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Direct uptake of organic carbon by grass roots and allocation in leaves and phytoliths: ^{13}C labeling evidence

A. Alexandre[1], J. Balesdent[1,4], P. Cazevieille[3], C. Chevassus-Rosset[3], P. Signoret[4], J.-C. Mazur[1], A. Harutyunyan[2], E. Doelsch[3], I. Basile-Doelsch[1], H. Miche[1], and G. M. Santos[2]

[1]Aix-Marseille Université, CNRS, IRD, INRA, CEREGE UM34, 13545 Aix en Provence, France
[2]Department of Earth System Science, University of California, B321 Croul Hall, Irvine CA 92697-3100, USA
[3]CIRAD, UPR Recyclage et risque, 34398 Montpellier, France
[4]INRA UR 1119 GSE, 13100 Aix-en-Provence, France

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Correspondence to: A. Alexandre (alexandre@cerege.fr)

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Abstract

In the rhizosphere, the uptake of low molecular weight carbon (C) and nitrogen (N) by plant roots has been well documented. While organic N uptake relatively to total uptake is important, organic C uptake is supposed to be low relatively to the plant's C budget. Recently, radiocarbon analyses demonstrated that a fraction of C from the soil was occluded in amorphous silica micrometric particles that precipitate in plant cells (phytoliths). Here, we investigated whether and in which extent organic C absorbed by grass roots, under the form of either intact amino acids (AAs) or microbial metabolites, can feed the organic C occluded in phytoliths. For this purpose we added ^{13}C- and ^{15}N-labeled AAs to the silicon-rich hydroponic solution of the grass *Festuca arundinacea*. The experiment was designed to prevent C leakage from the labeled nutritive solution to the chamber atmosphere. After 14 days of growth, the ^{13}C and ^{15}N enrichments (^{13}C-excess and ^{15}N-excess) in the roots, stems and leaves, and phytoliths, as well as the ^{13}C-excess in AAs extracted from roots and stems and leaves, were quantified relatively to a control experiment in which no labelled AAs were added. The net uptake of ^{13}C derived from the labeled AAs supplied to the nutritive solution (AA-^{13}C) by *Festuca arundinacea* represented 4.5 % of the total AA-^{13}C supply. AA-^{13}C fixed in the plant represented only 0.13 % of total C. However, the experimental conditions may have underestimated the extent of the process under natural and field conditions. Previous studies showed that ^{15}N and ^{13}C can be absorbed by the roots in several organic and inorganic forms. In the present experiment, the fact that phenylalanine and methionine, that were supplied in high amount to the nutritive solution, were more ^{13}C-enriched than other AAs in the roots and stems and leaves strongly suggested that part of AA-^{13}C was absorbed and translocated in its original AA form. The concentration of AA-^{13}C represented only 0.15 % of the phytolith C (phytC) but was still 1.5 times higher in phytoliths than in leaves supporting that, ultimately, AA-^{13}C was preferentially fixed in organic compounds subject to occlusion in phytoliths. Plausible forms of AA-^{13}C and

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AA-^{15}N absorbed and translocated, AA-^{13}C fixation in phytoliths and implication of our finding for our understanding of the C cycle at the plant-soil interface are discussed.

1 Introduction

In the rhizosphere, there are numerous known interactions between carbon (C) and nitrogen (N) processes that have yet to be accurately assessed in qualitative and quantitative terms for their consideration in carbon cycle models (Heimann and Reichstein, 2008). Among those interactions the uptake of low molecular weight C and N (e.g. organic acids, sugars and amino acids (AAs)) by plant roots (both mycorrhizal and non-mycorrhizal plants) has been well documented through experiments using hydroponic solutions, artificial substrats or soils (e.g. Chapin et al., 1993; Bardgett et al., 2003; Kuzyakov and Jones, 2006; Persson et al., 2006; Jones et al., 2009a; Näsholm et al., 2009; Sauheitl et al., 2009; Gioseffi et al., 2012 ; Moran-Zuloaga et al., 2015). The aim of most of these studies was to investigate in which extent and under which conditions, organic N could be utilized by plants as a direct source of N (i.e., without going through a mineralization step). The answers are still debated (Jones and Darrah, 1992; Jones et al., 2009a; Moran-Zuloaga et al., 2015) but it has been showed that the net uptake of AAs, that may reach half of the total N uptake of grasses (Sauheitl et al., 2009), occurred for a wide range of AA concentrations (Chapin et al., 1993; Jämtgård et al., 2008 ; Harrison et al., 2008; Sauheitl et al., 2009), and different levels of soil microorganism competition (e.g. Jones et al., 2005a; Sauheitl et al., 2009). This suggested that this process might be widespread in natural or field conditions. Significance of organic C absorption was also investigated, mostly through the estimation of the net uptake of glucose-C. Organic C uptake, which has been shown to be low relatively to the plant's C budget, was often interpreted as the recapture of roots exudates (Jones and Darrah, 1992, 1993, 1996; Kuzyakov and Jones, 2006; Jones et al., 2009a). However, very recently, radiocarbon analyses demonstrated that a fraction of C occluded in amorphous silica micrometric particles that precipitate in plant cells (phytoliths) came

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from old soil organic C, and possibly old AAs reached by the roots (Santos et al., 2012; Reyerson et al., 2015). Silicon (Si) is the second most abundant element of the earth surface after oxygen. Its uptake by plants is widespread and generates, at the ecosystem scale, important fluxes from the soil to plants (Conley, 2002). For instance, Si absorption represents 2 to 10 times the amount of dissolved Si exported to stream water in tropical ecosystems (Alexandre et al., 2011). If part of the organic C uptake is linked to Si uptake in the rhizosphere, the involved flux may thus also be significant.

Here, we aim to investigate whether and in which extent organic C absorbed by grass roots, either under the form of intact AAs or microbial metabolites, can feed the organic C occluded in phytoliths. For this purpose we added ^{13}C- and ^{15}N-labeled AAs to the Si-rich nutrient solution of the grass *Festuca arundinacea*. AAs are ubiquitous in soil organic matters of various residence times (Bol et al., 2009). The experiment was designed to prevent C leakage from the labeled nutritive solution to the chamber atmosphere. After two weeks of growth, the ^{13}C and ^{15}N enrichments in the roots, stems/leaves, and phytoliths of the grass (two replicates) were quantified, and expressed in ^{13}C-excess and ^{15}N-excess relatively to a control experiment in which no labelled AAs were added.

2 Material and methods

2.1 Hydroponic culture

Festuca arundinacea, commonly referred to as tall fescue, is widely distributed globally as a forage and an invasive species (Gibson and Newman, 2001) and can adapt to a wide range of conditions. *Festuca arundinacea* was grown in hydroponic conditions for 24 days using an experimental procedure adapted from RHIZOtest (Bravin et al., 2010), a plant-based test recently standardized (ISO 16198:2015). Seventy-two plant-receiving pots (i.e. a cylinder closed at the bottom with a polyamide mesh of 30 µm pore size, using an adjustable clamp) were inserted in three perforated platforms cov-

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ering three 12 L tanks containing the nutrient solutions (24 plant pots per tank) (Fig. 1). This assembly enabled close contact between seeds or seedling roots and the nutrient solutions. In order to prevent escape of the C- and N-bearing gas from the nutrient solution to the chamber atmosphere, O-rings sealed the plant pots to the perforated platform and the perforated platform to the tank. Additionally, the seeds were covered with agar-agar (polysaccharide agarose). Each tank was hermetically connected to two 20 L containers (an input container filled with the nutrient solutions and a waste container). Seeds were first germinated for 10 days in a germination solution and seedlings were then grown for 14 days in a growth solution. Both solutions were renewed continuously at a rate of 2 L/24 h using a peristaltic pump (Fig. 1). Additionally, the growth solution was entirely renewed once, after 8 days of growth.

Overall, 10 L of germination solution and 64 L of growth solution were used per tank. Germination and the growth nutrient solution composition were described in detail in Guigues et al. (2014). The nutrient solutions included 42 mg L^{-1} of inorganic N (KNO_3) and 18 mg L^{-1} of inorganic C in Ethylene diamine tetraacetic acid (EDTA), added to chelate metals the plant uses for growth. The solutions were also supplemented with 105 mg L^{-1} of SiO_2 (under the form of SiO_2K_2O). The growth chamber parameters were set at (day/night): 25/20 °C temperature, 75/70 % relative humidity and 16/8 h of day/night using a photon flux with a photon flux density of 450 µmol photons m^{-2} s^{-1} during the day.

At the end of the experiment, all samples were cleaned (to remove agar-agar), rinsed in deionized water and oven-dried at 50 °C (to constant mass). When the tanks were filled (1st and 8th days of growth) and emptied (8th and 14th days of growth), the growth solution was sampled and kept frozen for future analyses.

2.2 Isotope labeling

In the two first tanks (two replicates), a mixture containing four ^{13}C- and ^{15}N-labeled AAs was added to the growth solution containing only inorganic C and N (as described above). Those AAs were selected for their following characteristics: L-Alanine (l-ALA)

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(C/N = 2.6) is ubiquist and occurs in high proportions in soils and plants proteins. The D-enantiomer of Alanine (D-ALA) which is present in natural soils (Hill et al., 2011), was expected to be more resistant to degradation and, if absorbed, less subject to metabolization. Consequently, we speculated that D-ALA may accumulate as a waste product first in cell vacuoles and later in phytoliths. L-Phenylalanine (L-PHE) (C/N = 7.7) comprises a phenolic ring resistant to decomposition by microorganisms in soils, solutions or plants. L-Methionine (L-METH) (C/N = 4.3) is a sulfur amino acid expected to be recovered at low abundance in plants, but is easily identifiable in Gas Chromatography- Isotope Ratio Mass Spectrometry (GC-IRMS). Commercial 97–99 % ^{13}C and ^{15}N molecules (Eurisotop) were diluted with non-labeled amino-acids to reach the following atom abundances: L-PHE (19.51 % ^{13}C; 19.13 % ^{15}N); L-METH (19.87 % ^{13}C; 19.49 % ^{15}N); L-ALA (22.05 % ^{13}C; 16.26 % ^{15}N); D-ALA (7.43 % ^{13}C; 0.37 % ^{15}N). All AAs were uniformly labeled except (i) D-ALA, which was not ^{15}N-labeled but was ^{13}C-labeled on one atom (C-2) and, (ii) an equivalent fraction of L-ALA because the labeled D-ALA was provided as a racemic mixture (DL-ALA). In each tank, the following amounts of AAs were added to the growth solution: PHE 249.9 mg; MET 125.1 mg; L-ALA 150.3 mg; D-ALA 125.2 mg. The total mixture (two tanks) represented 322 mg of C (26.8 mmol) and 75.7 mg of N (5.4 mmol) with average atom abundances of 18.15 % ^{13}C and 13.43 % ^{15}N. The maximum AAs concentration in the growth solution on the first or 8th days of the growth period was 0.225 mmol L^{-1}, equivalent to 6.7 mg L^{-1} of ^{13}C and 1.6 mg L^{-1} of ^{15}N.

The third tank was only filled with the growth solution, without labeled AAs. It served as a control experiment to calculate the ^{13}C and ^{15}N enrichments of the plants from the labeled tanks, and verify that ^{13}C and ^{15}N derived from AAs (AA-^{13}C and AA-^{15}N), that may have contaminated the chamber CO_2, were not photosynthesized by the plants.

2.3 Analyses

For each tank (one control tank and two labeled tanks) stems/leaves were separated from the roots into two samples. The six resulting samples were ground finer than

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200 µm. After alkaline fusion, they were analyzed in SiO_2, using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

For each sample, total C, $^{13}C/^{12}C$, total N and $^{15}N/^{14}N$ were determined after dry combustion by IRMS using a Carlo Erba NA 1500 elemental analyzer (EA) coupled to a Thermo-Finnigan Delta-plus mass-spectrometer. Solutions were also analyzed for total C and $^{13}C/^{12}C$ by IRMS, after evaporation by dry combustion in tin capsules.

Proteic carbon was analyzed as the sum of 19 individual AAs representing ca. 95 % of all AAs. Quantification and $^{13}C/^{12}C$ determination of individual AAs were performed using a GC-IRMS (Thermo Fisher Scientific). The extraction and purification procedure was a slightly modified version (Rubino et al., 2014) of the protocol developed by Amelung et al. (2006). Briefly, dry plant samples were hydrolyzed in 6 M HCl (20 h, 100 °C). AAs were purified on Dowex 50 W X8 cation exchange resin (100–200 mesh size, Arcos Organics, Thermo Fisher Scientific), dried by rotary evaporation, re-dissolved in 0.1 M HCl and dried again by speed-vacuum evaporation. AAs were separated and quantified as tert-butyl dimethyl silyl derivatives (TBDMS-aa): AAs were dissolved in N-Methyl-N-(tbutyldimethylsilyl) trifluoroacetamide (MTBSTFA) mixed with 1 % trimethylchlorosilasane (TMCS) (Sigma-Aldrich Co. Ltd.) and acetonitrile and heated at 120 °C for 1 h. One µL of TBDMS-AA solution was injected into the GC through a GCCIII combustion interface (Thermo Fisher Scientific). TBDMS-AA were separated on a DB5 column (30 m, 0.25 mm i.d., 0.25 µm film thickness) with helium as a carrier gas. AA identification and quantification were performed using commercial mixtures of 20 proteinogenic AAs (Sigma Aldrich). Norvaline (Sigma-Aldrich Co. Ltd.) was added to plant samples before hydrolysis as an internal standard for quantification. Due to the addition of non-labeled carbon by TBDMS, AA ^{13}C enrichment was subsequently calibrated for each AA (Shinebarger et al., 2002). Briefly, this calibration was based on the independent measurement of ^{13}C of the TBDMS-derivatives of the commercial AA and an additional set of four ^{13}C-labelled AA (Rubino et al., 2014). The calibration equation involves the number of carbon atoms added as TBDM and the isotopic composition of the latter. This isotopic composition term

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disappears in the calculation of the isotope excess (cf Eq. 1 below). When multiple peaks were encountered for a single AA, the main or the best individualized peak was chosen for both quantification and isotope ratio determination. The isotope ratios were calculated using ammonium sulfate IAEA-N1 ($\delta^{15}N = 0.43 \pm 0.07$ ‰) and IAEA-N2 ($\delta^{15}N = 20.41 \pm 0.07$ ‰), polyethylene IAEA CH7 ($\delta^{13}C = -32.15 \pm 0.05$ ‰) as secondary standards. The sucrose standard IAEA CH6 used as a control yielded a mean value of 10.43 ‰.

Phytoliths were extracted from plants using a high purity protocol based on acid digestion and alkali immersion steps previously described in detail by Corbineau et al. (2013). Phytolith samples were observed in natural light microscopy to determine their morphological assemblage and check for the absence of residual organic matter particles. An additional purity check was done via Scanning Electron Microscopy (SEM) (Corbineau et al., 2013; Reyerson et al., 2015). Then, phytolith samples were analyzed for their C and N contents, as well as their ^{13}C and ^{15}N abundances by EA (Fisons NA 1500NC) coupled to a continuous flow IRMS (Finnigan Delta-Plus). About 6–10 mg of phytolith concentrates were weighed using a pre-calibrated microbalance (Sartorius AG, Göttingen, Germany) into tin capsules (5 × 9 mm capsules, Costech Analytical Technologies Inc., Valencia, CA, USA) and pre-baked at 100° C for 2 h to remove extraneous contaminants. To assure accurate integration and linearization of the raw analytical data obtained from the lower C and N peaks, we decreased the helium carrier flow rate and measured several aliquots of in-house collagen L-Cystine 99 % ($\delta^{13}C = -28.74 \pm 0.13$ ‰ and $\delta^{15}N = -6.14 \pm 0.07$ ‰; from Sigma Aldrich Co. Ltd) and Atropina ($\delta^{13}C = -21.30 \pm 0.06$ ‰ and $\delta^{15}N = -2.90 \pm 0.10$ ‰; from Costech 031042) as well as the internationally certified reference materials (e.g. Graphite USGS24 $\delta^{13}C = -16.05 \pm 0.07$ ‰ and, Amonium sulfate – IAEA-N1 $\delta^{15}N = +0.43 \pm 0.07$ ‰). Aliquots of baked-clean silicon dioxide (SiO_2; mesh# −325, Sigma Aldrich, St. Louis, MO, USA) and fossil phytoliths (MSG70; Crespin et al., 2008) were also analyzed to provide independent blank data (Santos et al., 2010) and to check accuracy. To serve as quality assurance, note that the reproducibility obtained on the phytolith laboratory

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standard MSG70 was SD ± 0.2 ‰ for $\delta^{13}C$ and $\delta^{15}N$, SD ± 0.01 % for C and N, and SD ± 0.09 for C/N.

2.4 Calculations

For the control tank (no labeling), results are reported as δ values in ‰ relative to the Vienna Pee Dee Belemnite (V-PDB) for $\delta^{13}C$, and in ‰ relative of the atmospheric N_2 for $\delta^{15}N$. For the labeled tanks, concentration, recovery and net uptake of C derived from the supplied labeled AAs were calculated using the ^{13}C-excess of a sample (^{13}C-excess$_{sample}$) expressed in Eq. (1)::

$$^{13}\text{C-excess}_{sample}\ (\%) = {}^{13}\text{C atom}_{sample}\ (\%) - {}^{13}\text{C atom}_{control}\ (\%) \quad (1)$$

where ^{13}C atom$_{sample}$ is the ^{13}C atom abundance of a sample (stems and leaves, roots or phytoliths) from a labeled tank and ^{13}C atom$_{control}$ is the ^{13}C atom abundance of the same sample from the control tank.

The concentration of AA-^{13}C in a sample, expressed in % of total C in the sample, was calculated using Eq. (2):

$$[\text{AA-}^{13}\text{C}_{sample}]\ (\%) = \frac{{}^{13}\text{C} - \text{excess}_{sample}\ (\%)}{{}^{13}\text{C atom}_{solution}\ (\%) - {}^{13}\text{C atom}_{control}\ (\%)} \times 100 \quad (2)$$

where ^{13}C atom$_{solution}$ is the ^{13}C atom abundance in the labeled solution at the beguinning of the growth phase.

Recovery of AA-^{13}C in a sample, expressed in $\mu g\,g^{-1}$ of the dry matter weight (d.wt), was calculated using Eq. (3):

$$\text{Recovery AA-}^{13}\text{C}(\mu g\,g^{-1}) = [\text{AA-}^{13}\text{C}_{sample}] \times [\text{C}]_{sample}\ (\mu g\,g^{-1}) \quad (3)$$

where $[C]_{sample}$ is the concentration of total C in the sample.

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Net uptake of C derived from the supplied AAs (AA-^{13}C uptake), expressed in % of AA-^{13}C initially supplied to the solution, was calculated using Eq. (4):

$$\text{AA-}^{13}\text{C uptake (\%)} = \frac{\text{Recovery AA-}^{13}\text{C (mg g}^{-1}\text{)} \times \text{d.wt}_{\text{sample}}\text{ (g)}}{\text{AA-}^{13}\text{C}_{\text{max, supplied}}\text{ (mg)}} \quad (4)$$

where d.wt$_{\text{sample}}$ is the dry weight of sample and AA-^{13}C$_{\text{max, supplied}}$ is the maximum AA-^{13}C supplied to the solution (322 mg of AA-^{13}C).

The same calculations were applied to ^{15}N.

3 Results

3.1 Total C, N and AAs concentrations in the plants

After 14 days in the growth solution, the above-ground part of the plants of *Festuca arundinacea* were 30 cm high but had not reached maturity or flower development (Fig. 1). The C/N ratios of the stems and leaves were similar in the labeled and control plants (8.4 and 9.0), whereas the C/N ratios of roots were higher in labeled (11.0) than in control (4.2) plants (Table 1; Fig. 2). Recovered AAs accounted for 52 mg C g^{-1} of the dry matter in both roots and leaves. This is a high AAs content, also attested by the low C/N ratio, in agreement with the high N level requirement of young plants sufficiently fertilized to support rapid protein synthesis (Mattson, 1980). However AA-^{13}C and AA-^{15}N only accounted for ca. 13–14 % of total C, and 40 % of total N. This N contribution was lower than what might be expected. Indeed, at any stage of growth, AAs (in the form of protein or free molecules) should account for more than 50 % of grass N. This discrepancy can be attributed to an underestimation of AAs by the extraction-purification process (i.e., incomplete hydrolysis recovery due to recombination into strong acids, and incomplete silylation). An underestimation of the AAs is consistent with the fact that amino-acid TBDMS-derivatives, such as tryptophane or cystine (the

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cystein-dimer), could not be recovered. However such an underestimation should not bias the measured relative proportions of METH, PHE, and ALA.

3.2 Excess, uptake and recovery of AA-^{13}C and AA-^{15}N in the plants

The δ values of the roots and aerial parts of the control plants were respectively −31.0 and −31.7 ‰ for δ^{13}C and 13.8 and 14.5 ‰ for δ^{15}N. These values were in the range of the ones measured for C_3 grasses in natural conditions (δ^{13}C from −22 to −34 ‰; e.g. O'Leary, 1988), ensuring that CO_2 potentially produced by decomposition of the ^{13}C-labeled molecules inside the tanks did not contaminate the growing chamber atmosphere. The amount of labeled C recovered in plants and phytoliths was thus considered as exclusively resulting from root uptake.

Relative to plants from the control tank, plants from the labeled tanks were enriched by 0.05 % in ^{13}C and 0.5 % in ^{15}N in the roots and by 0.02 % in ^{13}C and 0.3 % in ^{15}N in the stems and leaves (Table 1).

Overall, the net uptake of AA-^{13}C and AA-^{15}N by the plant represented respectively 4.5 and 46.9 % of the AA-^{13}C and AA-^{15}N added to the solution (Table 1; Fig. 2). Whereas 35 % of absorbed AA-^{13}C and 19.2 % of absorbed AA-^{15}N were stored in the roots, 64.4 and 81.1 % of the absorbed AA-^{13}C and AA-^{15}N, respectively, were allocated to the stems and leaves (after Table 1). The associated AA-^{13}C/AA-^{15}N ratios were 0.8 in the roots and 0.3 in the stems and leaves (Table 1).

Concentrations of AA-^{13}C and AA-^{15}N represented only 0.13 % of total C and 2.8 % of total N of the plant, respectively (Table 1; Fig. 3). This contribution was higher in roots (0.28 % of C and 4.1 % of N) than in stems and leaves (0.10 % of C and 2.6 % of N). In roots, AA-^{13}C was more concentrated in AAs than in total plant matter (0.70 vs. 0.28 % of C) (Table 1). When translocated to leaves, AA-^{13}C concentration in AAs decreased to reach that of the bulk leaf matter (0.10 % of C) (Table 1).

Among the measured AAs in plant, ALA and PHE were more abundant by more than a factor 10 relative to MET (Table 1). However, ALA was not more enriched in ^{13}C than

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most of the AAs (Fig. 4). Instead, PHE and MET were significantly more enriched in ^{13}C than other AAs in roots, stems and leaves (Fig. 4).

3.3 Concentrations of phytoliths, phytC, phytN, and AA-^{13}C and AA-^{15}N in phytC and phytN

Silica content measured by ICP-AES accounted for 0.08 and 0.26 % of the dry weight (d.w.) of roots and stems/leaves respectively, which is lower than the > 1 % d.w. concentration previously measured for this species harvested 8 weeks after sowing (Hartley et al., 2015), possibly due to the fact that the plants did not reach maturity or/and that the volume of the roots in contact with the Si-enriched solution was small, limited by the RHYZOtest configuration. Most of the stems and leaves silica was in the form of phytoliths (0.19 % d.w.) (Table 2) that constituted a morphological assemblage characteristic of the Festucoideae grass subfamily which *Festuca arundinacea* belongs to (Honaine et al., 2006) (Fig. 5). Root phytoliths were not abundant enough to be quantified. Occluded C (phytC) and N (phytN) represented 0.51 and 0.10 % d.w. of phytoliths, respectively (Table 2), in the range of values previously measured for phytC) (Santos et al., 2010; Alexandre et al., 2015; Reyerson et al., 2015)

Interestingly, phytoliths were 1.5 times more enriched in ^{13}C (^{13}C-excess of 0.03 %) than the leaves (^{13}C-excess of 0.02 %), or leaves AAs (^{13}C-excess of 0.02 %) (Table 1). The AA-^{13}C concentration in phytoliths represented 0.15 % of phytC which is low but still 1.5 times higher than the concentrations in the bulk matter and AAs of stems and leaves (Table 1). The AA-^{13}C/AA-^{15}N ratio in phytoliths was low (0.8) but higher than in bulk stems and leaves (0.3) (Table 1; Fig. 3).

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4 Discussion

4.1 Plausible forms of AA-^{13}C and AA-^{15}N absorbed and translocated

Festuca arundinacea may have absorbed AA-^{13}C and AA-^{15}N from the labeled solution in several organic and inorganic forms described below. The AA-^{13}C/AA-^{15}N ratios of roots and leaves (0.8 and 0.3, respectively) that were significantly lower than C/N ratios of the supplied AAs (from 2.6 to 7.7), suggested that the grass absorbed ^{15}N already mineralized in the tank preferentially to ^{15}N still in AA form. Similarly, biological and chemical degradation of AAs in the solution probably produced AA-$^{13}CO_2$. Additionally, under non-sterile conditions, microbial activity around roots can biodegrade the AAs in a range of hours (Jones et al., 2005; Kielland et al., 2007; Jones et al., 2009) and produce derived metabolites still containing AA-^{13}C and AA-^{15}N. At least, intact AAs can also be directly absorbed (Whiteside et al., 2009).

Inside the plant, absorbed AAs can be distributed to the whole plant by AA transporters or be rapidly subject to deamination. AA-^{13}C and AA-^{15}N absorbed in other forms than AAs can be used in the build-up of new molecules or as energetic resources (e.g. Näsholm et al., 2009). AA-^{13}C and AA-^{15}N can also be lost through respiration (Gioseffi et al., 2012) or exudates (e.g. Jones and Darrah, 1993). At least, rhizospheric and endophytic microorganisms, that acquired their labeled signature from the labeled solution, may also account for the AA-^{13}C and AA'^{15}N recovery in the plant. All these processes may have accounted for the ^{13}C and ^{15}N enrichments measured in the roots. However, the fact that ^{13}C was more concentrated in the extracted AAs than in bulk root matter (Table 1; Fig. 3), and further, that ^{13}C-excess values of MET and PHE were significantly higher than ^{13}C-excess values of other AAs in the roots (Fig. 4), supported that some AA-^{13}C was absorbed by and fixed in the roots in their original AA form.

From roots to leaves, the concentration of AA-^{13}C (relative to total C) in AAs decreased significantly (Fig. 3). This suggests that plants metabolized AA-^{13}C during the translocation to aerial parts. Deamination of labeled AAs, probably recombined into other AAs, sugars or other molecules constituting the plant matter may also be in-

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voked. At least some AA-^{13}C may have been lost via respiratory processes overtaking N losses, as suggested by the decrease of the AA-^{13}C/^{15}N ratio from roots to stems and leaves (Table 1). Those three processes probably occurred jointly.

Thus, the present labeling experiment does not allow to precisely trace the molecular form under which the AA-^{13}C and AA-^{15}N were absorbed and fixed in roots, stems and leaves, as recently done using a position-specific C and N labeling technique (Moran-Zuloaga et al., 2015). However the fact that PHE and METH that were supplied in high amount to the nutritive solution were significantly more ^{13}C-enriched than other AAs in the roots and stems and leaves (Fig. 4) strongly suggests that part of AA-^{13}C was absorbed and translocated in its original PHE and METH form. On the other hand, the low ^{13}C-enrichement in ALA suggests that this molecule was rapidly degraded or metabolized in the solution instead of being absorbed.

4.2 AA-^{13}C fixation in phytoliths

In agreement with the radiocarbon evidence for soil organic C occlusion in phytoliths (Reyerson et al., 2015), AA-^{13}C accounted for a measurable part of phytC. Due to the very small concentration of phytC, isotopic analysis at the molecular level was impossible. The phytolith C/N value (5.0) was close to a value previously measured in cultivated wheat phytoliths (3.7; Alexandre et al., 2015) and in the range of C/N values characteristic of AAs (4–5, Jones et al., 2009). However, the AA-^{13}C/AA-^{15}N ratio (0.8) was far from this range. The form in which AA-^{13}C, and most generally phytC, has been occluded in the silica structure thus remains unknown.

Previous Nano Secondary Ion Mass Spectrometry (NanoSIMS) investigation of phytC indicated that at least part of phytC is continuously distributed in the silica structure, at the sub-micrometric scale (Alexandre et al., 2015). This has been further supported by Raman spectroscopy mapping (Gallagher et al., 2015). The process of silica precipitation has been investigated by environmental scanning electron microscope (ESEM) and TEM-EDX analyses that highlighted that silica first precipitates in the in-

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ner cell wall, probably triggered by the presence of callose or lignin, then infills the cell lumen in a centripetal way, until most of the cell becomes silicified (e.g. Perry et al., 1987; Motomura, 2004; Laue et al., 2007; Law and Exley, 2011; Zhang et al., 2013). During this process, an organic template probably participates in the silica formation (Harrison, 1996; Laue et al., 2007). When the cell silicification is complete, residual organic compounds that were not already occluded probably gather in any remaining spaces within the cell and delimitate micrometric central cavities characteristic of most phytoliths (Alexandre et al., 2015). In the present case, the higher concentration of AA-^{13}C (relatively to total C) in phytoliths than in leaves, supports a preferential fixation of AA-^{13}C in these residual organic compounds subject to occlusion in the silica structure. There are two plausible hypotheses for this preferential fixation. The first hypothesis is that AA-^{13}C may be associated with Si when absorbed by the roots, translocated in the plant and introduced into the cells. However, ^{29}Si NMR spectroscopy of ^{29}Si-labeled exudate of wheat xylem previously indicates only the occurrence of the dissolved forms of Si (Casey et al., 2004). Although this does not preclude the subsequent formation of organo-silicate complexes it weakens the hypothesis of Si and C being associated since their uptake by the roots. Additionally, in our experiment, the roots that contain the lowest amount of Si also contain the highest amount of AA-^{13}C which is not in agreement with AA-^{13}C and Si being associated when absorbed by the roots. The second hypothesis is that AA-^{13}C may be isolated as an unwanted substance in cell vacuoles and subsequently trapped in the silica structure. In order to check this hypothesis we used D-ALA, expected to be less metabolized than L-AAs, although recent investigations suggest that plants are able to utilize D-AAs at rate comparable to those of other N forms (Hill et al., 2011). D-ALA was not specifically taken-up or retained as an intact molecule (Fig. 4) and cannot account for the AA-^{13}C measured in phytC. D-ALA may thus not be appropriate for tracing unwanted substances. Further investigations, including the use of spectroscopies relevant for characterizing phytC at the molecular level, are necessary to support or refute one or more of these other hypotheses.

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4.3 Implication for our understanding of the C cycle at the plant-soil interface

In the experiments presented here, the net uptake of AA-^{13}C by *Festuca arundinacea* represented 4.5 % of AA-^{13}C supplied to the nutrient solution, part of it being absorbed as intact AA molecules (here methionine and phenylalanine). AA-^{13}C fixed in the plant represented only 0.13 % of total C, the root absorption of AA-^{13}C being clearly marginal compared to photosynthesis. However, our experiment may underestimate the extent of the process under natural and field conditions. Indeed, AAs uptake was shown to be inhibited by the high concentrations of mineral N preferred by the plant (Paungfoo-Lonhienne et al., 2008; Sauheitl et al., 2009; Gioseffi et al., 2012). In the present case, mineral N may come from KNO_3 initially presents in the original nutrient solution and from the supplied AAs dissociated by microbes, mycorrhizas or root exsudates (Paungfoo-Lonhienne et al., 2008; Keiluweit et al., 2015). The maximum AA content in the growth solution (0.2 mmol L^{-1}) was higher than AAs concentrations that have been measured in soil solutions (from 0 to 0.1 mmol L^{-1}; Vinolas et al., 2001; Jämtgård et al., 2010). The probable high concentration of inorganic N in the solution, partly coming from the mineralization of AAs supplied in high concentration, may consequently have inhibited AAs uptake. Additionally, due the RHIZOtest configuration, roots were confined to a small volume, and their contact with the renewed labeled solutions and AA-^{13}C available for uptake was limited. Thus, the range of AA-^{13}C uptake and allocation in roots, stems, leaves and phytoliths of the grass grown in the present labeling experiment may underestimate what may happen in field conditions.

The comparison with soil conditions is not straightforward due to the diversity of C and N biogeochemical exchanges in soils. However, to gain an idea on the order of magnitude of the C flux that may occur from soil to plant, we used the 0.13 % AA-^{13}C concentration in plants obtained in the present experiment and extrapolated that value to the grassland ecosystem scale. Grasslands cover a global surface of 2.4×10^9 ha (Scurlock and Hall, 1998) and are characterized by a Net Primary Production (NPP) ranging from 7 to 20×10^9 t C ha^{-1} yr^{-1} (Scurlock et al., 2002). The global grassland

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productivity thus ranges from 16.8 to 48 10^9 t C yr^{-1}. The obtained flux of soil-derived C absorbed by grasses then ranges from 21.8 to 62.4 × 10^6 t C yr^{-1}. This is nonsignificant when compared to the 2.6 × 10^9 t C yr^{-1} estimate for the land C sink (IPCC Staff, 2007), or to the 0.4 × 10^9 t C yr^{-1} estimate for the global long term soil C accumulation rate (Schlesinger, 1990). It is however higher than a possible CO_2 phytolith biosequestration flux (e.g. Parr and Sullivan, 2005; Parr et al., 2010; Song et al., 2014). The CO_2 phytolith biosequestration concept is based on the assumptions that phytC is exclusively derived from atmospheric CO_2, and has a long residence time in soils. A recent re-examination of the CO_2 biosequestration flux by phytoliths, in the light of the lower and more realistic estimates of phytolith residence time in soils, yielded a value of 4.1 × 10^4 t C yr^{-1} for the world grasslands (Reyerson et al., 2015). The present study further minimizes the significance of CO_2 biosequestration by phytoliths showing that it could be counteracted by the flux of C potentially mobilized from soils by grass root uptake.

Recent experiments have demonstrated that root exudates promote a net loss of soil C previously assumed to be stable at the millennial scale thanks to its protection by mineral constituents (e.g. clays or amorphous minerals). Root exudates would stimulate microbial and fungi digestion (priming effect) (Fontaine et al., 2003, 2011) and promote dissolution of the mineral phase through oxalic acid production (Keiluweit et al., 2015). From the present experiment, we suggest that direct uptake of organic C by roots, in conjunction with the N uptake, should be accounted for when investigating the role of roots in soil C mobilization. Radiocarbon dating of phytC (the ultimate fixation of soil C absorbed by the roots) that produced ages of several thousand years (Reyerson et al., 2015) strongly suggests that old pools of soil organic C are being accessed by root uptake.

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5 Conclusions

In agreement with previous studies, the present labeling experiment supports that AA-C absorbed by grass roots and allocated to stems and leaves can partly preserve its original molecular form (here methionine and phenylalanine). Moreover, the experiment shows for the first time that AA-C absorbed by grass roots and allocated to stems and leaves can partly feed the C ultimately fixed in organic compounds subject to occlusion in stem and leaf silica. Further analyses are required to identify the form in which phytC has been occluded. This finding complements previous radiocarbon evidence of soil C (and more particularly old soil C) contribution to phytC (Santos et al., 2012; Reyerson et al., 2015) and raise questions about the mechanisms that drive soil C mobilization by plant roots, for a better understanding of soil/plant interactions involved in the terrestrial C cycle.

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Table 1. Allocation of AA-^{13}C and AA-^{15}N in *Festuca arundinacea* grown in labeled and control solutions. Mean values and standard deviations (numbers in brackets) are given. The numbers in bold refer to the percentage of the individual AA applied. Un. is for unanalyzed.

	Dry weight	Total elements			Isotopic composition				Label concentration	
	d.wt g	[C] mg g^{-1} d.wt	[N]	C/N	δ^{13}C ‰	^{13}C-excess Atom%	δ^{15}N ‰	^{15}N-excess Atom%	[AA-$^{13}C_{sample}$] % plant C	[AA-$^{15}N_{sample}$] % plant N
Control tank										
Arial Part	16.2	389.4	43.4	9.0	31.7		13.8			
Phytolith	0.03	0.0	0.0	5.2	28.8		3.2			
Roots	4.4	270.3	63.9	4.2	31.0		14.5			
Labeled tank-Aerial parts										
Total	23.9	395.0	46.8	4.9		0.017		0.338	0.10	2.6
	(0.5)	(15.0)	(4.0)			(0.001)		(0.047)	(0.003)	(0.4)
Phytolith	0.040	5.1	1.05	4.9		0.026		0.118	0.15	0.9
	(0.001)	(0.9)	(0.07)	(1.2)		(0.002)		(0.038)	(0.000)	(0.3)
Sum AAs		52.5	13.2	4.0		0.020			0.10	Un.
		(6.4)	(1.6)						(0.000)	
Phenylalanine (PHE)		5.4	0.7	7.7		0.038			0.20	Un.
		(0.5)	(0.1)			(0.010)			(0.000)	
Methionine (MET)		0.3	0.1	4.3		0.187			1.10	Un.
		(0.2)	(0.0)			(0.027)			(0.200)	
Alanine (ALA)		4.7	1.8	2.6		0.012			0.07	Un.
		(1.1)	(0.4)			(0.001)			(0.000)	
Labeled tank-Roots										
Total	4.9	376.0	34.3	11.0		0.048		0.534	0.28	4.1
	(0.5)	(13.0)	(3.6)			(0.000)		(0.100)	(0.002)	(0.8)
Sum AAs		52.0	14.6	3.6		0.112			0.70	Un.
		(5.3)	(1.5)						(0.100)	
Phenylananine (PHE)		2.3	0.3	7.8		0.499			2.90	Un.
		(0.2)	(0.0)			(0.155)			(0.900)	
Methionine (MET)		0.3	0.1	4.3		0.546			3.20	Un.
		(0.1)	(0.0)			(0.049)			(0.300)	
Alanine (ALA)		3.8	1.5	2.6		0.101			0.60	Un.
		(0.2)	(0.1)			(0.005)			(0.000)	
Labeled tank-total										
Total plants	28.8	391.5	44.7	8.8		0.022		0.371	0.13	2.8
		(14.1)	(4.1)			(0.000)		(0.060)	(0.010)	(4.3)

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Table 1. Continued.

	Recovery of label from solution			Label net uptake	
	Recovery AA-^{13}C µg C g^{-1} d.wt	Recovery AA-^{15}N µg N g^{-1} d.wt	AA-^{13}C/^{15}N	AA-13C uptake % supplied C	AA-^{15}N uptake % supplied N
Control tank Arial Part Phytolith Roots					
Labeled tank-Aerial parts					
Total	385.0 (1.7)	1201.7 (66.4)	0.3	2.86 (0.05)	37.94 (1.23)
Phytolith	0.02 (0.003)	0.0002 (0.00006)	0.8	2×10^{-7} (4×10^{-8})	1×10^{-8} (3×10^{-9})
Sum AAs	50.7 (0.0)	N.D.		**0.38** **(0.000)**	Un.
Phenylalanine (PHE)	12.1 (3.2)	N.D.		**0.18** **(0.047)**	Un.
Methionine (MET)	3.2 (0.5)	N.D.		**0.15** **(0.022)**	Un.
Alanine (ALA)	3.3 (0.3)	N.D.		**0.07** **(0.006)**	Un
Labeled tank-Roots					
Total	1065.0 (43.6)	1387.2 (117.5)	0.8	1.61 (0.24)	8.98 (1.74)
Sum AAs	347.0 (49.6)	N.D.		**0.53** **(0.075)**	Un.
Phenylananine (PHE)	66.9 (20.8)	N.D.		**0.20** **(0.063)**	Un.
Methionine (MET)	9.8 (0.9)	N.D.		**0.10** **(0.009)**	Un.
Alanine (ALA)	22.6 (1.2)	N.D.		**0.10** **(0.005)**	Un
Labeled tank-total					
Total plants	500.7 (22.0)	1232.3 (79.0)	0.4	4.5 (0.197)	46.9 (3.0)

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Table 2. Concentration of phytoliths, phytolith occluded C ([PhytC]) and phytolith occluded N ([PhytN]) in *Festuca arundinacea* grown in labeled and control solutions. Numbers in italics refer to the standard deviation associated with the averaged values (one value per labeled tanks).

	Phytolith	[PhytC]	[PhytN]
	% d.wt plant	% d.wt phytolith	
Control tank			
Aerial part	0.102	0.88	0.17
Roots	0.075		
Labeled tank			
Aerial parts	0.19	0.51	0.1
	(0.002)	(0.08)	(0.007)
Roots	0.014		
	(0.01)		

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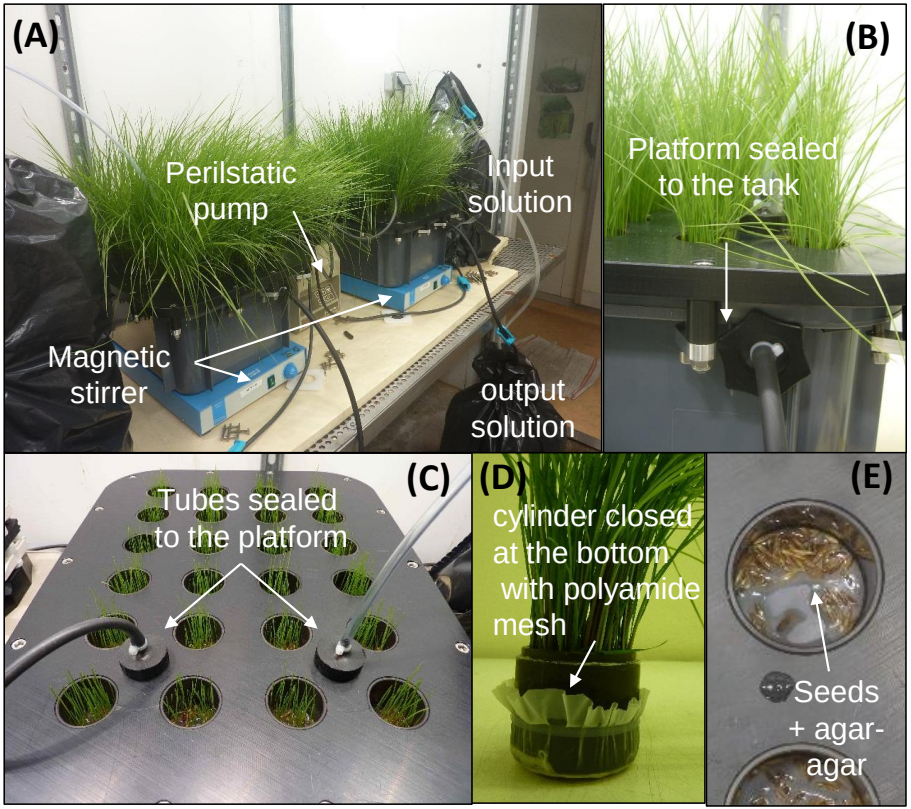

Figure 1. The labeling experiment in the growth chamber. **(a)** The two labeled tanks are connected to the solution containers. A perilstatic pump facilitates the solution renewals. **(b)** A platform is sealed to each tank. **(c)** Plant-receiving pots are cylinders closed at the bottom with a polyamide mesh. **(d)** Twenty-four plant-receiving pots are inserted into each platform. **(e)** In each pot, seeds are covered with agar-agar to limit gas exchanges.

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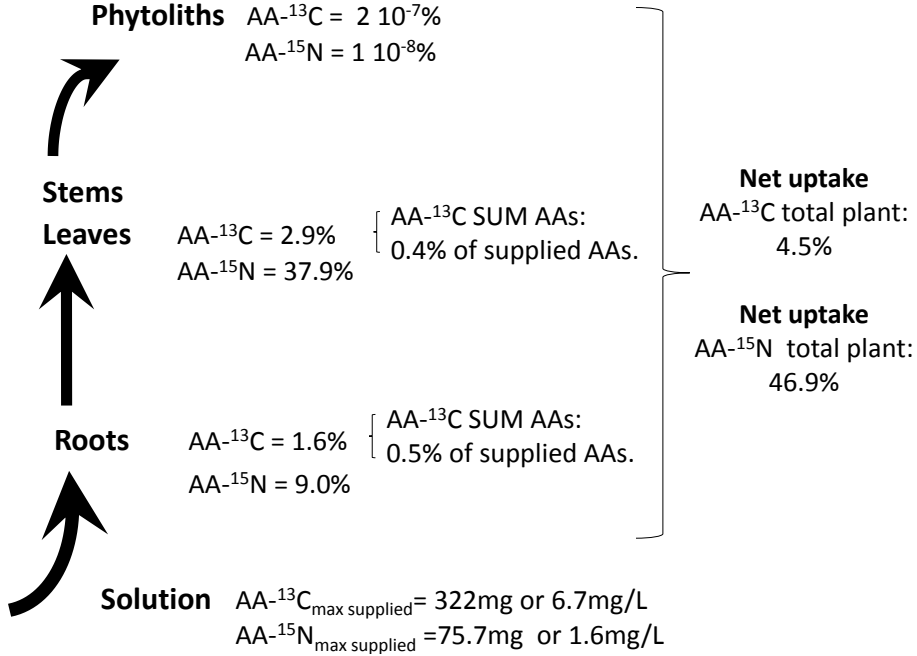

Figure 2. Net uptake of AA-^{13}C and AA-^{15}N by *Festuca arundinacea* grown in labeled tanks (in % of maximum ^{13}C and ^{15}N supplied to the nutritive solution).

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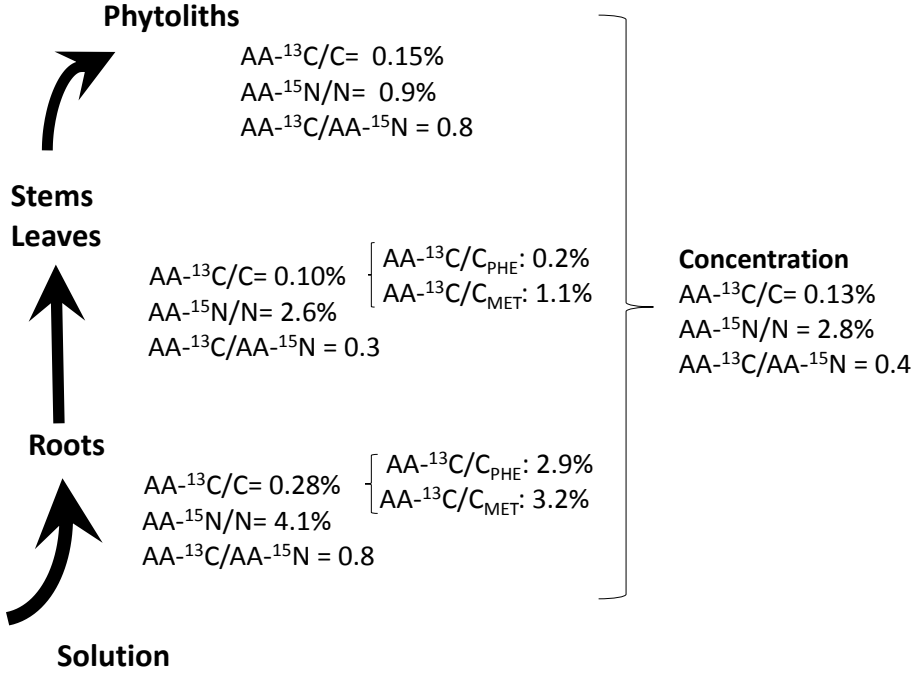

Figure 3. Concentration of AA-^{13}C and AA-^{15}N in bulk matter, phenylalanine (PHE) and methionine (MET) of roots, stems and leaves and phytoliths of *Festuca arundinacea* grown in labeled tanks (in % of bulk C, N, Phe and Met respectively).

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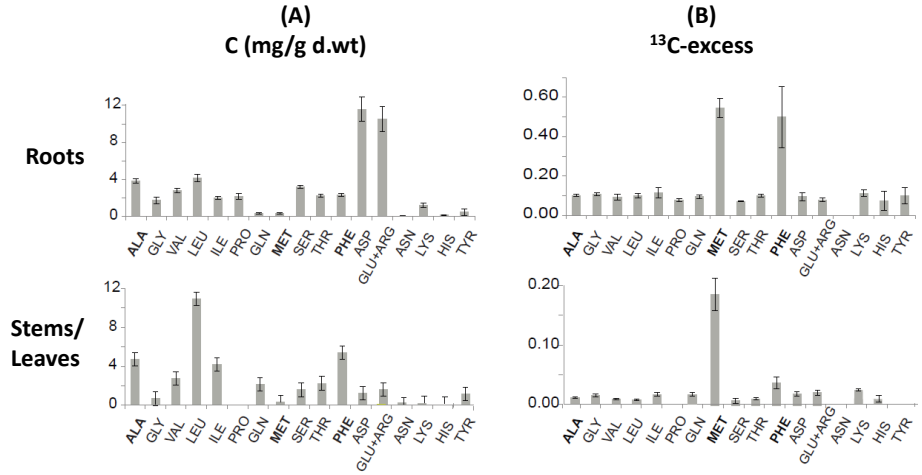

Figure 4. Concentration **(a)** and ^{13}C-excess **(b)** of amino acids (AAs) measured by GC-IRMS in roots, stems and leaves. Bars stand for one standard deviation of 4 replicates (2 tanks × 2 AAs extractions).

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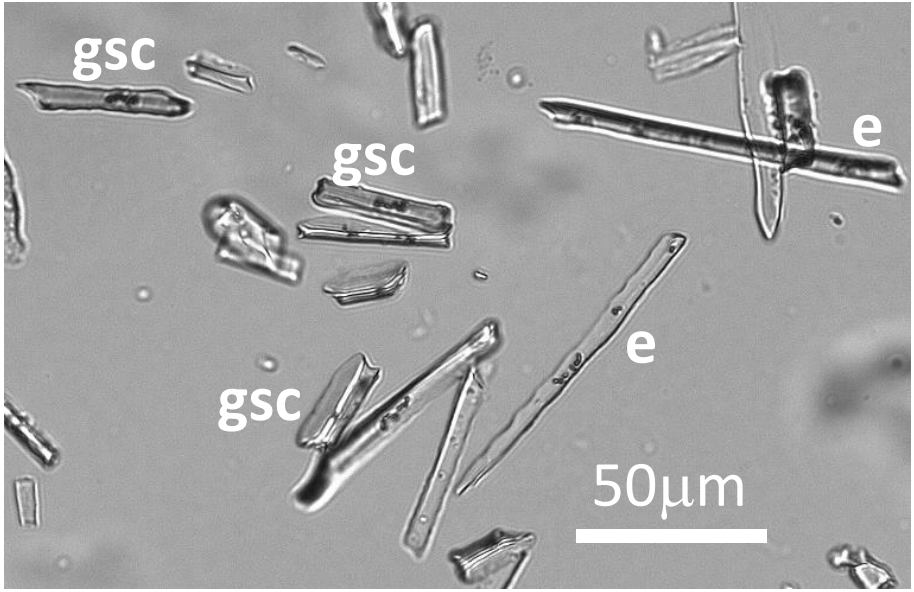

Figure 5. Natural light microscopy image of the phytolith assemblage produced by the stems and leaves of *Festuca arundinacea* dominated by (e) the elongate type and (gsc) the grass short cell trapeziform type (Madella et al., 2005).

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