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A method for ^{13}C-labeling of metabolic carbohydrates within French bean leaves (*Phaseolus vulgaris* L.) for decomposition studies in soils

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The molecular composition of plant residues is suspected to largely govern the fate of their constitutive carbon (C) in soils. Labile compounds, such as metabolic carbohydrates, are affected differently from recalcitrant and structural compounds by soil-C stabilisation mechanisms. Producing ^{13}C-enriched plant residues with specifically labeled fractions would help us to investigate the fate in soils of the constitutive C of these compounds. The objective of the present research was to test ^{13}C pulse chase labeling as a method for specifically enriching the metabolic carbohydrate components of plant residues, i.e. soluble sugars and starch. Bean plants were exposed to a $^{13}CO_2$-enriched atmosphere for 0.5, 1, 2, 3 and 21 h. The major soluble sugars were then determined on water-soluble extracts, and starch on HCl-hydrolysable extracts. The results show a quick differential labeling between water-soluble and water-insoluble compounds. For both groups, ^{13}C-labeling increased linearly with time. The difference in $\delta^{13}C$ signature between water-soluble and insoluble fractions was 7‰ after 0.5 h and 70‰ after 21 h. However, this clear isotopic contrast masked a substantial labeling variability within each fraction. By contrast, metabolic carbohydrates on the one hand (i.e. soluble sugars + starch) and other fractions (essentially cell wall components) on the other hand displayed quite homogeneous signatures within fractions, and a significant difference in labeling between fractions: $\delta^{13}C = 414 \pm 3.7$‰ and 56 ± 5.5‰, respectively. Thus, the technique generates labeled plant residues displaying contrasting ^{13}C-isotopic signatures between metabolic carbohydrates and other compounds, with homogenous signatures within each group. Metabolic carbohydrates being labile compounds, our findings suggest that the technique is particularly appropriate for investigating the effect of compound lability on the long-term storage of their constitutive C in soils. Copyright © 2009 John Wiley & Sons, Ltd.

Understanding the origin of stable organic carbon (C) in soils is a crucial element for devising successful strategies to improve C sequestration in soils, and thereby mitigate anthropogenic increases in atmospheric CO_2. Soil organic matter (SOM) essentially derives from the input of plant residues to soil and three types of mechanisms have been invoked so far to explain the persistence of organic carbon in soils for long periods.[1] Organic compounds can be selectively preserved, due to their molecular-level characteristics such as elemental composition, presence of certain functional groups or molecular conformation that restrict their decomposition, this process being named recalcitrance. Organic compounds may also be protected from decomposition due to their interaction with minerals (i.e. adsorption, complexation) or by being inaccessible to the decomposers due to their location in the soil complex architecture (i.e. physical protection).[1]

The molecular composition of plant residues has long been considered to be a major control over the fate of their constitutive C but this hypothesis has actually proven difficult to demonstrate and the importance of recalcitrance for SOM stabilization is being questioned.[2] Ideally, we would need to follow the fate of the constitutive C of selected types of plant molecules added to the soil as plant residues. This has not yet been truly accomplished. Up to now, tracing the fate in soils of plant-residue C at the molecular level has relied on three main types of proxies:

– The first method consists of measuring the mineralization rate of plant residues uniformly labeled with either ^{14}C or ^{13}C, and correlating this rate with the

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chemical composition of the plant residue.[3,4] An earlier and cruder version of this method consists of comparing non-labeled plant residue mineralization in soil with that of a control soil without residue.[5] This method is not appropriate to study the fate of a specific molecular group, which would only be approached through a range of plant residues varying only in the desired (and studied) compounds. Such material is unfortunately not available. However, this method has been used extensively to identify groups of compounds that best explain mineralization rates. For example, multiple studies have reported that a higher lignin concentration reduces mineralization rates[6,7] and that a higher content in soluble compounds increases mineralization rates.[5] These studies have heavily emphasized short-term mineralization rather than long-term C stabilization, while the latter is the relevant information in terms of C storage in soil. Incubation studies are generally conducted for periods of time too short to address C stabilization.[8] While the correlation between composition of plant residues and short-term mineralization is often high, the same is not true with longer-term stabilization.[9] This emphasis on short-term mineralization has led to misinterpretations. For example, lignin has long been considered as a major contributor to stable soil organic C because of its short-term chemical recalcitrance to mineralization, while recent studies suggest that its long-term stabilization is not especially high.[10,11]

- The second method consists of incubating pure compounds labeled with either ^{13}C or ^{14}C. These compounds can be artificially synthesized[12,13] or extracted from labeled plants.[14] The purification degree of the extracted compounds appears to affect their mineralization rates, such as for lignin.[14] Most importantly, other studies suggest that the structure of plant residues affects their decomposition. For example, residue particle size affects mineralization rates.[15–17] A potential explanation is that the decomposition of labile compounds within plant residues is reduced due to restricted access and protection by structural components.[7,18] Hence, these studies suggest that incubating isolated compounds does not yield the relevant information for understanding the fate of C contained in contrasting molecular families within the plant-residue structure.
- The third method is compound-specific ^{13}C-signature tracing of naturally or artificially labeled compounds.[19] This new method has attracted much attention recently, as it allows us to derive the total C budget for molecular families across the plant-soil continuum.[10] The main advantage of this method is that it allows us to follow the fate of plant-derived molecules *in situ* and over long periods of time, i.e. at least tens of years.[19–22] However, once a compound has lost its molecular identity, the fate of its molecular C can no longer be traced. For example, when a lignin structure has been decomposed, it is not possible to determine unequivocally whether the constitutive C has been lost as CO_2 or stabilized in the soil under a different molecular structure. It is thus important to differentiate between the stabilization of 'intact' molecules and the persistence of their constitutive carbon, which may be recycled as microbial molecules.

Compound-specific C stabilization data emerging from these three methods are not easily reconciled. The method based on correlation between mineralization rates and composition suggests that plant recalcitrant molecules, such as lignin, contribute most to stable C in soils. However, the recent compound-specific isotopic studies do not sustain this hypothesis. Indeed, these studies show that about 90% of plant-residue lignin is decomposed within 1 year in temperate soils,[10] and that the remaining fraction has an apparent residence time of about 20 years.[11,20] The idea emerging from these works is that the recalcitrance of plant compounds controls their decomposition rate in the short term, but that it does not contribute much to the longer-term stabilization of their constitutive C in soils.[2] We recently hypothesized that C from labile molecules can be preserved in soils as much as other plant molecular groups.

Metabolic carbohydrates are highly labile compounds, composed of cold-water-soluble sugars and hot-water-soluble starch. Soluble sugars are considered highly labile compounds. Indeed, glucose has been used abundantly in incubation studies as the archetype of a labile compound.[13,23,24] Starch, a polymer of glucose units, is insoluble in cold water whilst soluble in hot water. It is easily hydrolysable and is rapidly hydrolyzed at night in plant leaves and released as soluble sugars in the cell cytoplasm.[25,26] Starch exhibits similar decomposition properties to soluble compounds in soil incubation studies of plant residues.[12] Gaillard *et al.*[27] suggested that easily decomposable components of the soluble fraction of plant residues might preferentially contribute stable C in soil, despite their lability, i.e. intrinsic chemical biodegradability, because of their ability to be transported to the soil matrix where they could be protected by adsorption to or occlusion in the soil matrix.

Plant residues with a differential ^{13}C-isotopic labeling of the metabolic carbohydrate fraction from the remaining one would be necessary to probe the potentially contrasting fate of their constitutive C. In practice, both the metabolic carbohydrate and the remaining fraction need to have a fairly homogeneous ^{13}C signature in order to solve the resulting two-pool isotopic equation. When the residues are incubated in soils, SOM emerges as a third isotopic pool. Therefore, the SOM degradation rate and SOM-$^{13}CO_2$ value must be determined beforehand in a separate experiment with homogeneously labeled residues. Our preliminary results from a parallel experiment demonstrate the feasibility of this technique. Here, we hypothesize that plant residues with a differential labeling of the metabolic carbohydrate fraction can be produced because the different compounds constitutive of this fraction follow similar production kinetics and metabolic pathways.

During photosynthetic carboxylation, CO_2 is assimilated first as trioses and a fraction of this carbon is transformed to hexoses such as fructose and glucose, which form sucrose (disaccharide), the main sugar circulating in the phloem. Trioses and hexoses are also quickly used to build up C reserves, such as starch, in the cell. Hexoses are later combined to form cell wall polymers, such as cellulose. On the basis of these two distinct steps of anabolism, we hypothesized that a short labeling with ^{13}C-CO_2 would preferentially label trioses, hexoses, sucrose and starch, relative to structural compounds and particularly to cell wall constituents.

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The main objective of the present study was to determine if the metabolic carbohydrate fraction of a plant like bean could be labeled specifically and homogeneously compared with the remaining fraction by a ^{13}C pulse chase and to determine the conditions for obtaining such differentially labeled material.

EXPERIMENTAL

Plant material and growth conditions

Bean plants were chosen for these experiments because: (a) bean leaves are rich in the metabolic water-soluble fraction (27%) and easily decomposed in soils (C/N ≈10), (b) they are easy to grow in a glasshouse, and (c) ^{13}C-labeling is known to be quite reproducible on bean leaves from different plants.[28]

French bean plants (*Phaseolus Vulgaris* L.), 'Flavert' variety, were cultivated for 6 weeks in a glasshouse in May and June 2005 under natural light and CO_2. The pots were filled with compost-based soils. The ^{13}C-labeling was conducted on 6-week-old plants, which were at the flowering stage with first-generation leaves appearing mature, both cotyledonary and first trifoliate leaves.

After labeling, leaves and stems were immediately harvested and the leaves were separated into different categories: 'mature-leaves', 'young-leaves' and 'leaves with an intermediate age'. Mature leaves were the first-generation leaves, both cotyledonary and trifoliate. From observations and measurements at the time of sampling they did not grow any more. Young leaves were last-generation trifoliates, located at the end of branches and still growing. Intermediate leaves were the leaves as an intermediate position between these two groups. This last category was established in order to have good separation of mature from young-leaves but it was not considered for measurements in this study. Leaves were immediately frozen in liquid nitrogen and kept at −20°C before freeze-drying.

Pulse chase $^{13}CO_2$ labeling system

Plants to be labeled were introduced for the desired time into the growth chamber in the morning. Early light-period exposure was chosen because metabolic carbohydrate reserves are then generally at a minimum level due to night respiration. The potential for untranslocated metabolic carbohydrate accumulation in leaves was therefore considered highest. The growth chamber was a 'C_2-3A' derived from the model introduced by André *et al.*,[29] and now fully regulated by a microprocessor controller and an environmental data acquisition system. The chamber was 0.48 m^2 by 0.90 m tall. Light is generated by four lamps, OSRAM HQI-BT model (400 W/D). The temperature (24 ± 0.2°C) and humidity (65 ± 3%) were both regulated by a cooling circulator with a heat exchanger inside the growth chamber. A mixing gas system consisting of three mass flow controllers allowed us to regulate the injection of CO_2-free air (Bronkhorst, Montigny Les Cormeilles, France, mass flow meters: EL-Flow F201C 0-8 L/mn), normal CO_2 (EL-Flow F201 0-30mL/mn) and ^{13}C-labeled CO_2 (EL-Flow F200C 0-0,75mL/mn) to the growth chamber. The rates of air and CO_2 supplies are computer-controlled to obtain a molar fraction of 400 μmol/mol inside the chamber with the ^{13}C-labeled CO_2 (^{13}C abundance 10.69 ± 0.01%) flow rate maintained at 8% of the total CO_2 flow (^{13}C abundance 1.072 ± 0.002%), and to obtain a final $\delta^{13}C$-CO_2 of about 670 ± 6‰ (^{13}C abundance 1.840 ± 0.003%) as input into the growth chamber. The CO_2-free air was injected at a rate of 0.9 L min^{-1}. The air was only removed from the chamber at a rate of 0.6 L min^{-1} to maintain inside the chamber a slight positive pressure relative to ambient atmospheric pressure and thereby avoid atmospheric CO_2 contamination. The CO_2 input flow rate was regulated to match the CO_2 assimilation rate of plants, which was about 6 mL min^{-1} when plants were initially placed into the growth chamber.

Labeling experiment

The production times for trioses and hexoses during carboxylation are known to be very short, less than 1 min.[30] Since by using 1-min labeling we could not obtain enough labeled material, different labeling times were tested to determine the labeling duration necessary to both (i) maximise ^{13}C-labeling of metabolic carbohydrates in leaves and (ii) minimise the labeling of water-insoluble compounds. On that basis, the kinetics of labeling were determined by exposing bean plants to ^{13}C-CO_2 in the growth chamber for 0.5, 1, 2, 3 and 21 h in separate experiments.

The natural $\delta^{13}C$ values of leaves are mainly dependent on leaf photosynthetic activity and stomatal conductance, and thus are related to leaf age and also to the irradiance received by the leaves.[31–33] We selected mature leaves in order to maximise the difference in $\delta^{13}C$ between metabolic carbohydrate and the remaining fractions for further biodegradation experiments in soil. We assumed that these leaves no longer grew and therefore incorporated less labeled ^{13}C in the water-insoluble fraction than the young leaves did. We observed that mature leaves were situated lower in the bean canopy, and therefore had a reduced exposure to light compared with younger leaves; this should also restrict their growth. The study was focused on mature leaves, but young leaves were also measured in order to compare their $\delta^{13}C$ values with those of the mature leaves, and to verify our hypothesis for the mature leaf selection.

Labeling reproducibility

The ^{13}C-CO_2 value in the middle of the empty growth chamber was nearly constant during a 14-h test period, i.e. 553.6 ± 1.4‰ (n = 4). It was tested using 100-mL flasks placed in the chamber beforehand. Using the glove-box system, flasks were regularly filled with air sampled in the centre of the chamber before being sealed and reserved for further ^{13}C analysis.

Potential sources of variability of the labeling are: (a) heterogeneity in our batch of mature leaves due to their position in the growth chamber, (b) the extraction protocol, and (c) the instrumental precision. We used the 21-h ^{13}C-labeled foliar material to quantify the impact of these different sources of variability on $\delta^{13}C$ values. (i) The variability in our batch was determined by using six subsamples of mature leaves. Freeze-dried leaves were cut into pieces of 1 to 2 mm. (ii) The variability on cold-water extraction was determined by repeating the extraction three times on the same leaf batch with leaf powder ground under

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500 μm. (iii) Instrumental precision was calculated by measuring six times the same sample (i.e. leaf powder) on our elemental analyser-isotope ratio mass spectrometer (EA-IRMS) system.

Extraction of metabolic carbohydrates

Metabolic carbohydrates were extracted according to a two-step procedure. First, the water-soluble fraction was extracted, and the soluble sugar components were quantified and their ^{13}C signature determined, compared with that of the rest of the soluble fraction. Second, starch was extracted from the remaining water-insoluble fraction, and quantified and analyzed for its ^{13}C value, as explained below.

Cold-water extractions were conducted according to the procedure of Duranceau *et al*.[34] as modified by Bathellier *et al*.[35] Briefly, 100 mg of leaf powder were shaken three times with 1 mL ultra-pure and cold water for 1 min and kept in ice bath for 10 min between each operation. The material was then centrifuged for 10 min at 16 000 *g* at 4°C. The supernatant containing the water-soluble fraction was collected by filtering on a 0.7-μm glass fibre disc (filter Sartorius, Minisart GF, Palaiseau, France). Aliquots of this water-soluble fraction and of the extraction residue, i.e. the non-water-soluble fraction, were freeze-dried and weighed for C and ^{13}C bulk analysis.

In a second step, metabolic carbohydrates were extracted from both the water-soluble and the water-insoluble fractions. We divided the water-soluble fraction into major sugars (glucose, fructose, sucrose), proteins and undetermined water-soluble compounds (organic acids and amino acids mainly), and the water-insoluble fraction was divided into starch and non-starch constituents (cellulose and lignin mainly).

The water-soluble fraction was subsampled, and proteins were precipitated in an aliquot heated at 100°C, collected and freeze-dried for C, N and ^{13}C analyses. The protein-free water-soluble extract was filtered (filter HV 0.45 μm type; Nihon Millipore Kogyo, Osaka, Japan) prior to separation by high-pressure liquid chromatography (HPLC) using a Sugar Pak1 column (6.5 mm diameter and 300 mm length; Waters, Milford, Ma, USA), to both quantify and separate the principal soluble sugars (i.e. sucrose, glucose and fructose). In addition to sugar peaks, a large 'undetermined compounds' peak was also observed. Although not precisely determined, this peak corresponds mostly to organic and amino acids. Each of these four fractions (sucrose, glucose, fructose and undetermined solubles) was transferred into tin capsules (Courtage Analyses Services, Mont Saint-Aignan, France) and oven-dried at 50°C before C and ^{13}C analysis.

The water-insoluble fraction was separated into starch and HCl-insoluble residues, mainly cellulose and lignin. Starch extraction was conducted according to Duranceau *et al*.[34] Briefly, the water-insoluble fraction was washed three times with 1 mL ethanol 95% (v/v), at 0°C, to remove the pigments. The water-insoluble fraction was then suspended twice with 1 mL HCl (6 N), transferred to a 10-mL tube, and kept 1 h at 5°C to solubilize the starch. After 20 min centrifugation at 14 000 *g* and 5°C, the supernatant comprising the dissolved starch was retrieved and poured into a 50-mL tube, and another 1 mL of 6 N HCl was added to the pellet to repeat the preceding step, and recover the remaining starch. Pure methanol (four times the total supernatant volume) was added to the 50-mL tube containing the dissolved-starch supernatant. This mix was kept at 5°C overnight to precipitate starch, which was then collected by centrifugation. The starch was dried, weighed, and transferred into tin capsules for C and ^{13}C analysis, according to Wanek *et al*.[36] The quantity of HCl-insoluble residues was calculated by subtracting the water-soluble fraction and the starch from the leaf total organic C.

Carbon analysis

All bulk leaf material and separated fractions, as well as individual sugars, proteins, starch and HCl-insoluble residues, were weighed in tin capsules for EA-IRMS analysis. The EA-IRMS hyphenated system (elemental analyser NA1500 or 2500, Carlo Erba, Milan, Italy – isotope ratio mass spectrometer, Sira 10 or Optima, GV Instruments, Villeurbanne, France) provides both elemental (C and N) and isotopic analysis on the same sample. Carbon-isotope compositions were calculated as deviations of the C isotope ratio ($^{13}C/^{12}C$, called R) from the international standard (Vienna Pee Dee Belemnite, V-PDB).

$$\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 1000$$

The Sira 10 dual-inlet isotope ratio mass spectrometer was used for its high precision: about 0.06‰ standard deviation measured on pure standard compounds.[37] The precision on the Optima-IRMS instrument is lower, less than 0.5‰, but such continuous flow IRMS instruments can run very small samples (until ≈25 μg C) which was especially necessary for sugars collected from HPLC separation.

RESULTS

Elemental composition of leaf material

Selected mature bean leaves represented approximately 44% of the total aerial biomass and 71% of the total leaf biomass. The rest of the total leaf biomass was composed of leaves identified as 'young' (8%) or of 'intermediate age' (21%). The moisture content of the fresh leaves was 88%.

The total C content of the mature leaves was 390 ± 14 mg C g^{-1} DM with a C/N ratio of 9.2 ± 0.4 g g^{-1} (Table 1). About 27% of this total was contained in the water-soluble C fraction, which displayed a C/N ratio of 7.6 ± 0.7 g g^{-1}. The extracted soluble sugar-C represented 19% of the water-soluble C extract, i.e. 5% of the total leaf C. Starch, extracted by HCl hydrolysis, accounted for 8% of the total leaf biomass, expressed on a C content basis. In total, the metabolic carbohydrates contained 51.7 ± 5.0 mg C g^{-1} DM, i.e. 13.2% of total leaf C (Table 1).

Labeling reproducibility

Sources of ^{13}C variability are illustrated with 21-h labeled material (Table 2). The variability was found to be: heterogeneity inside the batch > extraction > instrumental precision. The labeling variability among leaves was evaluated through $\delta^{13}C$ measurements conducted on subsampled individual leaves. The lowest $\delta^{13}C$ values were

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Table 1. C content of different extracted fractions for mature and young leaves at different labeling times. Cold-water-soluble fraction is given separately. Metabolic carbohydrates and remaining C are calculated from corresponding above fractions. Measured values are means ± SD (n = 3)

Mature leaves	1/2 hour	1 hour	2 hours	3 hours	21 hours
Leaf total C (mg C/g DM)	385.5	390.4	389.5	388.5	399.6
Carbon fractions	C (% of total)	C (% of total)	C (% of total)	C (% of total)	C (% of total)
Cold water-soluble fraction	27.0	27.6	26.3	28.0	24.0
s.d.	1.2	0.5	0.8	2.3	0.7
Sucrose	2.4	3.2	4.2	3.8	2.6
s.d.	0.03	0.1	0.2	0.1	0.1
Glucose	0.4	0.6	0.5	0.7	1.5
s.d.	0.02	0.01	0.04	0.04	0.04
Fructose	0.6	1.0	0.9	1.1	1.7
s.d.	0.02	0.1	0.1	0.04	0.1
Starch	9.0	6.8	8.3	8.3	8.5
s.d.	1.6	0.8	1.2	2.3	0.3
$\sum$ = Metabolic carbohydrates	12.5	11.6	13.9	13.9	14.3
s.d.	1.7	1.0	1.5	2.4	0.5
Proteins	1.8	0.8	1.7	1.3	0.9
s.d.	0.1	0.1	0.3	0.2	0.1
Undetermined water solubles	21.8	22.0	19.1	21.1	17.3
s.d.	1.3	0.6	0.7	1.1	0.7
Water and HCl-insoluble fraction	64.0	65.7	65.4	63.7	67.5
s.d.	0.8	0.9	1.5	1.5	1.0
$\sum$ = Remaining fractions	87.5	88.4	86.1	86.1	85.7
s.d.	2.2	1.6	2.4	2.7	1.8

Young leaves	1/2 hour	1 hour	2 hours	3 hours	21 hours
Leaf total C (mg C/g DM)	420.8	421.9	422.4	429.4	419.3
Carbon fractions	C (% of total)	C (% of total)	C (% of total)	C (% of total)	C (% of total)
Cold water-soluble fraction	22.3	24.0	23.1	21.9	23.4
s.d.	0.8	1.0	2.6	1.7	2.2
Sucrose	1.0	1.7	1.4	1.8	2.2
s.d.	0.04	0.1	0.2	0.1	0.1
Glucose	1.5	2.1	1.9	2.3	2.4
s.d.	0.1	0.2	0.3	0.2	0.2
Fructose	1.6	2.3	2.3	2.3	2.1
s.d.	0.1	0.2	0.4	0.2	0.1
Starch	5.8	5.8	6.0	6.6	10.1
s.d.	1.2	0.7	0.4	1.7	0.3
$\sum$ = Metabolic carbohydrates	9.9	11.9	11.6	13.0	16.9
s.d.	1.5	1.1	1.4	2.3	0.7
Proteins	2.9	2.5	1.9	2.3	1.5
s.d.	0.1	0.2	0.2	0.2	0.2
Undetermined water solubles	15.3	15.4	15.6	13.2	15.2
s.d.	1.0	0.9	1.8	0.6	1.8
Water and HCl-insoluble fraction	71.9	70.2	70.9	71.5	66.5
s.d.	1.6	0.7	2.6	1.7	2.4
$\sum$ = Remaining fractions	90.1	88.1	88.4	87.0	83.1
s.d.	2.6	1.8	4.6	2.4	4.4

observed in cotyledonary leaves, (i.e. oldest leaves and lowest in canopy), with mean value of 46 ± 12‰ (n = 5) at 21 h; and the maximum values in young trifoliate leaves (i.e. leaves close to the light, non-shaded and still growing), with mean values of 189 ± 16‰ (n = 3) at 21 h.

Nature and kinetics of ^{13}C-labeling

The δ^{13}C values increased significantly with labeling duration in bulk leaf material and also in water-soluble extracts and water-insoluble residues (Table 3). Unlabeled leaves used as control (Table 3, time zero before labeling) showed a δ^{13}C difference of 3.5‰ between water-soluble and water-insoluble fractions, which was always much lower than the differences observed for the labeled leaves.

We calculated Δ_{sol} as the ^{13}C-enrichment in the water-soluble extract in comparison with that of the water-insoluble fraction, $\Delta_{sol} = \delta^{13}C_{soluble} - \delta^{13}C_{insoluble}$. The ^{13}C-enrichment appeared rapidly, with water-soluble δ^{13}C values increasing

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Table 2. Variability analysis ^{13}C-labeling after 21 h: (a) among-plants variability within a selected batch (n = 6); (b) extraction variability for water-soluble and -insoluble fractions (n = 3); and (c) instrumental variability (EA-IRMS) (n = 6)

	C-content		δ^{13} C		
Variability source	(%)	S.D.	(‰)	S.D.	n
a) Labeling of mature leaves	40.0	1.3	116.1	11.5	6
b) Cold water extraction on one leaf powder:					
- water-soluble extract	26.8	0.8	165.6	6.4	3
- water-insoluble residue	45.5	0.5	95.8	7.0	3
c) Instrumental precision for one leaf powder	40.0	0.2	124.7	0.9	6

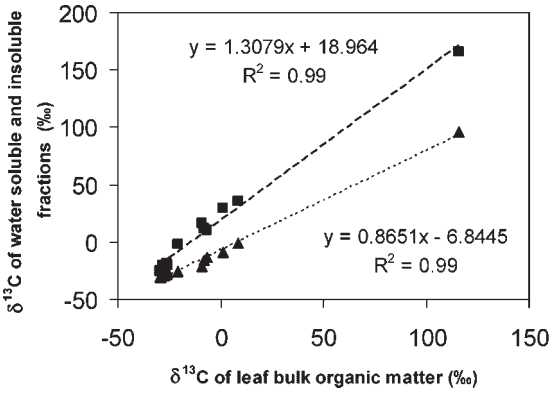

Figure 1. δ^{13}C values of water-soluble (closed squares) and water-insoluble (closed triangles) fractions plotted versus those of leaf bulk organic material. Dashed and dotted lines correspond to the linear regressions for water-soluble and insoluble fractions, respectively. Data points are those obtained during different labeling times. Regressions without the distant dots gave similarly results with $R^2 = 0.97$. Therefore, regressions were kept on datasets with all dots.

by 7‰ after only 30 min of labeling. The water-soluble fraction was always more enriched than the water-insoluble one, but the water-insoluble fraction also exhibited relative ^{13}C-enrichment with time, compared with time zero. Δ_{sol} reached 37.6‰ after 2 h and 69.8‰ after 21 h of labeling.

Weight-average δ^{13}C values of metabolic carbohydrates were calculated from results obtained after extraction of sugars and starch (Table 3). The metabolic carbohydrates displayed the highest rate of ^{13}C-enrichment, while water-HCl-insoluble residues had the lowest. When the δ^{13}C values of the water-soluble and insoluble fractions were plotted versus the δ^{13}C of the leaf bulk organic material, two distinct linear relationships ($R^2 = 0.99$) were observed with different slopes (Fig. 1), indicating that the labeled-C entering each fraction was fully proportional to the ^{13}C-enrichment in bulk leaf material, at least during the 21-h labeling.

The water-soluble fraction of mature leaves was composed of 18 ± 5% w/w major sugars, 5 ± 2% proteins, and 77 ± 4% undetermined water-soluble compounds. For young leaves we obtained 25 ± 4% w/w for the major sugars, 9 ± 3% for proteins and 66 ± 4% for undetermined water-soluble compounds. At all labeling times, sucrose was the most abundant sugar in mature leaves (Fig. 2). By contrast, young leaves contained about equal proportions of sucrose, glucose and fructose. Water-soluble compounds were proportionally more abundant in mature leaves (26.6 ± 1.6%) than in young leaves (22.9 ± 0.9%). Metabolic carbohydrates were present in both leaf categories in equivalent proportions, i.e. about 13% w/w.

All fractions exhibited ^{13}C-enrichment with time, particularly marked from 2 h. Contrasting ^{13}C-labeling was obtained for two groups of compounds: highly labeled water-soluble sugars and starch, i.e. metabolic carbohydrates, on the one hand, and weakly labeled proteins, undetermined water-solubles and HCl-insoluble residues, on the other (Fig. 3). Mature leaves were labeled more quickly than young leaves (Fig. 3). ^{13}C-enrichment was generally very low during the first hour, except for sucrose, glucose and starch in mature leaves with 10 to 20‰ ^{13}C-enrichment. Sucrose was always more enriched than other sugars. After 2 h of labeling, undetermined water-solubles, proteins and HCl-insoluble residues exhibited higher enrichments in young leaves than the mature leaves. As a result, after 21 hours of labeling these

Table 3. δ^{13}C values with labeling duration for mature bean leaves and corresponding water-soluble, water-insoluble and water HCl-insoluble fractions. δ^{13}C values of metabolic carbohydrates are weight-average values for sugars and starch. Δ_{sol} is the ^{13}C-enrichment in the water-soluble extract by comparison with the water-insoluble fraction, $\Delta_{sol} = \delta^{13}C_{soluble} - \delta^{13}C_{insoluble}$. Time zero corresponded to the measurements before labeling (i.e. control values)

Time	δ^{13}C (‰)					
		after cold water extraction			after major sugars and HCl extractions	
hour	leaf bulk organic matter	water-soluble carbon	water-insoluble carbon	Δ_{sol}	metabolic carbohydrates (extracted sugars + starch)	water HCl-insoluble residue
0	−30.0	−27.6	−31.1	3.5		
0.5	−27.9	−20.5	−27.6	7.1	−16.7	−29.5
1	−26.1	−18.5	−28.0	9.5	−13.1	−28.3
2	−8.8	16.8	−20.8	37.6	84.6	−24.9
3	8.5	35.3	−1.2	36.5	128.2	−13.6
21	116.1	165.6	95.8	69.8	414.0	53.4

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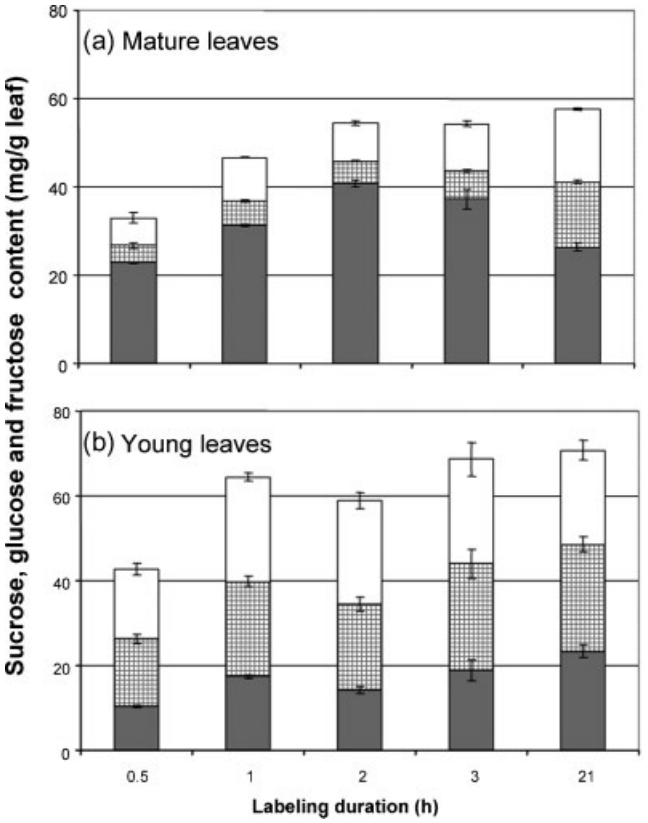

Figure 2. Individual water-soluble sugar contents (mg g^{-1} leaf dry matter) determined by HPLC at different labeling times for bean mature and young leaves (a and b, respectively). Values represent means ±SD (n = 3) for sucrose (grey), glucose (squared) and fructose (white).

fractions were more enriched in young leaves than the equivalent fractions in mature leaves, whereas sugars and starch displayed similar enrichment in both leaf categories. As expected, mature leaves showed higher $\delta^{13}C$ difference between HCl-insoluble residues and sugars (sucrose especially) than young leaves (Fig. 3).

Proteins were the least enriched compounds of the water-soluble fraction, generally less than the HCl-insoluble residues except for mature leaves after 1 and 2 h of enrichment (Fig. 3).

The C contents and $\delta^{13}C$ results for the different fractions were used to back-calculate the $\delta^{13}C$ values for bulk mature leaves. This back-calculation ignores the pigments that were removed during the analytical procedure. These photosynthetic pigments account for about 3% of the leaf biomass,[38] and therefore our analysis is coherent at about 97%. As a means of data quality control, these back calculated values were then compared with direct measurements conducted on the bulk samples (Table 4). A similar procedure was applied to $\delta^{13}C$ values of water-soluble and water-insoluble extracts, which were back-calculated from the sum of their components and compared with direct measurements of the entire fraction. Even without the extracted pigments, these comparisons showed good agreement between measured and recalculated values (Table 4). This confirmed the consistency of quantities and $\delta^{13}C$ results determined on the different components after extractions.

DISCUSSION

Experimental material

Before being exposed to ^{13}C-CO_2, leaf extracts displayed a $\delta^{13}C$ difference between water-soluble and water-insoluble compounds of 3.5‰. A similar difference was reported in previous studies.[39,40] A larger $\delta^{13}C$ difference was also reported when comparing sugars with lipids or lignin.[40] The bulk leaf $\delta^{13}C$ value, -30.0 ± 0.3‰, was consistent with the observed proportions of water-soluble C, 19% leaf-C vs. water-insoluble-C, 81% leaf-C, and $\delta^{13}C$ values measured, -27.6 ± 0.7‰ and -31.1 ± 0.5‰, respectively (n = 8).

Sources of variability in ^{13}C-labeling

Our aim was to obtain quite homogeneous ^{13}C-labeled leaf batches. Both leaf age and position in the growth chamber potentially influence the photosynthetic capacity, which can affect the ^{13}C-isotopic signature.[41] Selecting and preparing mature leaves separately from other leaves yielded a batch with a reasonable $\delta^{13}C$ standard deviation (Table 2). The $\delta^{13}C$ values at 21 h for water-soluble and water-insoluble compounds were well characterized with a standard deviation of 6 or 7‰, much lower than the difference in ^{13}C value between water-soluble and insoluble fractions, i.e. 70‰.

The time of day when the plants had been labeled also seemed to play an important role. When we repeated the same labeling but later in the course of the photoperiod, i.e. with much more starch reserves (results not shown), the labeling for bulk leaf OM was very low (around −25‰ after 2-h labeling) compared with the present results obtained just at the end of the night (around −9‰ for the same labeling duration). We hypothesize that labeling was higher immediately following the night period because leaf starch reserves have been depleted by night-time respiration and need to be reconstituted.[26] The increase in soluble sugar content of bean leaves after 0.5 h light (Fig. 2) supports this hypothesis. Indeed, soluble sugar stocks in plant leaves are generally depleted during the night period, although much less so than leaf starch reserves.[42] This suggests that our method of labeling right at the onset of the light period does indeed maximize ^{13}C-labeling of the metabolic carbohydrate fraction. Providing constant photosynthetic activity, labeling later in the course of the light period should lead to higher enrichment of stems and roots under enhanced translocation. Although we did not measure the ^{13}C signatures of these organs, our results suggest that the nycthemeral cycle should be considered carefully in compound-specific labeling experiments. A possible way to limit dilution of new carbon (labeled) by old carbon (unlabeled) would be to drastically consume starch reserves with a 2-day dark period for plants.[28]

^{13}C-labeling of the metabolic carbohydrates

Our experiments at the metabolite level showed that both the water-soluble and the water-insoluble fractions were isotopically heterogeneous upon such labeling, which agrees with some reported results.[28] By contrast, the metabolic carbohydrate fraction was quite homogeneously labeled, as the starch was as much labeled as the soluble sugars. In

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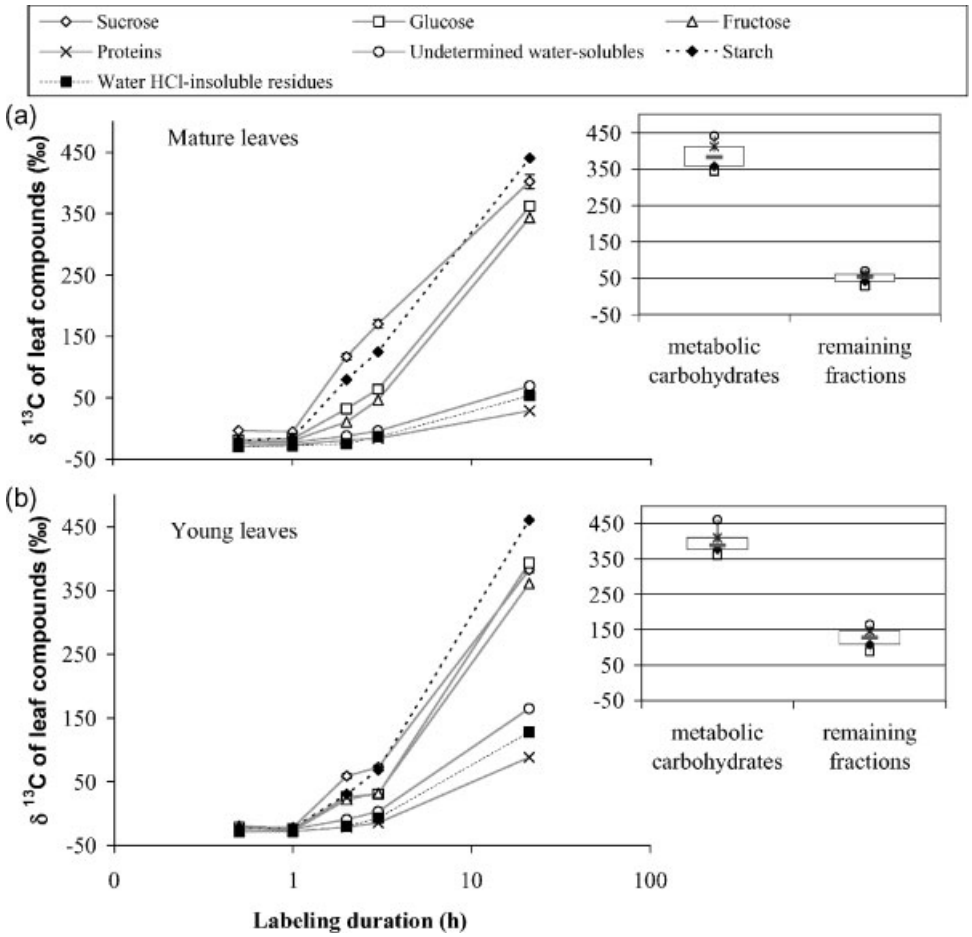

Figure 3. $\delta^{13}C$ values of water-soluble (open symbols) and water-insoluble (closed symbols) compounds isolated from bean mature (a) and young (b) leaves at different labeling times. X-axis is on logarithmic scale. Corresponding box plots for metabolic carbohydrates (sugars and starch) and remaining fractions (undetermined water-solubles, proteins, water HCl-insoluble residues) at 21 h. Symbols for box plots represent min, Q1, median, Q3, max. SD values (n = 3) are shown only when larger than the symbols.

addition, proteins and undetermined water-soluble compounds (i.e. organic and amino acids) had a degree of labeling close to that of HCl-insoluble residues. The rapid and strong labeling of starch is related to its rapid synthesis during photosynthesis,[28] whereas soluble proteins and undetermined water-soluble compounds are not immediately synthesized from the first carboxylation products.

Our method produced a clear differential ^{13}C-labeling of the metabolic carbohydrates vs. the rest of the leaf compounds. Weight-average ^{13}C signatures of the 21-h labeled compounds were 414‰ and 56‰ for the metabolic carbohydrates and the remaining fraction, respectively. The analytical variability on these measurements was low, with a coefficient of variation of about 1%. There was a certain spread in $\delta^{13}C$ value among compounds within each group, with starch displaying a $\delta^{13}C$ value about 13% higher than the average of the metabolic carbohydrates (Fig. 3). However, this spread was limited compared with the large difference in

Table 4. $\delta^{13}C$ values for bulk leaf material, water-soluble and -insoluble fractions measured either directly after first extraction or calculated using the values measured on individual components obtained after the second extraction (n = 3)

Mature leaves	$\delta^{13}C$ (‰) leaf bulk O.M.		$\delta^{13}C$ (‰) water-soluble		$\delta^{13}C$ (‰) water-insoluble	
time (h)	measured	recalculated	measured	recalculated	measured	recalculated
0.5	−27.9 (±0.2)	−26.7 (±0.3)	−20,5	−22.6 (±0.1)	−27,6	−28.3 (±0.4)
1	−26.1 (±0.1)	−25.2 (±0.1)	−18,5	−20.4 (±0.2)	−28,0	−27.1 (±0.1)
2	−8.8 (±1.9)	−7.2 (±1.4)	16,8	9.6 (±0.7)	−20,8	−13.2 (±2.1)
3	8.5 (±0.4)	8.1 (±2.8)	35,3	22.8 (±1.4)	−1,2	2.3 (±3.6)
21	116.1 (±0.9)	107.5 (±6.9)	165,6	141.5 (±3.7)	95,8	96.9 (±8.1)

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$\delta^{13}C$ values obtained between the two groups (Fig. 3). As long as the difference in $\delta^{13}C$ values between groups is large compared with the variability within the group, we estimate that the resulting material is appropriate for C-tracing in soils. Indeed, most labeling techniques suffer from a within-fraction variability in the $\delta^{13}C$ values. The extensively applied ^{13}C-natural abundance method is no exception, with a difference in ^{13}C signature between C_3 and C_4 plants that approximates to 15‰,[41,43] while the spread of ^{13}C-compound-specific signatures within a species can reach up to 7‰.[41,42]

CONCLUSIONS

Stable isotope analysis is a major tool used to establish pathways and rates of C fluxes between various ecosystem components. Developing stable isotope techniques is still needed to investigate C dynamics in soils. Here, we successfully tested a pulse ^{13}C-CO_2 method for the differential labeling of the metabolic carbohydrate vs. the remaining fraction of bean leaves. Our method strongly labeled the metabolic carbohydrate fraction, which is composed of simple sugars and starch. This fraction is both labile and non-structural, and therefore immediately or rapidly mobile in soils. This first success suggests that other categories of molecules could be specifically labeled depending on research needs. For example, multiple methods could be tested to specifically label simple sugars exclusive of starch. Labeling could be conducted on starch-less mutants of Arabidopsis.[26] Labeling could potentially be conducted towards the end of the light period, when starch reserves have been replenished. The chosen plant material could be sugar-translocating stems rather than leaf-accumulation starch. Even with the best methodology, a certain compound-specific heterogeneity in the ^{13}C-labeling will remain unavoidable, for example among sugars. The advantage of the present pulse labeling method vs. previous approaches is that it allows us to follow the fate of molecular groups in undisturbed plant residues, which is especially useful for C tracing in decomposition studies.

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