deeper understanding of the modifications of the nitrogen isotope signatures, we determined $\delta^{15}N$ in transport saps and in different fractions of leaves, axes and roots during a diel course along the plant axis. The most significant diel variations were observed in xylem and phloem saps where $\delta^{15}N$ was significantly higher during the day compared with during the night. However in xylem saps, this was observed only in the canopy, but not at the hypocotyl positions. In the canopy, $\delta^{15}N$ was correlated fairly well between phloem and xylem saps. These variations in $\delta^{15}N$ in transport saps can be attributed to nitrate reduction in leaves during the photoperiod as well as to ^{15}N-enriched glutamine acting as transport form of N. $\delta^{15}N$ of the water soluble fraction of roots and leaves partially affected $\delta^{15}N$ of phloem and xylems saps. $\delta^{15}N$ patterns are likely the result of a complex set of interactions and N-fluxes between plant organs. Furthermore, the natural nitrogen isotope abundance in plant tissue is not constant during the diel course – a fact that needs to be taken into account when sampling for isotopic studies.

Key-words: day/night cycle; isotope fractionation; nitrogen; transport.

INTRODUCTION

Nitrogen isotopes are well-recognized tools in plant physiology and eco(physio)logy (Högberg 1997; Robinson 2001; Dawson *et al*. 2002). The natural nitrogen isotope composition ($\delta^{15}N$) is now being widely used in research on N cycling in organisms and ecosystems. ^{15}N natural abundances are used in fundamentally different ways, from traditional ^{15}N tracers by integrating N cycle processes via N isotope fractionations and the mixing of various N-containing pools (Robinson 2001). Nitrogen isotope composition of plant material is determined by the isotope ratio of the external nitrogen source (nitrate, ammonium, amino acids and/or N_2) and physiological mechanisms within the plant like assimilation events, loss of nitrogen, resorption and reallocation of nitrogen (Högberg 1997; Robinson, Handley & Scrimgeour 1998; Comstock 2001; Evans 2001; Robinson 2001; Dawson *et al*. 2002; Werner & Schmidt 2002; Craine *et al*. 2009). The bulk nitrogen pool of plant organs contains multiple N species such as inorganic nitrogen, amino acids, proteins and chlorophylls. Variations in $\delta^{15}N$ can thus also be attributed to different mixing ratios of different N species, each of which could potentially have a distinct $\delta^{15}N$ (Werner & Schmidt 2002; Tcherkez 2011; Gauthier *et al*. 2012). Consequently, there is substantial variation in $\delta^{15}N$ values between ecosystems, plant species, plant individuals or plant parts and biochemical fractions. Handley *et al*. (1999) and Craine *et al*. (2009) reported that foliar $\delta^{15}N$ increased with decreasing mean annual precipitation and with increasing mean annual temperature. The variation range in plant $\delta^{15}N$ is generally –10 to +10‰ (Evans 2001) with quite large differences between plants with nitrate as the sole nitrogen source (Högberg 1997). It is believed that nitrate availability contributes to this variability, plant cultivated under low nitrate concentration being less depleted in ^{15}N than those cultivated under higher nitrate supply (Evans 2001). In case of low nitrate concentration in the soil, the efflux of (^{15}N-enriched) nitrate from the root to the soil is limited, and as a result, all nitrate taken up will be assimilated. Additionally, at higher nitrate supply, more nitrate is transported to the shoots for assimilation (Peuke *et al*. 1996), which may affect the $\delta^{15}N$ of the shoots. Furthermore, there are differences in $\delta^{15}N$ between plant organs, reported both in the lab and in the field (Högberg 1997), with shoots being generally ^{15}N-enriched compared with roots (Yoneyama *et al*. 1997; Peuke, Gessler & Rennenberg 2006). It is believed that this difference in $\delta^{15}N$ value is caused by the isotope fractionation against ^{15}N during nitrate reduction: nitrate molecules left behind after reduction in roots are ^{15}N-enriched and translocated to shoots (for a review, see Tcherkez & Hodges 2008). As a matter of fact, the allocation of nitrogenous compounds of contrasted $\delta^{15}N$ is thus certainly the cornerstone causing differences in $\delta^{15}N$ between plant parts.

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Quite generally, there are considerable disparities among plant metabolites: nitrate is typically ^{15}N-enriched while proteins, chlorophylls or alkaloids are ^{15}N-depleted (for a review, see Werner & Schmidt 2002). This is caused by isotope fractionation during plant nitrogen metabolism. Virtually all enzymatic steps that break or make C-N, O-N or N-H bonds fractionate between nitrogen isotopes (Werner & Schmidt 2002) and so is the case of key enzymes involved in plant primary nitrogen metabolism (for a specific review, see Tcherkez 2011). As a consequence, primary nitrogen assimilates like amino acids are believed to have contrasted $\delta^{15}N$ values (Hayes 2001; Tcherkez & Hodges 2008; Gauthier *et al*. 2012). Under the assumption that nitrogenous compounds exchanged between plant parts via xylem and phloem path have a $\delta^{15}N$ value different from that of total organic matter, this may contribute to isotopic differences between organs (Comstock 2001).

However, little is known about the $\delta^{15}N$ value of phloem metabolites in the phloem. The small amount of available data indicate little difference in $\delta^{15}N$ between total N in leaves and total phloem N (Yoneyama *et al*. 1997). The comparison of plant parasites that rely on phloem N from hosts showed no difference between host and parasite $\delta^{15}N$ (Tennakoon, Chak & Bolin 2011), suggesting that phloem nitrogen might be on average isotopically similar to primary nitrogen assimilates. Nevertheless, leaf nitrogen metabolism follows a diel rhythm so that N reduction and assimilation mainly occurs in the light and, therefore, the composition and $\delta^{15}N$ in phloem may change along the day/night cycle. In fact, phloem metabolic composition is believed to follow a diurnal course, with less sugars and amino acids at night (Sharkey & Pate 1976; Peuke *et al*. 2001). This composition effect may be accompanied by changes in metabolic flux rates that also impact on $\delta^{15}N$, because isotope fractionations are flux-dependent. Metabolic flux-related changes in phloem isotopic composition have been demonstrated for $\delta^{13}C$ recently (Gessler *et al*. 2008).

As an aid in clarifying ^{15}N-distribution patterns in plants, we investigated the isotope composition in leaf, xylem and phloem sap, stem, and root, during a full day/night cycle in *Ricinus communis* (Euphorbiaceae). We show clear $\delta^{15}N$ changes in both xylem and phloem sap along the day/night cycle, in contrast with leaf, stem or root tissues. Xylem and phloem $\delta^{15}N$ were loosely, but significantly correlated, suggesting nitrogen exchange between them. Most correlations between $\delta^{15}N$ and %N in stem and leaf fractions were poor, indicating that metabolic composition, rather than nitrogen concentration itself, is crucial to explaining ^{15}N-patterns.

MATERIAL AND METHODS

Plant material

Seeds of *R. communis* L. were germinated in vermiculite moistened with 0.5 mM $CaSO_4$. After 13–15 days, plants were transferred to substrate, each plant in a 5 L pot. The substrate was made of two parts commercial potting mix (Floradur; Floragard GmbH, Oldenburg Germany) and one part Perlite (Perligran G, Deutsche Perlite GmbH, Dortmund, Germany). Pots were placed in a randomized manner and their position was changed daily. Every three days, plants were watered with tap water and after the first month, were supplied every second week with a commercial fertilizer (0.3% Hakaphos Blau, Compo GmbH, Münster, Germany: 4.5% N-nitrate, 10.5% N-ammonium, 10% P_2O_5, 15% K_2O, 2% MgO and in traces B, Cu, Fe, Mn, Mo and Zn). The $\delta^{15}N$ value of the fertilizer (bulk) was $+0.57 \pm 0.11$‰ and that of the ammonium and nitrate moiety was -1.27 ± 0.13 and $+4.88 \pm 0.12$‰, respectively. The $\delta^{15}N$ of the ammonium moiety was obtained via ammonium purification by quantitative precipitation as phosphoromolybdate (Tave 1969). The $\delta^{15}N$ of nitrate was then calculated with mass-balance from bulk $\delta^{15}N$. Plants were cultivated for 35–40 days in the greenhouse (26 ± 5 °C) with a 16 h photoperiod provided by natural daylight supplemented by mercury-vapour lamps (Osram HQL 400; Osram, Munich, Germany) so as to reach a minimal light level of 300–500 μmol photons $m^{-2}\,s^{-1}$.

Sampling of phloem and xylem saps

Phloem sap was collected at six time points (00:10, 02:59, 10:25, 12:08, 16:37 and 19:05 h mean time), according to Pate, Sharkey & Lewis (1974), by shallow incisions of the bark. A total of 21 plants were sampled and for each, there were six collection-positions along the shoot axis (see Fig. 1, position p-a. to p-f.). The collection of phloem saps from each single plant lasted 45–60 min.

Xylem sap was sampled at the same six time points during the diurnal course by applying pneumatic pressure to the root enclosed in a pressure vessel (Passioura 1980; Jeschke & Pate 1991a; Peuke 2000). From each individual plant, seven samples of xylem sap were collected first from cut leaf midribs and then from tissue flaps of stem internodes (see Fig. 1, position x-a. to x-g.). Pressurizing the roots up to 0.35 ± 0.04 MPa caused exudation at all sampling points, enabling sufficient sap volumes to be collected. Sap samples were kept on ice and stored at –80 °C.

Tissue sampling

All of the seven mature leaves (Fig. 1 L1 to L7), stem segments (corresponding to phloem sap collection positions) and fine roots were harvested after having finished phloem sap sampling and frozen in liquid nitrogen, for the $\delta^{15}N$ analysis in total (bulk), water-soluble and water-insoluble fractions. The samples were homogenized with a mortar and pestle in liquid nitrogen. The water-soluble fraction was prepared with 1.5 mL of deionized water added to 0.1 g of plant material. After agitation for 1 h at 4 °C, the extract was heated at 100 °C for 1 min to precipitate proteins and centrifuged (12000 *g*, 5 min, 4 °C). The supernatant was the water-soluble fraction that was composed mainly of sugars, some organic acids and amino acids (Brandes *et al*. 2007). The pellet was used as the water-insoluble fraction. We acknowledge that any kind of extraction procedure might suffer from incomplete extraction and background contamination, which will affect the isotopic composition of the different biochemical fractions (Richter *et al*. 2009). The fact that the sums of

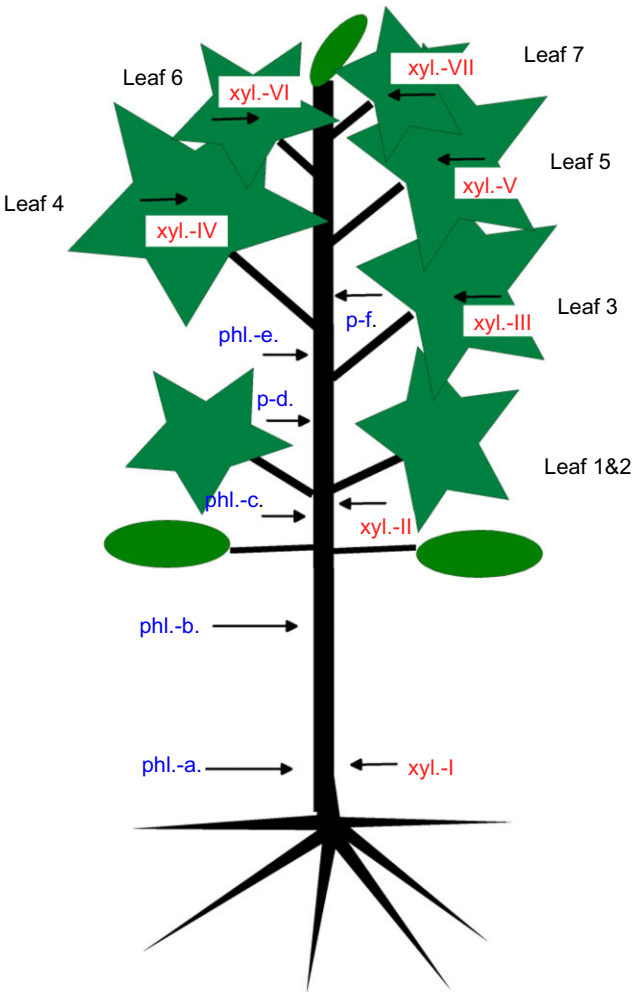

Figure 1. Scheme of the plants used in the experiments with sampling positions. For the analysis of ^{15}N in phloem sap organic matter, phloem saps were collected from six positions along the stem (phl.- to p–f) at six time points (00:10, 02:59, 10:25, 12:08, 16:37 and 19:05 h ±). From the same position axis, sections were harvested after phloem sampling. The cotyledons (Cot1 and Cot2) were already shed at the start of the experiments. Additionally all fully developed leaves (L1+2 – L7). In addition, at all six time points, fine root samples were harvested. Xylem sap was obtained from cut midribs of the leaves (xyl.-III to xyl.-VII) and from tissue flaps of stem internodes (xyl.-I and xyl.-II) at the six time points.

the $\delta^{15}N$ values weighted for the nitrogen contents of the water-soluble and water-insoluble fractions were comparable with bulk material, shows that no losses of nitrogen compounds occurred during the extraction.

Measurement of $\delta^{15}N$ values

Homogenized bulk samples, water extracts, and phloem and xylem saps were oven-dried at 60 °C for 12 h. Oven drying at that temperature was shown to have no effect on the $\delta^{15}N$ values of biological material compared with freeze-drying (Hobson, Gloutney & Gibbs 1997; Kaehler & Pakhomov 2001). Moreover, no effect of drying temperatures in a range of 40–105 °C on $\delta^{15}N$ were observed for fungal fruit bodies (Taylor *et al.* 1997). Samples were weighted and combusted in tin capsules (IVA Analysentechnik, Meerbusch, Germany) in an elemental analyser (NA 2500; CE Instruments, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta Plus; Finnigan MAT GmbH, Bremen, Germany) via a Conflo II interface (Finnigan MAT GmbH). $\delta^{15}N$ values reported here were defined as:

$$\delta^{15}N\,(‰) = (R_{sample}/R_{standard}) - 1 \quad (1)$$

where R_{sample} and $R_{standard}$ (0.0036765) are $^{15}N/^{14}N$ ratios in sample and atmospheric N_2, respectively. We used IAEA-600 (caffein), IAEA-N-1 (ammonium sulphate), IAEA-NO-3 and various in-house standards as reference material.

Statistics

Phloem saps were collected from six positions along the stem at six time points from three to four plants and, afterwards, axis sections were harvested (Fig. 1). Additionally, samples from all fully developed leaves (L1+2 – L7) and from fine root samples were taken at all six time points. Xylem sap was obtained from cut midribs of the leaves and from tissue flaps of stem internodes at the six time points. All sap collections and tissue harvests were repeated with three to four individual plants. Statistical calculations were performed with SAS release 9.2 (SAS Institute Inc., Cary, NC, USA). Differences in $\delta^{15}N$ and total nitrogen between time and sampling positions were determined using analysis of variance (ANOVA) in which the position was nested within a time point [ANOVA model: 'time', 'position (time)']. Additionally, time points in the dark on one hand and time points in the light were pooled and a nested model ['light', 'position (light)'] was calculated to figure out differences between night and day. Analyses were performed by the procedure general linear model (GLM). The adjustment of multiple comparisons according to Tukey was chosen for *P*-values and confidence limits for the differences of least square means (LS means). Correlation analyses were performed on the whole dataset using the CORR procedure of SAS (Kp: and *P*-value for H_0: Kp = 0). Regression analyses were performed with the REG procedure of SAS (H_0: estimates=0, minimum r^2 set at 0.5) using mean values over the different sampling point at the same sampling time.

RESULTS

$\delta^{15}N$ in xylem and phloem sap

$\delta^{15}N$ values in xylem sap varied with time and sampling positions (Fig. 2a), both effects being significant. However, $\delta^{15}N$ values were relatively low in the hypocotyl positions (I and II) and the diel pattern was relatively flat, peaking before noon. $\delta^{15}N$ values were mostly higher in leaf xylem sap in the light, with a clear day/night cycle (^{15}N-enrichment in the light). In the night, there was a substantial variability of $\delta^{15}N$ values between sampling positions.

A clear day/night pattern was visible for $\delta^{15}N$ in the phloem sap, with an increase in ^{15}N in the light, regardless of the sampling position (sampling position did not cause a

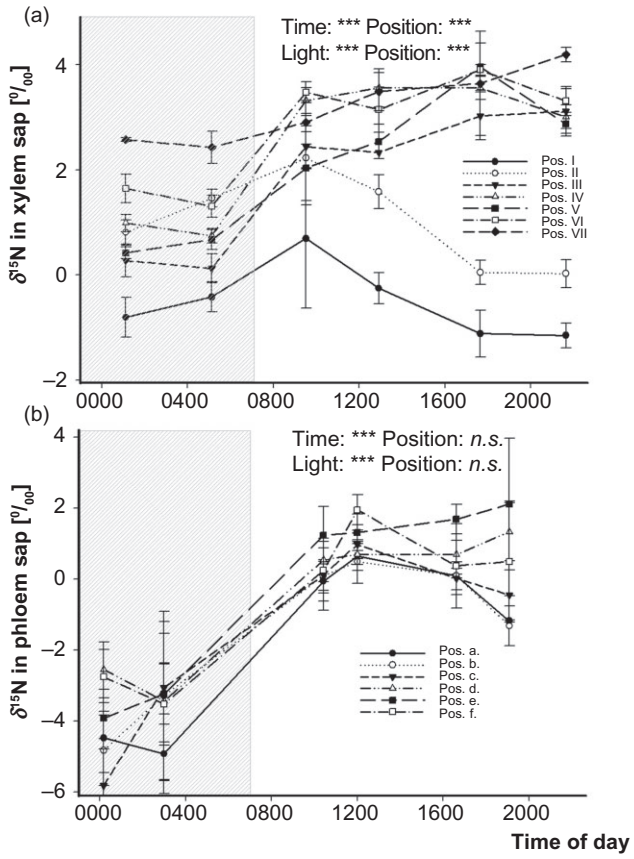

Figure 2. $\delta^{15}N$ in xylem (a) and phloem sap (b) of 35- to 40-day-old *Ricinus communis* during the diel course collected from seven different positions (xylem: I–VII; phloem: a–f) along the axis/leaf number. Data shown are mean values of three to four plants. Significant effects of the main factors time or light and position (of collection/harvest) are given by asterisk in the table. n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

statistically significant effect; Fig. 2b). Night $\delta^{15}N$ values ranged between –5.8 and –2.5‰ while day $\delta^{15}N$ values were between –1.3 and +2.1‰. Sampling time had a statistically significant effect on N concentration in phloem sap, in contrast to the position of sampling (Supporting Information Table S1). During the light period, the N concentration decreased in phloem saps from morning to evening and was highest in the night. However, when individual sampling positions were considered, there was no significant relationship between $\delta^{15}N$ and the N concentration in phloem saps (not shown).

$\delta^{15}N$ in plant tissues

In bulk matter of roots and axis segments, both sampling time and sampling position had a statistically significant effect on $\delta^{15}N$ (Fig. 3a). That is, $\delta^{15}N$ values were slightly lowered (^{15}N-depleted) in the light than in the dark. Root and hypocotyl had lower $\delta^{15}N$ values (from –0.5 to +2‰) compared with epicotyl axis segments (canopy region of the plant axis; from +2.5 to +6.5‰). In contrast to roots and plant axis, there was no significant diel effect on $\delta^{15}N$ in leaf bulk matter (Fig. 4a).

In the water-insoluble fraction from plant axis and root, neither sampling time nor sampling position had an effect on $\delta^{15}N$ (Fig. 3b). The water-insoluble fraction was ^{15}N-depleted compared with bulk organic matter and to the soluble fraction by up to 14‰. The $\delta^{15}N$ in the water-soluble fraction was on average affected by sampling time and sampling position (Fig. 3c); but such effects were insignificant in roots. In the plant axis, the N concentration was significantly affected by sampling time and sampling position in both bulk and water-soluble organic matter, with higher N concentration in younger (apical) axis segments (Supporting Information Table S1).

In leaves, the $\delta^{15}N$ in bulk matter and in the water-insoluble fraction was affected by the position of harvesting only, and not by sampling time (Fig. 4a,b). By contrast, the water-soluble fraction appeared to be significantly ^{15}N-enriched in the light compared with in the dark (Fig. 4c), regardless of the sampling position. There was substantial $\delta^{15}N$-variation among leaves, but bottom leaves (L1+2) appeared to be ^{15}N-enriched compared with others. Leaves 3–5 showed a

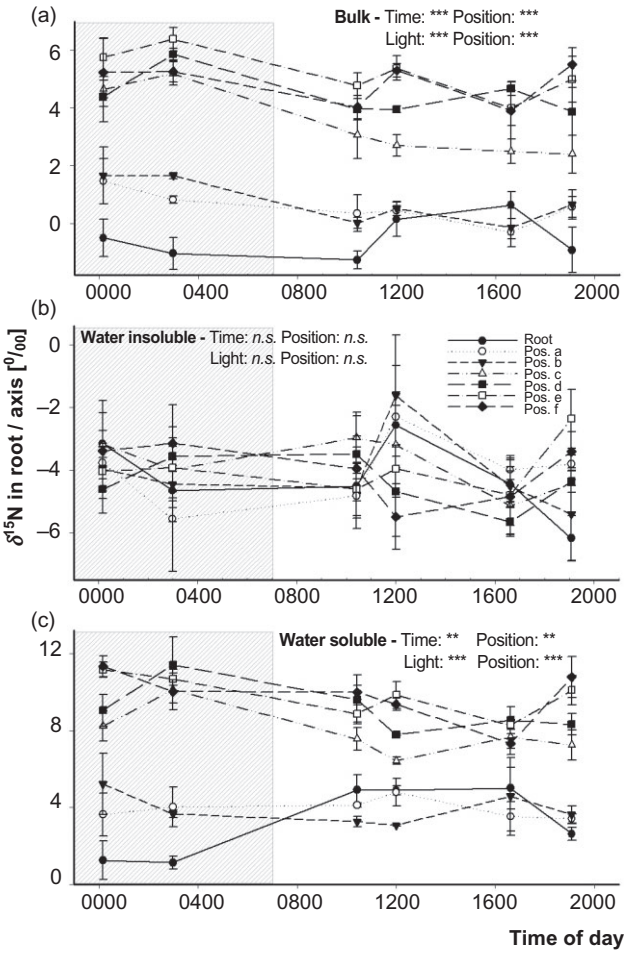

Figure 3. $\delta^{15}N$ in bulk material (a), water insoluble (b) and water extracts (c) from fine root and axis material of 35- to 40-day-old *Ricinus communis* during the diel course collected from seven different positions (a–f) along the axis/leaf number. For further details see legend to Fig. 2.

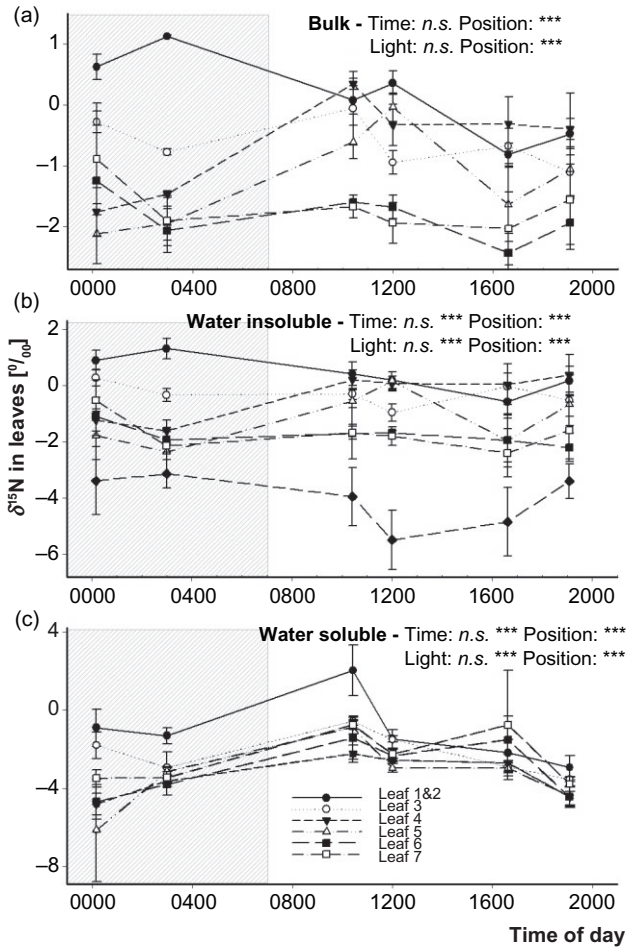

Figure 4. $\delta^{15}N$ in bulk material (a), water insoluble (b) and water extracts (c) from leaves of 35- to 40-day-old *Ricinus communis* during the diel course collected from seven different positions (leaves 1 + 2, leaves 3–7) along the axis. For further details see legend to Fig. 2.

^{15}N-peak before noon, which seemed to coincide with that in phloem (Fig. 2b). In contrast to what was observed in the plant axis, the $\delta^{15}N$ difference between bulk organic matter, water-insoluble and water-soluble fractions of leaves was rather small (up to 2‰ only). In leaf bulk organic matter and in the water-soluble fraction, N concentration was affected significantly by both sampling time and sampling position (Supporting Information Table S1) with a higher N concentration in young leaves.

Comparison of diel patterns

$\delta^{15}N$ data in Fig. 2 (xylem and phloem sap), 3 (root water-soluble organic matter) and 4 (leaf water-soluble organic matter) were redrawn in Fig. 5 so as to make $\delta^{15}N$ co-variations more apparent. Although somewhat lower $\delta^{15}N$ in the evening and at midnight was observed, the water-soluble fraction of leaves and roots was roughly constant with time. In xylem and phloem saps, $\delta^{15}N$ progressively increased in the light. Phloem sap eventually reached similar $\delta^{15}N$ values to that in the xylem in the evening. In the night, while $\delta^{15}N$ values in xylem were relatively close to 0‰, phloem sap had $\delta^{15}N$ values close to that in the water-soluble fraction of leaves. In other words, the apparent diel pattern in phloem was so that it fluctuated between the water-soluble fraction of leaves at night and xylem values in the light. Since both phloem and xylem tend to be ^{15}N-enriched in the light compared with in the night, such kinetics do not carry information on causality; however, there is presently no definite indication whether xylem influenced phloem $\delta^{15}N$ or the reverse.

Relationships between N concentration and $\delta^{15}N$

$\delta^{15}N$ of bulk organic matter was negatively correlated to total N concentration in leaves, but positively correlated in the plant axis (Table 1). That is, N-rich leaves were ^{15}N-depleted while N-rich axis segments were ^{15}N-enriched compared with the less N-containing tissues, indicating that the origin of the ^{15}N-enrichment was different in these organs and likely stemmed from the metabolic composition in nitrogenous compounds. In roots, $\delta^{15}N$ in the water-soluble fraction was better related to the N concentration of bulk material than to the N concentration in water-soluble extracts (Table 2). In the phloem, the correlation between N concentration and

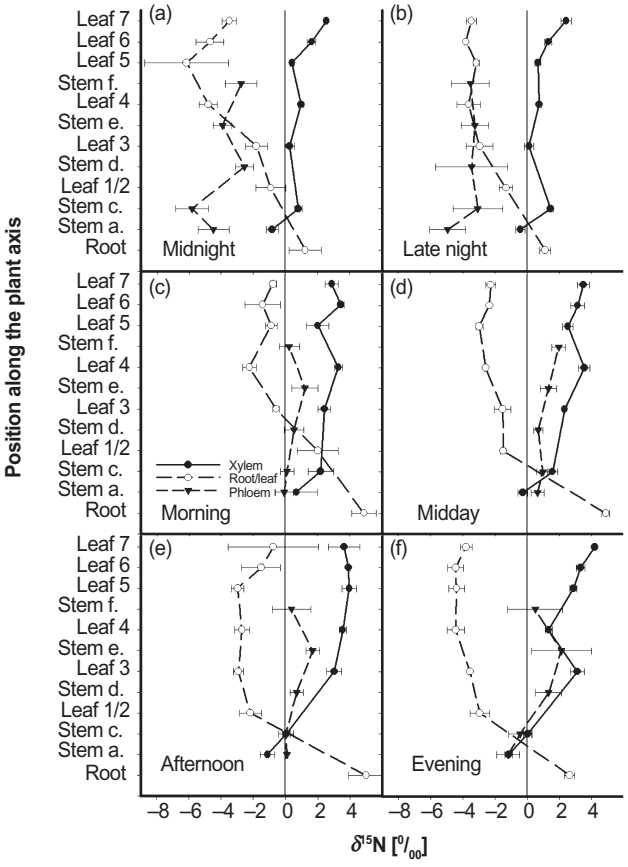

Figure 5. Diel time course of axial patterns of $\delta^{15}N$ in phloem, xylem and water soluble extracts from leaves and roots. Redrawn from Figs 2 (saps), 3 (root) and 4 (leaves).

Table 1. Correlation analysis between $\delta^{15}N$ and total N concentration in leaf, axis bulk material and water extracts (Wsl) and in phloem saps from 35- to 40-day-old *Ricinus communis* during the diel course collected from different positions along the axis/leaf number

	Bulk leaf ^{15}N	Bulk axis ^{15}N	Wsl. Leaf ^{15}N	Wsl. axis ^{15}N	Phl. ^{15}N	Bulk leaf N_{tot}	Bulk axis N_{tot}	Wsl. leaf N_{tot}	Wsl. axis N_{tot}	Phl. N_{tot}
Bulk leaf ^{15}N	–	–0.01	0.23	–0.12	0.22	**–0.57**	–0.33	–0.44	–0.25	–0.11
Bulk axis ^{15}N	n.s.	–	–0.25	**0.71**	–0.13	0.38	**0.56**	0.41	0.39	0.05
Wsl. Leaf ^{15}N	n.s.	n.s.	–	–0.36	–0.16	–0.27	–0.16	–0.07	–0.14	0.43
Wsl. axis ^{15}N	n.s.	***	n.s.	–	–0.09	0.35	0.42	0.34	0.34	–0.01
Phloem ^{15}N	n.s.	n.s.	n.s.	n.s.	–	–0.21	–0.32	–0.38	–0.18	–0.47
Bulk leaf N_{tot}	***	n.s.	n.s.	n.s.	n.s.	–	**0.69**	**0.75**	**0.54**	0.08
Bulk axis N_{tot}	n.s.	***	n.s.	n.s.	n.s.	***	–	**0.58**	**0.71**	0.22
Wsl. leaf N_{tot}	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	–	0.42	0.36
Wsl. axis N_{tot}	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	n.s.	–	0.22
Phloem N_{tot}	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	–

Significant ($P < 0.05$ for $H_0 : K_p = 0$). Pearson's correlation coefficients (K_p) >0.50 are indicated by bold numbers. n.s., not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2. Correlation analysis between $\delta^{15}N$ and total N concentration in root bulk material and water extracts (Wsl) from 35- to 40-day-old *Ricinus communis* during the diel course collected from different positions along the axis/leaf number

	Bulk root ^{15}N	Bulk root N_{tot}	Wsl. root N_{tot}	Wsl. root ^{15}N
Bulk root ^{15}N	–	0.42	0.03	0.16
Bulk root N_{tot}	n.s.	–	0.46	**0.59**
Wsl. root N_{tot}	n.s.	n.s.	–	0.42
Wsl. root ^{15}N	n.s.	**	n.s.	–

Significant ($P < 0.05$ for $H_0 : K_p = 0$). Pearson's correlation coefficients (K_p) >0.50 are indicated by bold numbers. n.s., not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

[Correction added on 25 June 2013 after first online publication: For Tables 1 and 2, en dashes have been inserted as separators to replace the incorrect "n.s." table entries.]

$\delta^{15}N$ was relatively weak, the Pearson's correlation coefficient being just below 0.5.

Regression analyses between $\delta^{15}N$ values

The relationship between $\delta^{15}N$ values of the different plant parts was investigated. The statistically significant and relevant results are shown in Fig. 6. $\delta^{15}N$ in water-soluble extracts from roots correlated with $\delta^{15}N$ in the phloem sap from the lower sampling positions (positions a and b) but not with those in xylem saps (positions I and II, Fig. 6a). For $\delta^{15}N$ in leaf water-soluble extracts, a negative correlation was found for leaves L3 and L4 with phloem sap from the corresponding sampling positions (d and e), restricted to day time measurements (Fig. 6b). There was no similar correlation for other leaves. $\delta^{15}N$ in the xylem sap was positively correlated to $\delta^{15}N$ in phloem (Fig. 6c) in the canopy region of the shoot (from leaf L3 to L5: phloem position d versus xylem position III, e versus IV and f versus V) but not in others (old leaves L1+2: phloem position c versus xylem position II; and hypocotyl: phloem position a versus xylem position I).

DISCUSSION

^{15}N-distribution within plants

In the present study, there were no such clear and straightforward ^{15}N gradients (Figs 3 and 4), as observed in other species (e.g. Peuke *et al.* 2006). On average, $\delta^{15}N$ of bulk matter was slightly more negative in leaves (–0.97‰) than in roots (–0.49‰). Higher $\delta^{15}N$-values were observed in the stem (+3.20‰ on average), with highest values in the upper canopy (+4.49‰). The lack of clear ^{15}N-enrichment in leaves is partially in contrast with previously published results in which leaves were shown to be up to 7‰ enriched compared with roots (Högberg 1997; Evans 2001; Peuke *et al.* 2006) – while a slight ^{15}N-depletion in leaves compared with roots has been observed by Haberer *et al.* (2007). Older leaves (1 and 2, and leaf 3) tended to have higher $\delta^{15}N$ in different biochemical fractions than younger ones. The $\delta^{15}N$ of older or

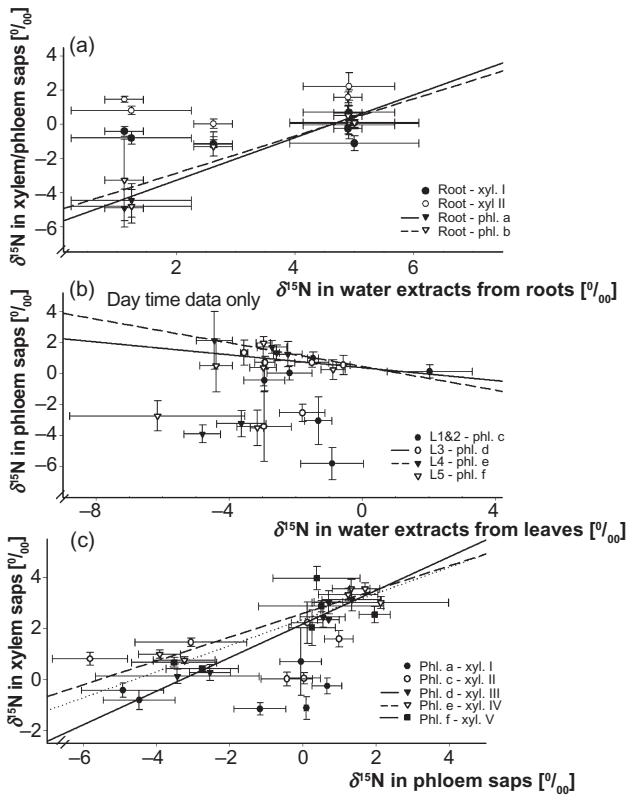

Figure 6. Regression analyses between $\delta^{15}N$ values: (a) water-soluble extract of root against xylem saps (position I or II) or phloem saps (position a or b); (b) water-soluble extract of leaves against phloem sap; (c) phloem sap against xylem sap. Data shown are mean values of three to four plants ± SE. Significant relationships ($P < 0.05$ and $r^2 > 0.5$) are plotted with continuous or dashed lines. For further details see legend to Fig. 2.

even senescent leaves may be affected by volatile losses of NH_3 (Högberg 1997). Here, plants were fed with a fertilizer that included ammonium in addition to nitrate and, thus, the contribution of nitrate reduction and allocation to ^{15}N-patterns (described later) is probably somewhat 'buffered' by ammonium assimilation. Nevertheless, *Ricinus* is a nitrate-preferring species and NH_4^+ is entirely assimilated by roots and not transported by xylem sap at all (Peuke and Jeschke 1993). Ammonium-derived assimilates are produced in roots, distributed among plant parts and thus 'dilute' the isotopic differences between organs and biochemical fractions caused by nitrate metabolism.

Still, we found here a small but visible ^{15}N-enrichment in the water-insoluble fraction of leaves ($\delta^{15}N$ comprised between –2 and +0.5‰) compared with stem and roots (near –4‰); and this pattern is reversed in the water-soluble fraction (near –3‰ in leaves and +3 to +4‰ in hypocotyl and roots). Considering that insoluble material mostly represents reduced (organic) N, it is likely that soluble compounds such as nitrate did contribute to the unusual ^{15}N-pattern in bulk matter. Actually, in most plants, nitrate storage account for less than 1% of total nitrogen content (Comstock 2001). In fact, nitrate is usually ^{15}N-enriched compared with other compounds (Yoneyama & Tanaka 1999). In other words, roots and stems would contain proportionally more nitrate than leaf blades under the conditions of our study. It has indeed been demonstrated for castor bean that nitrate transported aboveground via the xylem is mostly reduced by leaves (94%) with little nitrate accumulation, accounting for 0.2% only in leaves, 5.3% in roots and at most 7.8% in the axis (percentage of nitrate in total N; Peuke 2010). It is also likely that the ^{15}N-enrichment in the root and stem water-soluble fraction was caused by other compounds from N reduction and assimilation. In *Ricinus*, roots have been shown to reduce and assimilate 38% of absorbed nitrate to amino acids and amides (Peuke 2010). Because nitrate reduction fractionates against ^{15}N (Ledgard, Woo & Bergersen 1985), nitrate molecules left behind and, thereafter, translocated to shoots are thus ^{15}N-enriched. Furthermore, amidated nitrogenous compounds are ^{15}N-enriched compared with aminated compounds (Werner & Schmidt 2002; Tcherkez 2011; Gauthier *et al.* 2012) and therefore, nitrogen translocated from roots to shoots was probably somewhat ^{15}N-enriched. The quite high $\delta^{15}N$ value in stem water soluble fraction at the canopy level (up to +12‰, Fig. 3) may also point to the accumulation of nitrate and amides.

Because of the isotope fractionation associated with nitrate reduction, it is believed that $\delta^{15}N$ values are lower in phloem than in xylem saps (Evans 2001). Here, the $\delta^{15}N$ in phloem was on average –0.45‰, as compared with +1.67‰ in xylem sap (Fig. 2). Nevertheless, it should be noted that important diurnal variations were observed (addressed later) and that nitrogen exchange may have occurred between phloem and xylem. We further show that xylem sap from roots and hypocotyl is visibly ^{15}N-depleted compared with the xylem sap at the canopy level (Fig. 2a). The origin of the relative ^{15}N-enrichment in xylem sap in the canopy can either be the abstraction of ^{14}N by metabolic reactions (and associated ^{15}N fractionation) or the supply of ^{15}N from an enriched source. The transfer of ^{15}N-enriched molecules from the phloem to xylem seems likely to be involved in the observed isotopic patterns. In fact, it has been shown in *Ricinus* that organic nitrogen from older leaves can be loaded into the xylem, thereby sustaining nitrogen metabolism in younger leaves (Jeschke & Pate 1991b). Transfer cells, observed in the xylem of leaf traces exiting from nodal tissue, were suggested to be involved in this process (Jeschke & Pate 1991b). Glutamine, which is the major component of phloem nitrogenous compounds in this species, may have been exchanged from phloem to xylem. Similarly in grapevine, the concentration of organic N increased along the shoot axis and was greatest in the youngest measured leaf (Peuke 2000). Metabolic exchange would also contribute to explaining the relationship between $\delta^{15}N$ in xylem and phloem saps (Fig. 6).

It is further possible that the ^{15}N-enrichment in the root water-soluble fraction also came from the import of ^{15}N-enriched compounds from the phloem. It has indeed been suggested that up to 50% of the reduced N incorporated in roots is imported via the phloem (Jeschke & Pate 1991b).

Diel variations in $\delta^{15}N$ values

The most visible diel variation in nitrogen isotope composition was observed in xylem and phloem saps. Phloem nitrogen was ^{15}N-enriched during day time compared with the night time and so was xylem nitrogen (Figs 2 and 5). By contrast, there were little diel effects in bulk organic matter and in the water-insoluble fraction of leaves, roots and stems. In the water-soluble fraction of leaves and roots, there was a slight tendency to higher $\delta^{15}N$ values in the light.

The ^{15}N-enrichment in xylem sap in the light compared with in the dark most likely reflected the effect and timing of nitrate reduction by roots that fractionates against ^{15}N and possibly, the import of ^{15}N-enriched organic compounds from the phloem (see earlier). In fact, leaf nitrate reduction and assimilation is believed to occur mostly in the light (Stitt *et al.* 2002). In *Ricinus* leaves, nitrate reductase activity follows a diurnal cycle with a maximum around noon (Kandlbinder, Weiner & Kaiser, 2000). Additionally, diel cycles in loading of nitrate versus organic compounds might be involved in the control of the diurnal cycle of xylem sap ^{15}N enrichment. A relatively higher contribution of nitrate to phloem borne N during the day might cause increased $\delta^{15}N$ in the light compared with in the dark period. The relative ^{15}N-enrichment in the phloem sap in the light compared with in the dark was even larger (6‰) than in the xylem (4‰) and phloem eventually reached $\delta^{15}N$ values similar to that in the xylem sap (Fig. 5). The progressive ^{15}N-enrichment in phloem sap in the light may have come from (1) the ^{15}N-enrichment in nitrogen primary assimilates produced by leaves; (2) the entry of ^{15}N-enriched nitrates from the xylem sap; and (3) a change in phloem metabolic composition, with a progressive prevalence of ^{15}N-enriched compounds. Effect (1) is likely, simply because isotope fractionation associated with nitrate reduction causes a drift in the $\delta^{15}N$ in source nitrate (Rayleigh effect) and so in assimilates synthesized therefrom. There was indeed a ^{15}N-enrichment in the water-soluble fraction in leaves in the light (Figs 4 and 5). Effect (2) is probably of minor importance. In fact, in *Ricinus*, nitrate has been found to be loaded into the phloem in very low amounts only (Pate 1980; Jeschke & Pate 1991b; Peuke *et al.* 1996; Peuke 2010) and, furthermore, it has been shown to be simply absent in phloem sap (Hall & Baker 1972). That is, amino acids and amides are the prevalent N-compounds in phloem saps (Jeschke & Pate 1991b; Peuke 2010). Effect (3) may also contribute to enrich phloem sap in ^{15}N because total phloem amino acid concentration varied and peaked in the light period. Furthermore, glutamine abundance in phloem saps has been shown to change along a day/night cycle in *Ricinus*, with a progressive increase in the light (Gerendas & Schurr 1999; Peuke *et al.* 2001). Moreover, the ratio of ^{15}N-depleted amino acids to ^{15}N-enriched ones (e.g. Ser/Asp) decreased in the second half of the light period (amino acid spectrum taken from Peuke *et al.* 2001). This view would agree with the modest, but visible correlation between phloem $\delta^{15}N$ and N concentration (Table 1).

The modest ^{15}N-enrichment in the root water-soluble fraction in the light compared with in the dark may be associated with the control of root nitrogen metabolism. In fact, during the first part of the light period in tobacco roots (*Nicotiana tabacum*), it is believed that nitrate assimilation exceeds nitrate uptake, leading to a rapid depletion of nitrate and accumulation of ammonium and amino acids (Stitt *et al.* 2002). In *Ricinus*, it has been shown that nitrate uptake by roots was several-fold higher in the light compared with in the dark. However, nitrate uptake by roots and transport to the shoot are largely uncoupled (Herdel *et al.* 2001). That is, in the light, nitrate uptake is much larger than nitrate transfer to the shoot and thus root nitrate is either stored, lost (efflux) or reduced by root metabolism (to amino acids that are partly exported). Because the root water-soluble fraction tends to be ^{15}N-enriched compared with the dark (Fig. 4), the latter hypothesis is plausible. In the light, nitrogen metabolism in shoots, thus, mostly relies on nitrate absorbed and stored during the night. As this nitrate pool is consumed by leaf metabolism, there should be a progressive ^{15}N-enrichment in nitrate and nitrogenous compounds synthesized there from during the light period in leaves and phloem. In other words, the particular timing of metabolism and nitrate uptake in *Ricinus* leads to a transient imbalance between the rate of nitrate absorption, export in the xylem and reduction in the light by leaves. As a consequence, we observed a relative ^{15}N-depletion during the night in roots (due to ^{15}N fractionation and nitrate replenishment) and ^{15}N-enrichment during the day (when ^{15}N fractionation is limited by nitrate availability). We acknowledge that the ^{15}N enrichment in the light also requires a variation of phloem transport to the roots relative to root uptake and assimilation of nitrate over the day/night cycle.

Conclusions and perspectives

$\delta^{15}N$ patterns are likely the result of a complex set of interactions and N-fluxes between plant organs and compartments (summarized in Fig. 7); root total organic nitrogen is ^{15}N-depleted due to ^{15}N fractionation associated with nitrate reduction and shoots are ^{15}N-enriched as a result of the isotopic mass-balance (i.e. translocation of remaining nitrates from roots). By contrast, the root water-soluble fraction may be ^{15}N-enriched due to nitrate prevalence and possible backflow of enriched organic-N from shoots. Day/night cycling of $\delta^{15}N$ in xylem and phloem is caused by a combination of ^{15}N fractionation (by reduction), phloem-to-xylem exchange of ^{15}N-enriched compounds and diel changes in the phloem metabolite composition. The diel variations in $\delta^{15}N$, particularly in water soluble fractions as well as in xylem and phloem saps underline the differences in N-assimilation between day and night. Additionally, exchange of nitrogen compounds between phloem and xylem were shown.

We nevertheless recognize that the present explanation of $\delta^{15}N$ patterns requires further assessment, such as compound-specific $\delta^{15}N$ measurements and analyses of metabolic concentrations in both xylem and phloem at several sampling positions along plant stem. As already stated earlier, there may be strong $\delta^{15}N$ difference between nitrogenous compounds; and, therefore, metabolite allocation patterns should

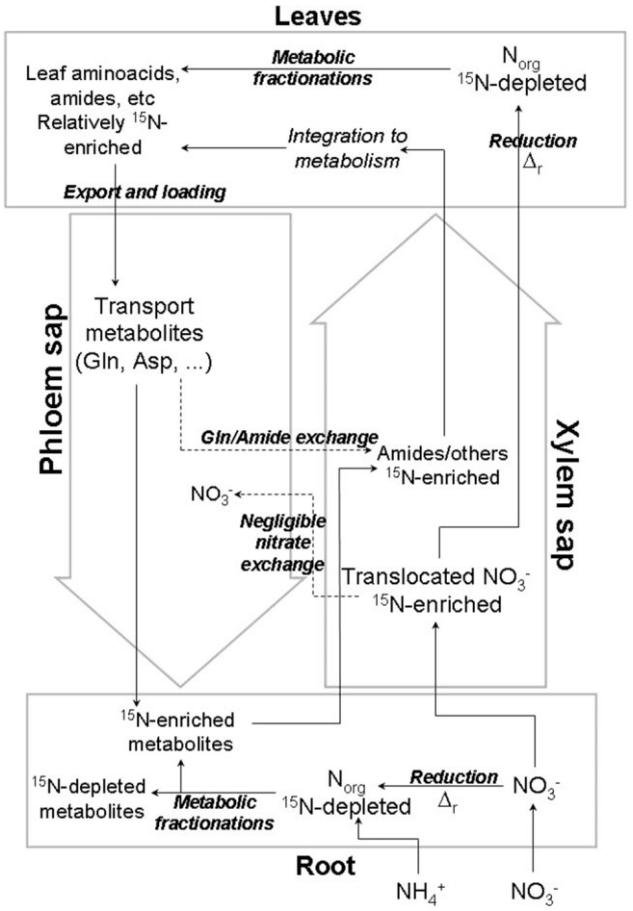

Figure 7. Simplified representation of ^{15}N isotopic fluxes that explain δ^{15}N values in *Ricinus* upon nitrate nutrition. Ammonium assimilation does not require reduction and therefore simply involves exchanges of organic nitrogen. Δ_r denotes the isotope fractionation associated with nitrate reduction. For simplicity, leaf canopy levels and the stem are not figured. Ammonia emission from the leaf as well as nitrate or ammonium efflux from the roots might additionally affect δ^{15}N of these tissues. See the text for further description.

influence the ^{15}N-distribution. Furthermore, our results show that the natural nitrogen isotope abundance in plant tissue is not constant along the diurnal course. In addition to explaining one part of the well-recognized δ^{15}N variability among plant samples (see references in the Introduction), this emphasizes the importance of taking into account the sampling time for plant isotopic studies.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Total nitrogen concentration in xylem, phloem saps and bulk as well as water extracts of fine roots, axis and leaves from 35–40 days old *Ricinus communis* during the diel course collected from different positions along the axis/leaf number. Shown are means ± standard errors of 3–4 replicates. Significant effects of the main factors time and position (of collection/harvest) are given by asterisk: n.s.: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; n.m.: not measured; n.c.: not calculated (SD, only 2 replicates).