Plant and Soil **267:** 243–253, 2004.
© 2004 *Kluwer Academic Publishers. Printed in the Netherlands.*

The dynamics of neutral sugars in the rhizosphere of wheat. An approach by ^{13}C pulse-labelling and GC/C/IRMS

D. Derrien[1,2], C. Marol[1] & J. Balesdent[1]
[1]*Laboratoire d'Ecologie Microbienne de la Rhizosphère, UMR 6191 CNRS/CEA/ Univ. Aix Marseille II, DEVM/DSV, CEA de Cadarache, 13108 Saint Paul lez Durance, France.* [2]*Corresponding author**

Received 13 January 2004. Accepted in revised form 29 March 2004

Key words: ^{13}C, GC/C/IRMS, rhizodeposition, rhizosphere, sugars, wheat

Abstract

Rhizodeposition, i.e. the release of carbon into the soil by growing roots, is an important part of the terrestrial carbon cycle. However the *in situ* nature and dynamics of root-derived carbon in the soil are still poorly understood. Here we made an investigation of the latter in laboratory experiments using $^{13}CO_2$ pulse chase labelling of wheat (*Triticum aestivum* L.). We analyzed the kinetics of ^{13}C-labelled carbon and more specially ^{13}C carbohydrates in the rhizosphere. Wheat seedlings-soil mesocosms were exposed to $^{13}CO_2$ for 5 hours in controlled chambers and sampled repeatedly during two weeks for $^{13}C/C$ analysis of organic carbon. After a two-step separation of the soil from the roots, the amount of total organic ^{13}C was determined by isotope ratio mass spectrometry as well as the amounts of ^{13}C in arabinose, fructose, fucose, glucose, galactose, mannose, rhamnose and xylose. The amount and isotopic ratio of monosaccharides were obtained by capillary gas chromatography coupled with isotope ratio mass spectrometry (GC/C/IRMS) after trimethyl-silyl derivatization. Two fractions were analyzed : total (hydrolysable) and soluble monomeric (water extractable) soil sugars. The amount of organic ^{13}C found in the soil, expressed as a percentage of the total photosynthetically fixed ^{13}C at the end of the labelling period, reached 16% in the day following labelling and stabilised at 9% after one week. We concluded that glucose under the form of polymers was the dominant moietie of rhizodeposits. Soluble glucose and fructose were also present. But after 2 days, these soluble sugars had disappeared. Forty percent of the root-derived carbon was in the form of neutral sugars, and exhibited a time-increasing signature of microbial sugars. The composition of rhizospheric sugars rapidly tended towards that of bulk soil organic matter.

Introduction

The release of organic compounds from growing roots into the soil, a phenomenon called rhizodeposition, is a very significant flow in the terrestrial carbon cycle. Expressed as a proportion of the net primary production of ecosystems, rhizodeposition has been reported to frequently reach values in the range 8 to 12% (Grayston et al., 1996; Nguyen, 2003). It is therefore expected that rhizodeposed carbon contributes in similar proportions to the soil carbon budget, i.e., to heterotrophic respiration, soil organic matter formation or primings (Kuzyakov, 2002). The fluxes of total carbon released into the soil by roots have been typically estimated in several ecosystems using isotopic labelling of assimilated CO_2 by ^{14}C (Nguyen, 2003; Warembourg and Paul, 1977) or ^{13}C (Palta and Gregory, 1997). To take into account this part of the carbon cycle in soil carbon models, the knowledge of the nature of rhizospheric compounds and their rates of biotransformations would ideally be required. Because rhizodeposition includes a great variety of low molecular weight compounds, proteins, mucilages, cell lysates and wall constituents (Rovira et al., 1979), there is a need of bulk characterization and quantification of the molecules that are released into the soil by the roots, and of their products of biotransformation.

*FAX No: +33 4 42 25 66 48.
E-mail : delphine.derrien@cea.fr

The bulk nature of low molecular weight exudates is better known at present by profiling approaches of root solutions in hydroponic cultures (Fan et al., 2001). However the extrapolation of such experiments to *in situ* conditions is limited by the absence of the soil environment, where the physical, nutritional, chemical and microbial factors have a determinant impact on the exudation (Meharg and Killham, 1995; Palta and Gregory, 1997). The extraction of the rhizosphere solution has brought important insights to the *in situ* nature of exuded compounds (Neumann and Römheld, 2001), but macromolecular and insoluble compounds are generally not quantified.

Our present study is an attempt to investigate the nature and dynamics of root-derived carbon in the soil by coupling pulse-chase isotope labelling of photoassimilated $^{13}CO_2$ with the subsequent quantitative analysis of labelled molecules in the soil. We investigated the pool of neutral sugars, which is cited as the most abundant family of exudates (Merbach et al., 1999), and constitutes a significant portion of soil organic matter (Oades et al., 1970). Winter wheat seedlings and the plowed horizon of a cambisol cultivated for continuous wheat were chosen as model materials. We carried out pulse-labelling which enables the access of the dynamic fluxes of carbon through the plant to the soil and in the rhizosphere. The use of ^{13}C allowed the determination of compound specific isotope ratios by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). The technique has been developed to separate molecular compounds in a gas chromatography column before oven combustion so as to analyze the $^{13}C/^{12}C$ ratio of each individual compound. The analysis of neutral sugars by gas chromatography requires a derivatization of the polar groups and involves the addition of several unlabelled carbon atoms per sugar. Different techniques have been applied to GC/C/IRMS for various monosaccharides in biogeochemistry. Alditol acetate derivatization was used by Macko et al. (1998) and the methylboronic derivatization, which reduces the number of added atoms was adapted recently by van Dongen (2001). Here we used trimethylsilylation. Despite the addition of numerous carbon atoms trimethylsilylation appeared convenient for labelling experiments (Derrien et al., 2003), and can be used in the common analysis of other polar compounds. We applied it to the study of the dynamics of neutral monosaccharides in two pools of rhizospheric carbon : soluble monomers and total hydrolysable sugars.

Materials and methods

Plant culture

The experiment was carried out in the laboratory with winter wheat (*Triticum aestivum* L. cv Taldor) on an eutric cambisol continuously cultivated for winter wheat for eight years at the experimental field of the Institut National de la Recherche Agronomique at Versailles (Ile-de-France, France). Topsoil is characterized by (g kg^{-1}): clays 0–2 μm (174 ± 9), fine silt 2–20 μm (208 ± 7), coarse silt 20–50 μm (323 ± 31), fine sand 50–200 μm (243 ± 25), coarse sand 200–2000 μm (52 ± 3), organic carbon (13.5 ± 1.2) and nitrogen (1.27 ± 0.06). Its water pH is 6.8 ± 0.2, its CEC 120 ± 3 meq kg^{-1}, the exchangeable cations are (meq kg^{-1}) Ca^{2+} (112 ± 3), Na^{+} (0.6 ± 0.3), Mg^{2+} (8.8 ± 0.5), and K^{+} (5.1 ± 1.1). The soil was sieved at 2 mm and adjusted at 0.18 g water g^{-1} dry weight. This water content corresponded to a matric potential of 0.1 MPa in this soil. Two hundred grams dry weight were then placed into cylindro-conical pots (height 180 mm, diameter 49 to 42 mm). One plant of wheat was sown per pot. The shape of the pot had optimized the development of a dense root net, what interested us in our study.

Nineteen plants were grown in a chamber equipped for automatic control of light, temperature, moisture, evapotranspiration, irrigation and CO_2 concentration (chambers developed by the Groupement de Recherches Appliquées en Phytotechnologie, CEA Cadarache, France). The day-night period was set at 12/12 hours, light was 400 μmol photons m^{-2} s^{-1}, day temperature was 23 °C and night temperature 20 °C, air moisture was adjusted at 80% and CO_2 concentration was maintained at 350 μL L^{-1}. At least, soil moisture was manually controlled.

The respect of CO_2 partial pressure was assured by gas injection when CO_2 was consumed by photosynthesis or by gas trapping when CO2 was released by respiration. Injections and trapping being measured, it was possible to quantify the wheat culture carbon fluxes. Three weeks after germination, the seedling at tillering stage photosynthetised 4 mL CO_2 $hour^{-1}$ $plant^{-1}$ and respired 0.5 mL $hour^{-1}$ $plant^{-1}$ at night.

$^{13}CO_2$ pulse labelling

The pulse labelling was proceeded three weeks after germination on fifteen of the wheat plants, chosen for

homogenous size. One plant was used for the reference of unlabelled isotopic composition. The plants were exposed during 5 h to CO_2 with an abundance of ca. 70% $^{13}CO_2$. The ^{13}C enrichment was performed as follow. First, the CO_2 concentration of the chamber housing the fifteen wheat plants was lowered to 80 μL L^{-1} by flushing it with CO_2-free air. After that, pure CO_2 with an abundance of 98.9% ^{13}C (Eurisotop) was quickly injected so that CO_2 concentration reached about 350 μL L^{-1}. This should happen rapidly (in 2 min) to prevent CO_2 lack stress. Then, during the 5 h pulse-labelling, $^{13}CO_2$ was injected at a constant rate to balance the net expected fixation of CO_2 (photosynthesis - respiration) of the 15 seedlings. At last, enriched CO_2 was instantaneously flushed by opening the chamber to room air and the chamber was thereafter continuously flushed with $^{13}CO_2$-free air to avoid photoassimilation of respired ^{13}C.

Plants and soil samples separation

To determine the kinetics of rhizodeposition, plants and soils harvests occurred at 5, 24, 48, 183 and 354 h after the beginning of the labelling. At each time, 3 pots were chosen randomly. The shoots were clipped and the roots were separated from the soil in a two step procedure. The method should allow to recover the soil material adhering to the roots, and avoid that fragments of roots remain in the soil, what would considerably dodge the estimation of rhizodeposition. At first, the rooting systems were manually separated from the soil and gently washed with ca. 40 mL K_2SO_4 0.025 *M* over a sieve. The rinsing suspension was added to the soil. Then, the soil was put in suspension K_2SO_4 0.025 *M* during 15 min and sieved over a 500 μm mesh sieve so that small roots were separated from soil. K_2SO_4 0.025 *M* was chosen as a dispersing agent because it is isotonic for root cells and achieves a good dispersion of clay material.

The K_2SO_4 solution was separated from the sediment by centrifugation (1600 $\times$ *g*) during 20 min. Both fractions were freeze-dried separately for further processing. The K_2SO_4 solution will be referred as the 'soluble' fraction. The total soil material was reconstituted from the two freeze-dried fractions pooled together and will be referred as 'soil'. Shoots and roots were oven dried at 60 °C and ground finer than 250 μm. Oven-dried shoots and roots like of soil fractions were intended for total C and ^{13}C analysis. Soil and the water-soluble fraction were moreover meant for sugar ^{13}C determinations.

Preparation of sugars

Prior to gas chromatograph analysis, soil sugars were hydrolyzed into their monomers. The hydrolysis of polysaccharides into monosaccharides should release a maximum of monosaccharides but has to minimize alteration and recombination of sugars with other organic compounds. We used the methods of Oades et al. (1970) and Larre-Larrouy and Feller (1997) recommending the use H_2SO_4 in sequential treatment. A first hydrolysis allowed the release of non-cellulosic carbohydrates, then in a second stage, cellulosic material was hydrolysed.

Soil (2.5 g) was refluxed with boiling 2.5 *M* H_2SO_4 for 20 min and vacuum-filtered through a glass fiber filter (GF/C Whatman cat no. 1822125). The soil remaining on the filter was then soaked in 2 mL H_2SO_4 12 *M* during 16 h, at room temperature. Then, after dilution of the acid to 0.5 *M*, it was refluxed for 5 h. The second hydrolysate was collected by the same filtration process than before and added to the first hydrolysate. Myoinositol was brought in the hydrolysate as an internal standard for further sugar quantification. Then SrCO3 was added to the warm solution in stoechiometric proportions to neutralize the solution. The $SrSO_4$ precipitate was rinsed several times on glass fiber filter with distilled water. This neutralization also stood for a purification of the hydolysate from the high quantities of sulfate and soil minerals-derived inorganic impurities such as Al, Si, Fe. Finally, the solution so obtained was dried under vacuum.

The soil hydrolysates corresponding to 0.5 g of soil or soluble fraction containing ca. 30 μg of carbohydrates were dissolved in 200 μL 1-methylpyrrolidinone (NMP) for 30 min at 60 °C for equilibration of anomeric forms. The derivatization of sugars was finally performed by adding hexamethyldisilazane (HMDS) in trimethylchlorosilane (TMCS) and pyridine (3:1:9 v/v/v; Supelco, sylon HTP, 250 μL) during 20 min at 60 °C.

Analytical methods

Total carbon and $^{13}C/C$ of solids (soil, water-extract, roots and shoots) were determined using an Elemental Analyser (elemental analyser Flash EA, ThermoFinningan) coupled to an Isotope Ratio Mass Spectrometer (IRMS, Deltaplus, ThermoFinnigan).

Sugars separation was achieved after Larre-Larrouy and Feller (1997) on a Trace GC Gas Chromatograph (ThermoFinningan). The capillary column was a DB5 (30 m $\cdot$ 0.25 mm i.d. $\cdot$ 0.25 μm film),

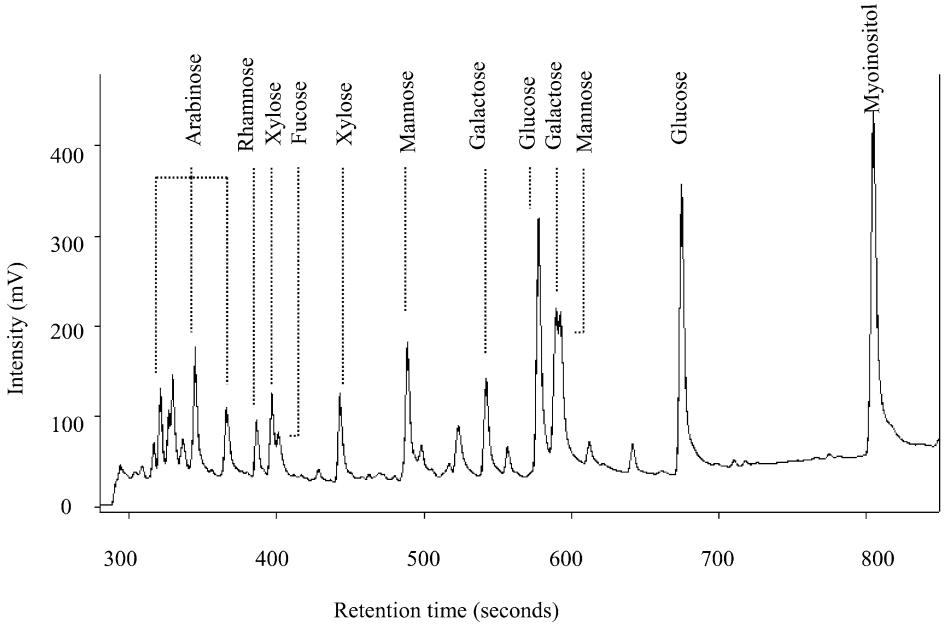

Figure 1. Chromatogram of soil sample hydrolysate (trimethylsilylated compounds).

using helium as carrier gas (constant column flow : 16.2 mL min^{-1}). The injector temperature was held to 265 °C in the split-mode (split ratio 1:40). The column temperature program started at 170 °C and ended at 230 °C with a 4 °C min^{-1} increment and a 4 minute step at final temperature. Isotope ratio mass spectrometer was the detector (Delta^{plus}, ThermoFinnigan).

An aliquot of 1 μL of derivatized sample was injected into the column. In the GC/C/IRMS analysis, the column effluent was driven to an oxidation furnace consisting in CuO, NiO and Pt wires at 950 °C. They allowed the combustion of molecules to CO_2, H_2O and nitrogen oxides. CO_2 entered the continuous flow IRMS and provided $^{13}CO_2$ enrichment values for each kind of molecule separed by GC. Standard sugars (arabinose, xylose, ribose, fucose, rhamnose, galactose, fructose, mannose, glucose and sucrose with myoinositol as an internal standard) prepared as soil samples were used for identification and calibration. These sugars in their various anomeric forms showed different retention times which were compared to those of soil samples (Figure 1). The concentrations of soil sample sugars so-identified were determined relatively to myoinositol, and corrected from the GC response factor. Some glucose- and rhamnose-spiked preparations were as well tested to check the effect of the neutralisation-derivatisation process on sugar extraction yields and to valid the non-dependence of the latter on sugars nature or amount. For the determination of isotopic ratios, the ^{13}C enrichment value adopted was the one of the best defined peak among the different anomers of each sugar.

Notations and calculations

The ^{13}C atomic abundance, expressed in percent and noted AT $^{13}C(\%)$, is defined as

$$AT^{13}C(\%) = {}^{13}C/({}^{13}C + {}^{12}C) \cdot 100.$$

In our experiment, we had artificially enriched the soil-plant system in ^{13}C. What interested us was also the part of the ^{13}C we had injected that remained in the system. That means the excess of the ^{13}C form compared to a natural unlabelled environment. The excess, noted E $^{13}C(\%)$ was calculated as

$$E^{13}C(\%) = AT^{13}C(\%)_{\text{labelled}} - AT^{13}C(\%)_{\text{unlabelled}}.$$

Consequently the ^{13}C quantity in whatever analysed fraction corresponds to the total carbon amount of a solid fraction (EA data) or of a molecular fraction (GC data) multiplied by the isotopic excess (given by the IRMS).

Trimethylsilylation involved the addition of several carbon atoms per sugar. These atoms have to be taken into account in the estimation of the carbon isotope ratio. In a previous study, we demonstrated that the number of analyzed methyl-silyl carbon atoms

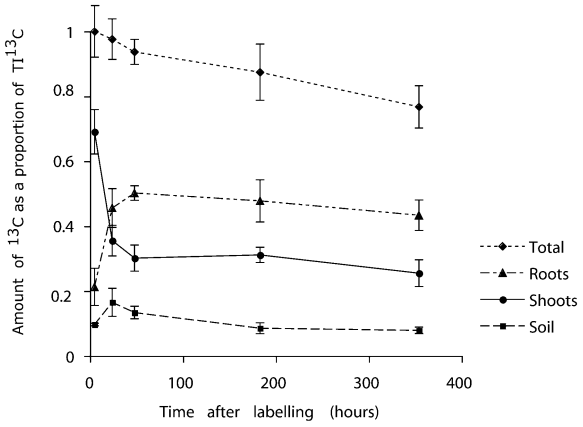

Figure 2. Dynamic allocation of fixed ^{13}C to plant and soil compartments. Time 0 refers to the beginning of the labelling period. 'TI ^{13}C' refers to the amount of organic ^{13}C (4 mg mesocosm^{-1}) at the end of the labelling period (5 h). Bars correspond to the standard deviation of 3 different mesocosms sampled at each date.

wasn't stoechiometric, due to incomplete derivatization and/or incomplete oxidation of methylsilyl carbon before IRMS (Derrien et al., 2003). We therefore calculated the isotopic ratio of each sugar using a specific calibration based on the analysis of standard and natural pentoses and hexoses of known ^{13}C enrichments. Using this calibration, the isotope excess of enriched samples could be determined with a relative error of 5% or less (Derrien et al., 2003), which was considered as sufficient for the present study.

Results

Allocation of photoassimilated carbon

During the two weeks of the experiment following pulse labelling, the wheat biomass had increased. The dried shoot mass passed from 176 ± 6 mg to 369 ± 22 mg whereas the dried roots mass raised from 138±24 mg to 330±47 mg. The regression of biomass over time indicates an average daily mass increase of 28 mg day^{-1} plant^{-1} during the experiment.

The kinetic allocation of ^{13}C photoassimilated carbon in shoots, roots and soil was determined by EA-IRMS. Respired labelled $^{13}CO_2$ wasn't measured in this experiment. In Figure 2 it can be seen that total organic ^{13}C decreased by ca. 20% throughout the experiment. This carbon loss can be attributed to respiration, but doesn't represent all the respiration of labelled C : most of plant respiration of fixed carbon generally occurs very rapidly (Warembourg et al., 2003), and part of it happened during the 5 h-long labelling phase. So the exact flow of gross photosynthetic fixation was unreachable here. For this reason, the amounts of ^{13}C in the various compartments are expressed as a portion of the total organic ^{13}C at the end of the 5 h labelling (Total Initial ^{13}C), noted TI^{13}C. This TI^{13}C corresponded to 4.0 ± 0.3 mg ^{13}C per plant.

Just after the assimilation the 4 mg of photoassimilated ^{13}C were mainly localised in shoots (70% of the total). However, a non-negligible proportion had already been transferred to the belowground compartment: 20% of ^{13}C were localised in roots, 10% in soil. The high proportion of ^{13}C in the shoots decreased rapidly during the first 48 h following the assimilation and was stabilized at 30% of the TI^{13}C until the end of the observation. On the contrary, an increase of ^{13}C content was observed in roots. Forty-eight hours after the beginning of labelling, the ^{13}C in roots represented 50% of the TI^{13}C. The tendency after 2 days was as well a stabilization of the ^{13}C quantity, with a very low decrease. Allocation of ^{13}C to soil reached 16% of the TI^{13}C 24 h after the pulse labelling, and stabilized thereafter to 9% of it. Finally, the distribution of ^{13}C in compartments two weeks after labelling, expressed as proportions of the total ^{13}C at this date was as follows : shoots 33 ± 5%; roots 56 ± 4%; soil organic 10 ± 1%.

Rhizospheric sugars identification

Once the carbon balance had been established on the different compartments of the system, we focused on the soil compartment. The gas chromatography calibrations that we performed allowed us to identify and quantify seven monosaccharides commonly attributed to the soil environment (Chantigny et al., 2000) (Table 1). The amount of sugars in soil samples exhibited no significant differences among dates and plants. This constancy is in agreement with the large and constant amount of soil organic matter concerning total sugars. So the identified monosaccharides totalized 2.6 mg of sugar per gram of soil, what is about 1.1 mg C g^{-1} of soil. They constituted almost 10% of soil organic matter here. Among them, glucose was the dominant moiety (37%). Mannose and galactose represented 17 and 15%, respectively of the analyzed sugars. Arabinose, xylose and rhamnose contributions were less important (12, 9 and 7%) while very few fucose (3%) and no fructose were identified.

Table 1. Identification and quantitfication of main soil and root monosaccharides. Amounts are expressed in mg.g^{-1} of soil or root dry matter. n.d.: not determined

	Arabinose	Xylose	Rhamnose	Fucose	Mannose	Galactose	Glucose
Soil	0.295	0.222	0.144	0.187	0.432	0.382	0.940
Root	39	169	<10	n.d.	<20	55	220

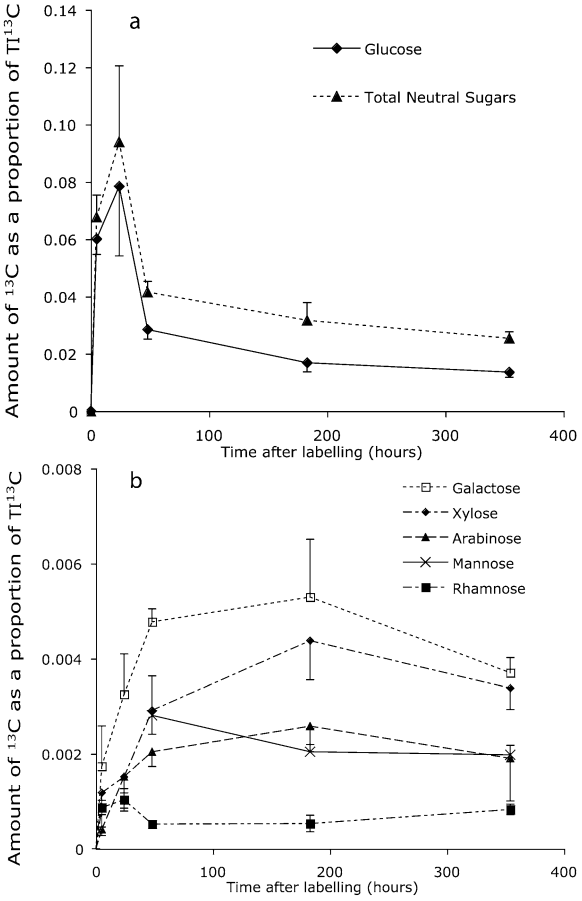

Figure 3. Dynamics of soil labelled sugars. a : Total soil carbon, sum of neutral sugars and glucose. b : Minor sugars. The two first points for mannose are not presented, due to possible confusion with fructose. Sames axes as in Figure 2. Bars correspond to the standard deviation of 3 different mesocosms sampled at each date.

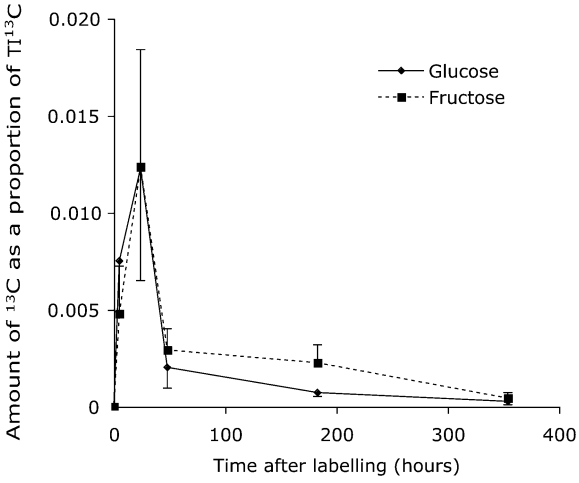

Figure 4. Dynamics of labelled monomeric sugars in the water soluble fraction. Same axes as in Figure 2. Bars correspond to the standard deviation of 3 different mesocosms sampled at each date.

Dynamics of rhizospheric sugars

The dynamics of soil labelled sugars are presented on Figures 3 a and b. Amounts of labelled glucose were 10 times larger than the ^{13}C from others monosaccharides. Labelled monosaccharides, i.e. monosaccharides built from rhizodeposed organic carbon, exhibited different dynamics. Labelled glucose already amounted to 6% of the TI^{13}C at the end of the 5 h labelling (Figure 3a). This proportion peaked at almost 8% one day after labelling, decreased very quickly the day after and remained stable at 2–3%.

Amounts of ^{13}C galactose, xylose and arabinose evolved in a different way and without sharp variations (Figure 3b). They reached their maxima one week after labelling (galactose corresponding to 5.10^{-3} of TI^{13}C, xylose to 4.10^{-3} of ^{13}C and arabinose to 3.10^{-3} of it). Very small declines of these proportions were observed during the second week of the experiment.

At last, even if very small amounts of labelled rhamnose had been detected (between 1.10^{-4} and 1.10^{-3} of the TI^{13}C), a small peak had been noted one day after labelling. ^{13}C fucose quantification wasn't possible because of too small quantities and consequently too bad defined peaks. Due to possible overlapping of peaks from fructose and mannose, the estimation of the ^{13}C amount of these sugars was more difficult (see discussion).

The analysis of the soluble fraction provided complementary information. Kinetics are reported on Fig-

ure 4. In this fraction we only identified some glucose and fructose at a ratio close to 1:1. Both of them behaved in the same way. After the end of the pulse labelling, soluble monomeric labelled glucose represented 7.10^{-3} of $TI^{13}C$ and soluble monomeric labelled fructose 5.10^{-3} of it. They both peaked at $1.2.10^{-2}$ of $TI^{13}C$ the day after and brutally decreased to 2.10^{-3} of $TI^{13}C$ 48 h after labelling. The amount of ^{13}C-sucrose in the 24 h extract was also determined and was almost nil. The amount of soluble monomeric glucose (sum of labelled and unlabelled) increased during the experiment from 4 to 7 μg g dry soil^{-1}, according to plant growth.

Discussion

Evaluation of the method

Characterization of labelled carbon left in the rhizosphere after pulse labelling experiments in real soil conditions, using either ^{14}C or ^{13}C, are virtually absent in the literature. Our case study based on ^{13}C labelling and IRMS can serve as an evaluation of the method we used for this characterization. In essence, we used hydrolysed soil organic matter as a diluter of the labelled rhizospheric sugars. This specific approach had advantages and possible drawbacks. One of the advantages was the ability to analyze, under real soil conditions, the total carbon in the soil independent of any method of extraction of the rhizospheric part of soil compounds. Another advantage concerned the sensitivity of the final result, i.e., the amount of ^{13}C-sugars. Due to the constancy of the amount of the diluter (here soil organic sugars) throughout the experiment, the estimate of labelled sugars depended only on the variations in the isotope ratio, and not on variations in the amount of the molecule, giving a high robustness in the kinetics. A disadvantage of this dilution was the difficulty in searching for labelled compounds that are present only in trace quantities in soil organic matter. This might be the case of ^{13}C-fructose, which was clearly detected in the water extract, but couldn't be assessed in the bulk sample due to the very small fructose content in soil organic matter. We also evaluated the risk of having missed some important ^{13}C labelled compounds in the trimethyl-silylated hydrolysate, by calculating the excess amount of ^{13}C over the whole GC chromatogram including the baseline. We could therefore estimate that the analyzed neutral sugars represented more than 90% of the labelled carbon in the GC chromatogram, and revealed a few highly labelled uncharacterized molecules (data not shown).

Trimethylsilylation of sugars presented the advantages of rapid preparation and of being a proven process. Numerous applications of this method exist for the identification of sugars in soils and sediments (Amelung et al., 1996; Larre-Larrouy and Feller, 1997; Ogier et al., 2001). The difficulty in the identification due to the presence of several anomers was partially overcome by the constancy of the ratio between anomers and the use of a single peak for the isotope ratio measurement. Finally, this analysis of silyl-derivatized compounds appeared convenient, but required a specific calibration of the number of methyl-silyl carbon atoms measured by IRMS (Derrien et al., 2003). The uncertainty on the isotope excess associated with this calibration was less than 5% relative to the estimated excess, and constant for a given sugar. We finally evaluated this error as negligible in comparison to other sources of variations or artifacts.

One possible source of error was the potential coelution of molecules at the same retention time in the GC, and risk of misinterpretation when a highly labelled minor compound is coeluted with a weakly labelled major one. This might have been the case for the couple fructose-mannose during the exudation phase. Another risk of artifact concerned the soil-root separation. This problem is of concern in all similar studies (Swinnen et al., 1994a). Here we minimized root tip injury and root cytoplasm extraction by a step by step procedure : separation of soil on the fresh, solid sample, gentle washing of the rooting system with 40 mL K_2SO_4, and separation of coarse particles from the soil by wet sieving. We determined the amount of fine root ^{13}C, which was recovered by wet sieving the soil. This accounted for 2% of the total root ^{13}C on average. This amount was very small when compared to the total root ^{13}C, indicating that the proportion of fine root or root debris broken through the first two separations was negligible. Nevertheless, the possible contribution of some root cytoplasmic content, root caps and root hairs in the 'soil' sample cannot be excluded. This question was mainly of concern for the interpretation during the first 48 h. After this time less ^{13}C was expected in root cytoplasms and root tips, and labelled border cells as well as root hairs have been naturally sloughed off into the soil (Nguyen, 2003).

Allocation of labelled carbon to the plant-soil compartments

To evaluate the extent to which the labelling phase was representative of the current plant metabolism, we compared the net fixation of ^{13}C to the biomass increase. We had evaluated the initial photosynthetic fixation as the ^{13}C content of the system at the end of the 5 h labelling (4 mg ^{13}C plant^{-1}), and the net fixation of assimilated ^{13}C as the amount of plant ^{13}C after one week, when re-allocation was complete (3.1 mg ^{13}C plant^{-1}). Considering both the isotope excess of chamber CO_2 (70%) and the duration of the labelling phase (5 h over 12 h of daylight) this net fixation of assimilated ^{13}C corresponded to 11 mg C day^{-1} plant^{-1}), or 24 mg dry matter day^{-1} plant^{-1}. The latter value was in general agreement with the plant biomass increase during the experiment (28 mg dry mass day^{-1} plant^{-1}), and proved the absence of stress during the labelling phase.

Most of the re-allocation of carbon between organs occurred within 24 h and was almost complete within 48 h (Figure 2). The remarkable feature was the unusually high root ^{13}C to shoot ^{13}C ratio (1.5 at 1 week). This high ratio could be related to both the tillering stage (Swinnen et al., 1994b) when carbon is reallocated belowground for further growth, and to the relatively low soil moisture (matric potential 0.1 MPa), known to favour root growth (Palta and Gregory, 1997). The labelled root to shoot ratio was higher than that of unlabelled C, which was 0.8 at labelling and 0.9 two weeks after at the end of the experiment. It was also higher than the ratio of root biomass increase to shoot biomass increase, which was equal to 1.0 throughout the experiment, indicating that the allocation to roots was highest at the beginning of the experiment and decreased thereafter. For these reasons, the belowground allocation of ^{13}C observed here can be considered to be particularly high. In a review of similar labelling experiments of annual plants, Nguyen (2003) reported a mean root to shoot allocation of labelled carbon of ca. 0.5.

We considered here that organic ^{13}C in soil was originated from root-derived organic carbon. It has been recently suggested that some ^{13}C might be fixed by direct incorporation of CO_2 carbon into microbial metabolism (Miltner et al., 2003). However this amount could be considered as minor in our case, according to the data of these authors.

The allocation of ^{13}C to soil was high when compared to the TI^{13}C and reached 16% at the maximum.

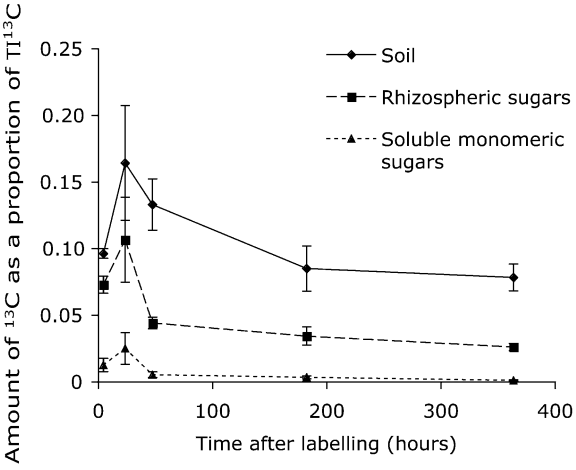

Figure 5. Contribution of neutral carbohydrates to labelled carbon in the rhizosphere. Amounts are expressed as a proportion of total photoassimilated ^{13}C, mean and standard deviation of thre mesocosms.

It is difficult to choose a basis for the comparison of fluxes of rhizodeposition in the literature. Nguyen (2003) proposed expressing the flow as a proportion of root growth. This appears convenient since this can be performed in *in vitro* studies and can be approximated in the pulse labelling experiments as the ratio of the labelled organic carbon in the soil to the labelled root carbon. In our case, this ratio peaked at 0.4 at 24 h and stabilized at 0.18 after 1 week. These values were in good agreement with the mean value of 0.33 reported in the literature by Nguyen (2003) from more than 60 data sets of pulse labelling of annual plants.

Water soluble ^{13}C accounted for the majority of labelled carbon in the soil at 24 h, but decreased sharply between 24 and 48 h, becoming negligible thereafter (Table 2). This decrease can be interpreted in several manners. Re-absorption of small molecules and sugars by the roots was demonstrated by Jones and Darrah (1996) in the case of maize. Root cytoplasmic soluble carbon, if any, would also have been allocated to other cell components. Finally biodegradation of soluble compounds in durations from hours to days is generally invoked as the main cause of soluble carbon decrease (Cheng et al., 1993; Warembourg and Billès, 1979).

Dynamics of sugars in the rhizosphere

Soil neutral sugars accounted here for 10% of the soil organic matter. This value, as well as the proportion of individual sugars, was in good agreement with other values in cultivated soils (Amelung et al.,

Table 2. Amount and repartition of labelled carbon in soil. Proportion of total ^{13}C, ^{13}C neutral sugars and ^{13}C glucose in the total soil and soluble fractions are expressed relatively to total labelled

Time after labelling (hours)	Total ^{13}C in soil (μg)	In soil (% of total soil ^{13}C)			In water extract (% of total soil ^{13}C)		
		^{13}C	Sugars ^{13}C	Glucose ^{13}C	Soluble ^{13}C	Soluble sugars ^{13}C	Soluble glucose ^{13}C
5	384	100	75 ± 4	63 ± 4	56 ± 5	13 ± 5	8 ± 3
24	655	100	65 ± 3	48 ± 4	67 ± 4	15 ± 4	7 ± 2
48	530	100	36 ± 4	23 ± 3	46 ± 12	4 ± 1	1 ± 1
183	339	100	41 ± 8	20 ± 4	39 ± 1	3 ± 1	1 ± 0
364	312	100	31 ± 1	16 ± 1	22 ± 9	1 ± 1	0 ± 0

1996; Larre-Larrouy and Feller, 1997; Oades et al., 1970). The first information obtained from this study concerned the contribution of neutral sugars to the rhizospheric carbon (Table 2). We demonstrated here, in the case of wheat, that they are the large dominant family of components released into the soil by living roots (Figure 5, Table 2: 65% of root-derived carbon was under the form of neutral sugars the first day after labelling). In the course of the experiment, the relative contribution of labelled sugars to ^{13}C in soil decreased. This might mean that a significant part of these sugars were metabolized by micro-organisms and their organic carbon bio-transformed into others microbial molecules. Another explanation would involve the delayed rhizodeposition or a longer life-time of some other root-derived molecules such as lipids, proteins, acidic sugars.

The difference between total sugars and soluble monomers (Table 2) corresponded to polymerized sugars. They might be present as homo-, heteropolysaccharides or glycosyled compounds. As we didn't hydrolyze the water soluble fraction, the soluble polymerized sugars weren't quantified. Nevertheless in Table 2, it can be seen at 24 h that the amount of polymerized sugars carbon (sugars ^{13}C – free sugars $^{13}C = 50\%$ of ^{13}C in soil) exceed the one of non-soluble carbon (33% of ^{13}C). This indicated that a substantial part of the sugarswere present here as soluble polymers. Free glucose and fructose contributed as well to the rhizodeposition flux during the day following the labelling. The soluble sugars accounted for ca. 22% of the total soluble ^{13}C 24 h after labelling. This tended to indicate that water-soluble rhizodeposits were dominated by combined sugars such as in mucilages and other molecules, possibly aminoacids, malate, acetate, or deoxymugineic acid as observed by Fan et al. (2001).

Among the neutral sugars we examined polymerized glucose was the dominant moiety derived from roots. Others sugars qualified from vegetal origin as arabinose and xylose (Chantigny et al., 2000) took a smaller place in our 2 week rhizodeposition study and were delivered more slowly to the soil.

The different dynamics of root-derived sugars we studied could be attributed to both their mechanism of rhizodeposition and fate. Thus the exudation *sensu stricto* of small compounds like free glucose and fructose occurred more rapidly after photoassimilation than the rhizodeposition of arabinose or xylose, which contribute to cell walls. Moreover, the degradability of molecules was controlled by the degree of polymerization. Soluble glucose and fructose disappeared quickly whereas xylose and arabinose included in polymerized cell wall constituents (Table 1) decreased slightly. The lack of polymerized fructose was in accordance with the generally observed absence of this sugar in mucilages (Osborn et al., 1999). The dynamics of sugars usually considered as microbial indicators (mannose, rhamnose and to a lesser extent galactose) were more complex. However, their proportions in soil increased in the course of the experiment, mainly in the first 48 h after labelling, in accordance with the consumption of exudates (Figure 6).

Another important point revealed by the study of root derived sugars dynamics is highlighted on Figure 6. We had noted the convergence of the distribution of labelled monosaccharides in our experiment to the one of soil organic matter. This tended to indicate that the signature of sugars in the soil organic matter pool was acquired rapidly after the degradation of initial organic inputs.

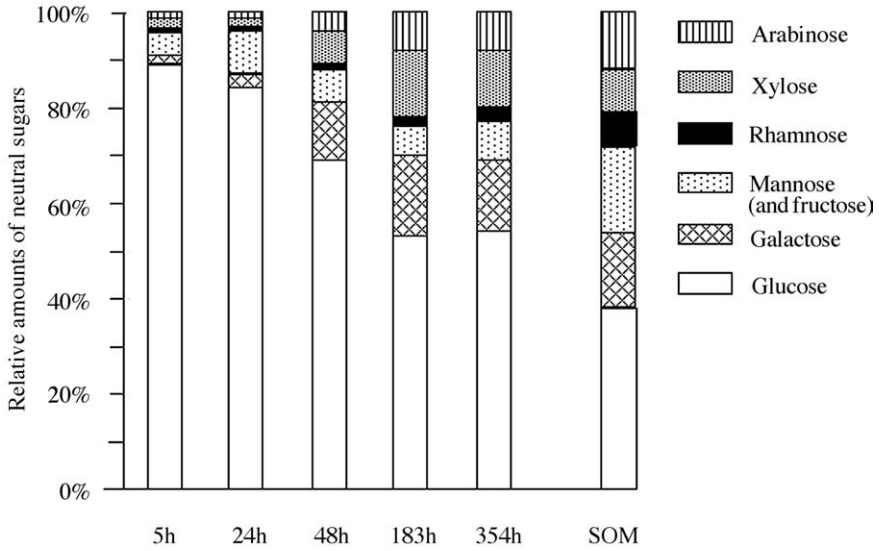

Figure 6. Pattern of distribution of neutral sugars in soil. The 5 first columns correspond to the evolution of the distribution in soil of root-derived labelled sugars between 5 and 354 h after a wheat pulse labelling. The last column represents the distribution of neutral sugars in the soil organic matter pool on a steady state. Fructose was present in samples 5 h and 48 h but wasn't quantified separately from mannose in soils.

Conclusions

To our knowledge, very few studies have coupled isotope pulse-labelling (^{13}C or ^{14}C) in real rhizosphere with chemical analyses of labelled organic C found in the soil (Ostle et al., 2003). We have showed here how the combined use of ^{13}C labelling and GC/IRMS can highlight this part of the carbon cycle in ecosystems. The present case study confirmed in soil that glucose stands for the main compound released into the soil by wheat, but is more in polymerized form than as soluble sugar. After the main peak of exudation, neutral sugars still constituted ca. 40% of rhizospheric carbon, as polysaccharides, with a predominant vegetal origin. Such an approach could usefully complete the knowledge obtained from chemical analyses of bulk exudates (Fan et al., 2001). Further knowledge of the nature of growing root derived carbon will obviously improve our prevision and modelling of its behaviour in the soil, i.e. its microbial transformations, fate, and contribution to the soil carbon budget.

Acknowledgements

We would like to thank the Groupe de Recherches Appliquées en Phytotechnologies of the DEVM in CEA Cadarache for providing plant cultures and labelling facilities, Harmony Guis for her friendly help with samples preparation and Saskia Meerding and Mike Salay for their comments on the manuscript.

References

Amelung W, Cheshire M V, and Guggenberg G 1996 Determination of neutral and acidic sugars in soil by capillary gas-liquid chromatography after trifluoroacetic acid hydrolysis. Soil Biol. Biochem. 28, 1631–1639.

Chantigny M, Angers D and Beauchamp C 2000 Decomposition of de-inking paper sludge in agricultural soils as characterized by carbohydrate analysis. Soil Biol. Biochem. 32, 1561–1570.

Cheng W, Coleman D C, Carroll C R and Hoffman C A 1994 Investigating short-term carbon flows in the rhizospheres of different plant species, using isotope trapping. Agron. J. 86, 782–788.

Derrien D, Marol C, Balesdent J and Santaella C 2003 Measurements of the $^{13}C/^{12}C$ ratio of soil-plant individual sugar by chromatography/combustion/isotope-ratio mass spectrometry of silylated derivatives. Rapid Commun. Mass Spectrom. 17, 2626–2631.

Fan T W M, Lane A N, Shenker M, Bartley J P, Crowley D and Higashi R M 2001 Comprehensive chemical profiling of gramineous plant root exudates using high-resolution NMR and MS. Phytochemistry 57, 209–221.

Grayston S J, Vaughan D and Jones D 1996 Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. Appl. Soil Ecol. 5, 29–56.

Jones D L and Darrah P R 1996 Re-sorption of organic compounds by roots of *Zea mays* L and its consequences in the rhizosphere. 3. Characteristics of sugar influx and efflux. Plant Soil 178, 153–160.

Kuzyakov Y 2002 Review: Factors affecting rhizosphere priming effects. J. Plant Nutr. Soil Sci. 165, 382–396.

Larre-Larrouy M-C and Feller C 1997 Determination of carbohydrates in two ferralitic soils : analysis by capillary gas chromatography after derivatization by silylation. Soil Biol. Biochem. 29, 1585–1589.

Macko S A, Ryan M and Engel M H 1998 Stable isotopic analysis of individual carbohydrates by gas chromatographic/combustion/isotope ratio mass spectrometry. Chemical Geology 152, 205–210.

Meharg A A and Killham K 1995 Loss of exudates from roots of perennial ryegrass inoculated with a range of micro-organisms. Plant Soil 170, 345–349.

Merbach W, Mirus E, Knof G, Remus R, Ruppel S, Russow R, Gransee A and Schulze J 1999 Release of carbon and nitrogen compounds by plant roots and their possible ecological importance. J. Plant Nutr. Soil Sci. 162, 373–383.

Miltner A, Richner H H, Kopinke F D and Kästner M 2003 Assimilation of CO_2 C in soil and its distribution on different soil organic matter components. *In* Proceedings of the international conference 'Mecanisms and regulation of organic matter stabilisation in soils', October 5–8, 2003, Munich, Germany, 21 pp.

Neumann G and Römheld V 2001 The release of roots exudates as affected by plant's physiological status. *In* The rhizosphere, biochemistry and organic substances at the soil-plant interface. Eds. R Pinton, Z Varanini and P Nannipieri. pp. 41–94. Marcel Dekker, Inc New-York, Basel.

Nguyen C 2003 Rhizodeposition of organic C by plants: mechanisms and controls. Agronomie 23, 375–396.

Oades J M, Kirkman M A and Wagner G H 1970 The use of gas-liquid chromatography for the determination of sugar extracted from soils by sulfuric acid. Soil Sci. Soc. Am. Proc. 34, 230–235.

Ogier S, Disnar J R, Alberic P and Bourdier G 2001 Neutral carbohydrate geochemistry of particulate material (trap and core sediments) in an eutrophic lake (Aydat, France). Organic Geochem. 32, 151–162.

Osborn H M I, Lochey F, Mosley L and Read D 1999 Analysis of polysaccharides and monosaccharides in the root mucilage of maize (*Zea mays* L.) by gas chromatography. J. Chromatogr. A 831, 267–276.

Ostle N, Whiteley A S, Bailey M J, Sleep D, Ineson P and Manefield M 2003 Active microbial RNA turnover in a grassland soil estimated using a (CO_2)-C-13 spike. Soil Biol. Biochem. 35, 877–885.

Palta J A and Gregory P J 1997 Drought affects the fluxes of carbon to roots and soil in ^{13}C-pulse labelled plants of wheat. Soil Biol. Biochem. 29, 1395–1404.

Pinton R, Varanini Z and Nannipieri P 2001 The rhizosphere, biochemistry and organic substances at the soil-plant interface. Marcel Dekker, Inc New-York, Basel, 424 pp.

Rovira A D, Foster R C and Matin J K 1979 Note on terminology: origin, nature and nomenclature of organic materials in the rhizosphere. *In* The soil-root interface. Eds. J L Harley and R S Russel. pp. 1–4. Academic Press London.

Swinnen J, Van Veen J A and Merckx R 1994a ^{14}C pulse-labelling of field-frown spring wheat: an evaluation of its use in rhizosphere carbon budget estimation. Soil Biol. Biochem. 26, 161–170.

Swinnen J, Van Veen J A and Merckx R 1994b Rhizosphere carbon fluxes in field-grown spring wheat: model calculation based on ^{14}C partitioning after pulse labelling. Soil Biol. Biochem. 26, 171–182.

van Dongen B E, Schouten S and Sinnoghe Damsté J S 2001 Gas chromatography/combustion/isotope-ratio-monitoring mass spectrometric analysis of methylboronic derivatives of monosccharides: a new method for determinignatural ^{13}C abundance of carbohydrates. Rapid Commun. Mass Spectrom. 15, 496–500.

Warembourg F R and Billès G 1979 Estimating carbon transfers in the plant rhizosphere *In* The soil-root interface. Eds. J L Harley and R S Russel. pp. 183–196. Academic Press London.

Warembourg F R and Paul E A 1977 Seasonal transfers of assimilated ^{14}C in grassland: Plant production and turnover, soil and plant respiration. Soil Biol. Biochem. 9, 295–301.

Warembourg F R, Roumet C and Lafont F 2003 Differences in rhizosphere carbon-partitioning among plant species of different families. Plant Soil 256, 347–357.

Section editor: H. Lambers