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Determination of Root Exudate Concentration in the Rhizosphere Using ^{13}C Labeling

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[Abstract] One of the most remarkable metabolic features of plant roots is their ability to secrete a wide range of compounds into the rhizosphere, defined as the volume of soil around living roots. Around 5%-21% of total photosynthetically fixed carbon is transferred into the rhizosphere through root exudates. Until recently, studies on the quantity and quality of root exudates were conducted mostly under axenic or monoxenic *in vitro* conditions. Today, *in situ* assays are required to provide a better understanding of root exudates dynamics and role in plant-microbe interactions. By incubating plants with $^{13}CO_2$ *in situ* for one week and quantifying ^{13}C enrichment from the root-adhering soil using mass spectrometry, we were able to determine root exudate levels. Indeed, labeled substrate $^{13}CO_2$ is converted into organic carbon *via* plant photosynthesis and transferred into the soil through root exudation. We assume that all ^{13}C increases above natural abundance are mainly derived from exudates produced by ^{13}C-labeled plants.

Keywords: Root exudates, Plant, Rhizosphere, $^{13}CO_2$ labeling, Root-adhering soil, ^{13}C-content, Isotope Ratio Mass Spectrometry, *Poaceae,* Monocotyledon, Legume, *Arabidopsis thaliana*

[Background] Through the exudation of a wide variety of compounds, plants communicate with the soil microbial community in their immediate vicinity, cope with herbivores, foster beneficial symbioses, change the chemical and physical properties of the soil, and inhibit the growth of competing plant species. Understanding how plants differ quantitatively in their root exudate patterns, according to their genotypes and traits as well as to environmental parameters and the soil microbial community is an exciting research challenge. This objective could be achieved by quantifying root exudate content using $^{13}CO_2$ plant-labeling coupled with a ^{13}C enrichment measurement from the root-adhering soil using mass spectrometry. This protocol was set up by using different plant species such as *Poaceae*, legumes (*Medicago truncatula)*, *Arabidopsis thaliana,* and Monocotyledon plants such as maize and wheat. As rhizodeposition also depends on plant species, the collection period was after 10 weeks for *Poaceae* and 4 weeks for the rest. This method can be used with other species, for which the collection period must be set up according to what is known about the studied plant.

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Materials and Reagents

1. 1 mm mesh size for soil sieving (Fisherbrand tamis, France)
2. Plastic pots for plant growing (7 x 7 x 6.4, black pots, France)
3. 5 x 8 mm “Ultra Clean” tin capsules (Elemental Microanalysis, Okehampton, UK, catalog number: D1034)
4. In the case of calcareous soils: 5 x 8 mm silver capsules (Elemental Microanalysis, Okehampton, UK, catalog number: D2009)
5. Soil and seeds for plant growing
   The soil used to set up this protocol was luvisol with no added nitrogen source collected at La Côte Saint-André (Isère, France) and which was continuously harvested with maize. It was a loamy soil composed of 16.2% clay, 45.4% loam and 28.4% sand, with a total nitrogen content of 1.9 $g \cdot kg^{-1}$
6. Reference materials for ^{13}C measurements: IAEA-CH3 (cellulose), IAEA-CH6 (sucrose) (Coplen *et al.*, 2006) and partially ^{13}C-labelled material [*e.g.*, D-Glucose (3-13C, 99%)] (Eurisotop, Saint-Aubin, France, catalog number: CLM-1393-0.25)
7. Calibration material for carbon (C) concentration measurements: aspartic acid (Elemental Microanalysis, Okehampton, UK, catalog number: B2042)
8. Distilled water
9. Liquid N_2
10. Pure $^{13}CO_2$ (> 99% atom ^{13}C; purchased from Cortec Net, Paris, France)
11. In the case of calcareous soils: hydrochloric acid (HCl) 37% (CAS number: 7647-01-0, *e.g.*, Sigma-Aldrich, Saint-Louis, MO, USA, catalog number: 30721-M)

Equipment

1. 50 ml beaker
2. Spatula and forceps
3. Desiccator (Freeze-dryer) (Alpha 1-4 LSC, Martin Christ®, Osterode am Harz, Germany)
4. Mixer mill grinder (Mixer Mill MM 200, Retsch®, Haan, Germany)
5. Laboratory balance with a resolution of 0.01 mg or better (XP6 microbalance, Mettler Toledo, Greifensee, Switzerland)
6. Isotope ratio mass spectrometer (Isoprime 100, Elementar UK Ltd., Cheadle, UK), coupled in continuous flow with an elemental analyzer (vario PyroCube in CN mode, Elementar Analysensysteme, Lagenselbold, Germany, or FlashEA 1112, ThermoElectron, Waltham, MA, USA)
7. Growth chamber equipped for automatic control of light, temperature, moisture, evapotranspiration, irrigation and CO_2 concentration

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Software

1. Isoprime 100 isotope ratio mass spectrometer operating software: Ionvantage® for Isoprime, build 1.6.1.0 (Elementar Analysensysteme, Lagenselbold, Germany)
2. Elemental analyzer operating software (pyrocube Software v4.0.1 was used in this protocol, Elementar Analysensysteme, Lagenselbold, Germany)

Procedure

1. Sieve the soil (1 mm mesh size) and adjust it to 0.17 g of water per gram of dry weight. Afterward, place 170 g of dry weight into pots. Place one seed per pot and do six replicates per plant species and six replicates for unplanted-soil (bulk soil).
2. Place the pots (six replicates per plant and six replicates for bulk soil) in a growth chamber and set up the day-night period, light intensity and daily temperature according to the studied plant (Guyonnet *et al.*, 2018b). For *Brasica napus*, *Arabidopsis thaliana*, *Triticum aestivum*, *Medicago truncatula* and *Poaceae* plants, for example, the day-night period is set at 8 h/16 h, respectively; light intensity is 13.5 klux; maximum daily temperatures ranges from 20 to 22 °C. Control and adjust soil moisture by weighing pots using a laboratory balance and adding distilled water every 2 days (Figure 1).
3. Three weeks after sowing seeds, inject pure $^{13}CO_2$ to three replicates per plant and bulk soil during active photosynthesis, according to Haichar *et al.* (2008 and 2012), in order to maintain a $^{13}CO_2$ level at 350 $\mu l \cdot L^{-1}$ for one week. Control $^{12}CO_2$ (soil respiration) and $^{13}CO_2$ concentrations in the growth chamber. The isotope excess of ^{13}C atoms in the chamber must be maintained at > 80% during the first 10 days and > 90% thereafter.
4. Cultivate the rest of the plants (three replicates per plant) and bulk soil (three replicates for unplanted soil) without ^{13}C-labeling in order to determine ^{13}C natural abundance in soil and plants.
5. At the end of labeling, separate the root system from the root-adhering soil (RAS) by shaking each plant and collect the root-adhering soil. Carefully separate the remaining fine roots from the RAS and freeze the RAS immediately in liquid N_2 before storing at -80 °C (Figure 1).
6. Lyophilise the RAS during 48 h and grind to a fine powder (~100 µm particle size; visual verification is sufficient) using a mixer mill to homogenize before measuring ^{13}C enrichment (Figure 2A). The duration of the grinding step should be adapted to each type of soil (approximately 1 min at 30 Hz; repeat if needed until a fine powder is obtained).

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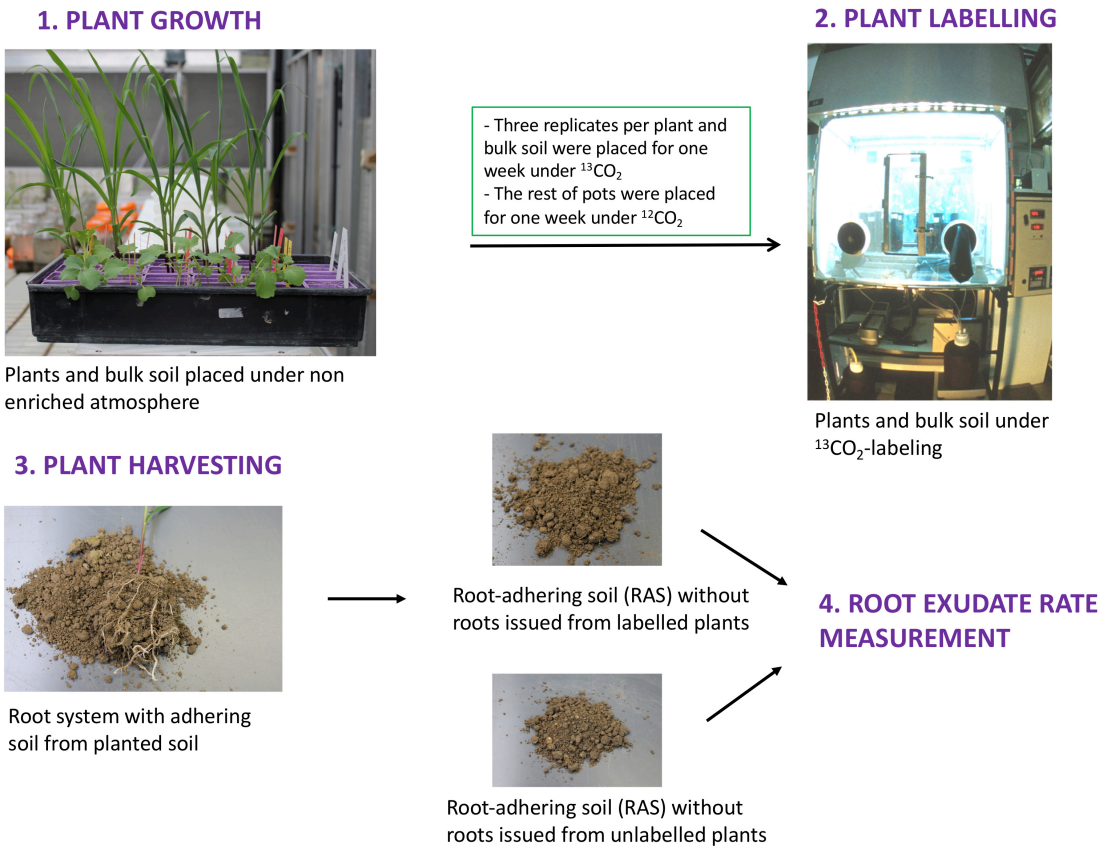

Figure 1. Plant growth, labeling and harvesting in order to measure root exudation rate

7. Weigh 8 mg (adjust the target mass to obtain a sufficient amount of carbon, commonly > 10 µg of C depending on the C concentration of the sample and IRMS sensitivity) of lyophilized RAS samples into 5 x 8 mm “Ultra Clean” tin capsules to the nearest 0.01 mg (Figure 2B).
   Note: In calcareous soils, inorganic carbon (carbonates) can distort the ^{13}C measurement of organic carbon. In this case, remove inorganic C by acid fumigation. Weigh soil samples into silver capsules instead of tin. Add a small amount (50-150 µl) of distilled water in each capsule to moisten the soil. Place each open capsule in a desiccator with a beaker of fuming (37%) hydrochloric acid (HCl) during 6 to 8 h. Dry at 60 °C. Crimp the capsule and place it in a new tin capsule.
8. Carefully seal the tin capsules using forceps into a cubical or spherical shape (Figure 2C).
9. Measure the $^{13}C/^{12}C$ ratio and C concentration of each sample by EA-IRMS (Figure 2D) after optimizing the source parameters and calibration according to good practice (*e.g.*, Dunn and Carter, 2018). Intersperse reference materials with the samples to allow for the normalization of the data and quality control.

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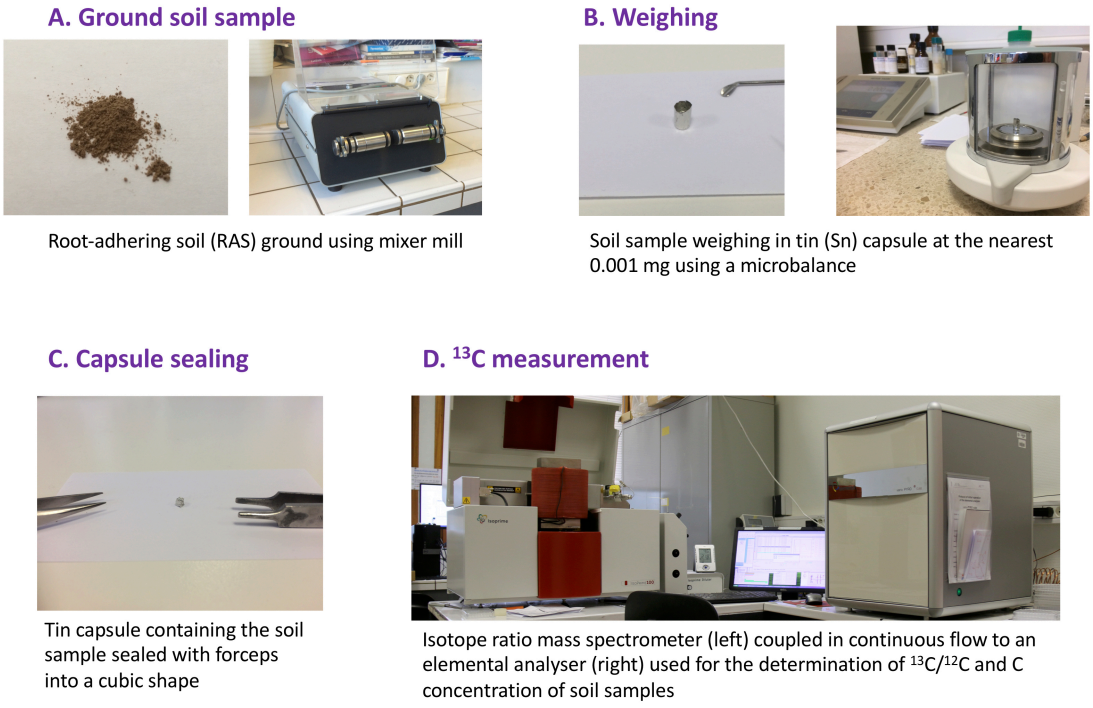

Figure 2. Scheme of soil sample preparation for the $^{13}C/^{12}C$ ratio measure using Elemental Analyser-Isotope Ratio Mass Spectrometer (EA-IRMS)

Data analysis

1. Apply blank corrections and normalize the C concentrations and $^{13}C/^{12}C$ ratio with the measured values of reference and calibration materials (Coplen *et al.*, 2006; Dunn and Carter, 2018).
2. C isotope composition is measured by IRMS as a $^{13}C/^{12}C$ ratio, expressed by the instrument software as $\delta^{13}C$ in ‰:

$$\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{PDB} - 1] \times 10^3$$

with $(^{13}C/^{12}C)_{sample}$, the $^{13}C/^{12}C$ ratio of the soil sample, $(^{13}C/^{12}C)_{PDB}$ is the $^{13}C/^{12}C$ ratio of PDB (Pee Dee Belemnite, the international standard used to anchor the $\delta^{13}C$ scale at 0‰) and equals 0.0112372.

3. Calculate the ^{13}C concentration in RAS using the equation:

$$[^{13}C] = [C] \times [(\delta^{13}C_t - \delta^{13}C_{t\text{-}0}) - (\delta^{13}C_{control} - \delta^{13}C_{control\text{-}0})] \times (^{13}C/^{12}C)_{PDB}/10^3$$

where,

$[^{13}C]$ estimates the concentration of exudates in the soil or the assimilation by microorganisms present in the soil in mg of ^{13}C by kg of soil (equivalent to dry weight),

[C] is the carbon concentration of the soil in $mg \cdot kg^{-1}$,

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$\delta^{13}C_t$ is the C isotope composition of organic carbon in the planted soil with ^{13}C-labeling, $\delta^{13}C_{t-0}$ is the C isotope composition of organic carbon in the planted soil without ^{13}C-labeling, $\delta^{13}C_{control}$ is the C isotope composition of the organic carbon ~~in~~ of the bulk soil with ^{13}C-labeling, $\delta^{13}C_{control-0}$ is the C isotope composition of the organic carbon in the bulk soil with ^{13}C-labeling. *Note:* $\delta^{13}C_{t-0}$ *and* $\delta^{13}C_{control-0}$ *(i.e., without* ^{13}C*-labeling) are determined in order to take into account the* ^{13}C *natural abundance in bulk and planted soils. In the majority of experiments, these values will be identical and can therefore be removed from the equation in step 3.*

Example of calculations:
For the soil planted with *Bromus erectus* with ^{13}C-labeling ($\delta^{13}C_t$), a $\delta^{13}C$ of 480.5‰ was measured for the root-adhering soil. The $\delta^{13}C$ of the bulk soil with ^{13}C-labeling ($\delta^{13}C_{control}$) was -5.5‰. Without ^{13}C-labeling, the $\delta^{13}C$ of the root-adhering soil ($\delta^{13}C_{t-0}$) was equal to -22.5 ± 0.3 ‰ and the $\delta^{13}C$ of the bulk soil ($\delta^{13}C_{control-0}$) was -22.7 ± 0.2 ‰. The C concentration ([C]) of the soil was 1.67 ± 0.2 % (16.7 x 10^3 mg·kg^{-1}).

Using the equation in step 3, a·concentration of exudates in the soil of 91.2 mg ^{13}C·kg^{-1} (dry weight) was measured.

Acknowledgments

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Competing interests

No competing interests to declare.

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