Rhizodeposition of organic carbon by plants with contrasting traits for resource acquisition: responses to different fertility regimes

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Abstract (200 words)

Background and Aims : Rhizodeposition plays an important role in mediating soil nutrient availability in ecosystems. However, owing to methodological difficulties (i.e. narrow zone of soil around roots, rapid assimilation by soil microbes) fertility-induced changes in rhizodeposition remain mostly unknown.

Methods : We developed a new long-term continuous ^{13}C labelling method to address the effects of two fertility levels on rhizodeposited C by plants different in nutrient acquisition strategies. .

Results : Experimental controls demonstrated that most of the biases related to the nature of this type of experiment (i.e., long-term steady-state labelling) were avoided. Our results showed that fertility-induced changes in rhizodeposition were modulated by root responses to nutrient availabilities rather than change in soil microbial biomass. Differences between species were mostly related to plant biomass, with higher total rhizodeposited C in species with higher total leaf and root biomass and higher specific rhizodeposited C (per gram of root) for species with lower root biomass.

Conclusions : These results suggest that the amount of C rhizodeposited under different levels of N were driven mainly by plant biomass and root morphology rather than microbial biomass and were more dependent on biomass allocation and morphological traits that on plant resource acquisition strategies.

Key words: carbon, exudation, leaf traits, nitrogen, plant nutrient use strategies, root traits.

Introduction

The availability and management of N is a major constraint on plant productivity in many agro-ecosystems worldwide (Passioura 2002; Goll et al. 2012). In most natural ecosystems and croplands which are not fertilized with inorganic amendments, nutrient availability to plants is largely determined by the saprotrophic activity of soil microbes, which decompose soil organic matter (SOM) into mineral carbon and inorganic nutrients (that are in turn readily taken up by plant roots and associated mycorrhiza; Molina and Smith 1998; Valé et al. 2005). At the same time, nutrient cycling in the rhizosphere can also be regulated by the provision of plant C to saprotrophic microbiota (Paterson 2003; Bahn et al. 2013). Rhizodeposition, that is, the release by plant roots of labile organic solutes and sloughed-off cell tissues, represents up to 30% C lost by plants (Jones et al. 2009, Neumann et al. 2009), and is believed to constitute a strategy whereby growing plants foster soil nutrient release by supplying additional energy to SOM decomposing microbes (Bardgett et al. 1998, 2008). Soluble compounds exuded by roots - mainly sugars, carboxylic acids and amino acids - account for 1 to 10% of rhizodeposition (Paterson 2003; Jones et al. 2004) and together with other rhizodeposits, they provide energy to soil microbiota for the mineralization of organic nitrogen (among other nutrients) from SOM (Paterson 2003; Jones et al., 2004; Kuzyakov & Xu 2013). Although mineralized N would initially be incorporated into microbial biomass, rapid turnover of microbial cells (relative to roots) ultimately makes it available to plants (Schmidt *et al*., 2007). Rhizodeposition may thus play an important role in speeding up the cycling of nutrients in the rhizosphere, and therefore plant growth itself (Lynch 1990; Bardgett et al. 1998).

The role of rhizodeposition in soil nutrient availability has previously been addressed in studies comparing plants grown under different fertility conditions (Paterson and Sim 1999; 2000; Denef et al. 2009). Nevertheless, the variability of the results obtained is high, which leads to significant discrepancies in the subsequent conclusions. For instance, in studies conducted with artificial substrates (such as hydroponics and axenic sand culture systems), the loss of C per unit weight of root increased with a low N supply (Paterson and Sim 1999; 2000). In contrast, in other studies carried out with soil, the effects of N fertilization on rhizodeposition were inconclusive with both positive and negative effects of low N supply on rhizodeposition (Jones et al. 2004; Denef et al. 2009). These contradictory findings may partly be due to methodological constraints. Rhizodeposited compounds are rapidly assimilated by soil microbes, thereby placing a limit on the possibility of estimating total plant C rhizodeposition during a given period of time (Paterson *et al.*, 2003; 2005; Dilkes et al. 2004). Furthermore, most studies investigating rhizosphere effects on SOM cycling have used continuous labelling or natural tracer techniques (by means of ^{13}C or ^{14}C isotopes) to differentiate plant-derived

CO_2–C from SOM-derived CO_2–C effluxes (Meharg 1994; Andrews et al. 1999; Nguyen et al. 1999). Continuous ^{14}C labelling is hazardous and requires special facilities that only exist in a few places around the world. Moreover, pulse labelling does not allow for the separation of total plant-derived CO_2–C from SOM derived CO_2–C since it does not uniformly label all plant C pools (Kuzyakov and Cheng 2004). Thus, the quantified release from roots is biased towards those pools receiving the greatest contribution from recent photoassimilates (Meharg 1994; Paterson et al. 2009).

Another aspect which may explain the above mentioned discrepancies could be the fact that those studies were carried out on plant species with differing nutrient acquisition strategies. Two of these strategies are broadly described in the literature and linked to soil nutrient availability (Tilman 1980, Grime 1979, Wright et al. 2004). Nutrient-poor ecosystems are dominated by species with low relative growth rate (RGR) coupled with low nutrient concentrations in tissues such as root nitrogen concentration (RNC) and leaf nitrogen concentration (LNC) (Lavorel and Garnier 2002; Roumet et al. 2006). Conversely, nutrient-rich ecosystems are dominated by species with high RGR and high LNC and RNC (Craine et al. 2001). Because of these contrasting strategies, fertility-induced change in root exudation may vary largely depending on plant species (Rovira 1969; Dakora and Phillips 2002). For instance, microbial activity in the rhizosphere is thought to be enhanced by plant species with high root exudation rates, which can be traced to rapid plant growth (entailing the disposal in large quantities of photoassimilates) and/or from root morphologies with high root biomass (Craine et al. 2001) and low levels of structural C (which do not limit the passive diffusion of exudates through root tissue) (Valé et al. 2005). Thus, species of nutrient-rich ecosystems may have the best potential to enhance C rhizodeposition (Van der Krift et al. 2001). In contrast, plant species with low relative growth rate which dominate nutrient-poor ecosystems may have a lower rate of C rhizodeposition in soil. Nevertheless, it has been suggested that these species might increase exudation to favour soil N mineralization (Van der Krift et al. 2001) even if the enhancement of soil N mineralization would be limited since under limiting nutrient conditions, both plant roots and active microbes compete for the available resources such as soil N, which could reduce N-acquisition by plants in the short term (Jones et al. 2004; Kuzyakov and Xu 2013).

In this study, we aimed to elucidate the effects of two fertility levels on rhizodeposited C - and its implication in soil microbiota - of four plant species collected from semi-natural grasslands with contrasting nutrient acquisition strategies (two exploitative species represented by *Dactylis glomerata*, *Lolium perenne*, and two conservative species represented by *Anthoxantum odoratum* and *Briza media*) (Quétier et al. 2007; Harrison and Bardgett, 2010). We hypothesised that (i) rhizodeposition rate were higher in plant with exploitative

strategies to stimulate microbial activities and that (ii) the changes in rhizodeposition rate of plant species in response to N fertilization were dependent of plant nutrient acquisition strategies. To do so, plants were submitted to continuous ^{13}C labelling during almost three months in growth chamber. Once steady state was reached, two harvests, 7 days apart, were performed in order to estimate C flows and mass-balance ^{13}C of the different soil-plant systems. These calculations allowed the estimation, over a period of 7 days, of ^{13}C rhizodeposition of the four species under both fertility regimes. This estimation was based on the hypothesis that ^{13}C rhizodeposition was equal to the sum over 7 days of (1) the cumulative labelling-derived ^{13}C content in labile soil C, (2) the cumulative labelling-derived ^{13}C content in microbial biomass and (3) the cumulative labelling-derived ^{13}C respired by microbial biomass. Additional soil and plant analysis (NH_4^+ and NO_3^- analysis, soil pH, $^{13}CO_2$ respired by the roots) were performed in order to identify underlying processes.

Material and methods

Plant culture

Two exploitative species (*Dactylis glomerata*, DG and *Lolium perenne*, LP*)*, and two conservative species (*Anthoxantum odoratum*, AO and *Briza media*, BM*)* were selected for this study, all of which belong to the *Poaceae* family. *Dactylis glomerata* and *Briza media* were collected from adult tussocks during July 2009 at the Lautaret pass (French Alps, 2000 m a.s.l., 45° 4' N - 6°34' E), and *Lolium perenne* and *Anthoxantum odoratum* were collected from adult tussocks in an English grassland (54°18' N - 2°5' W, Yorkshire Dales, United Kingdom) during the same period.

Upon receipt of the plants, they were immediately transplanted into pots. On each ramet, roots and leaves were cut at 5-cm from the base and planted individually in the pot. Each pot was filled with 220 g d.w. sandy grassland topsoil (0–30-cm) collected at the Helmoltz Zentrum experimental station in Scheyern (Germany, 479 m a.s.l., 48°30′N, 11°28′E), with a sandy soil texture (7.6% clay, 10.3% of silt and 82.1% sand). Total soil organic C and N contents were equal to 0.19g kg^{-1} and 0.01g kg^{-1} respectively. Soil pH_{H2O} was 6.5.

A total of 22 pots per species were prepared and placed in two environment-controlled chambers (Conviron E15, Controlled Environments Ltd., Winnipeg, Manitoba, Canada). In both growth chambers, the photoperiod was 10h, and mean air temperatures were equalled to 20°C with a relative air humidity maintained at 46%. Plants were supplied with a photosynthetic photon flux density (PPFD) of about 400 ± 30 µmol m^{-2} s^{-1} during the light period. Soil moisture content was corrected to 70% of soil water holding capacity (WHC) and maintained constant until the end of the experimental period (i.e. loss by evapotranspiration was counterbalanced each day by adding a given amount of water to the pots).

Eighteen pots without plants (“bare soil pot”) were placed in parallel with the plants as controls. Watering was conducted following the same protocol used for plants. In order to avoid any algal development in the upper soil layer, dark circle paper with a hole in the center was added to each pot.

Fertility treatment

The fertilization treatment aimed to simulate the higher level of fertility found in grasslands where the plant species were collected. For each plant species, half of the twenty-two replicates (i.e. eleven replicates) were N-fertilized (100 kg N ha^{-1}) using half-stretch Hoadland solution (i.e. NH_4NO_3; Arnon and Hoagland, 1939), whereas the other half received the same Hoadland solution without nitrogen (0 kg N. ha^{-1}), thereby avoiding any additional nutrient limitations. Bare soil pots followed the same protocol. Irrigating solution (with and without nitrogen) was provided at two application times, on the 30/10/09 and on the 06/11/09. For N-fertilized plants,

this corresponded to a supply of 40kg N ha^{-1} and 60kg N ha^{-1} respectively. The concentration of others macronutrients was (in mM): 0.7 K, 3.18 Ca, 0.55 P. The concentration of micronutrients was (in µM): 6 Cl, 14 B, 3 Zn, 0.7 Cu, 0.7 Mo, 0.1 Co and 200 Fe (as EDDHA).

Labelling procedure

In order to estimate rhizodeposited C, we conducted a steady state labelling in a $^{13}CO_2$ enriched atmosphere as previously described (Aljazairi et al 2014). The labelling procedure lasted in total 81 days, from the 02/11/09 until the 21/01/10. Sixteen replicates per species (n=8 per fertilization level) and twelve 'bare soil' pots (n=6 per fertilization level) were placed in the growth chamber with a ^{13}C-labelled atmosphere (total of 76 pots). Another six replicates per species (n=3 per fertilization level) and six "bare soil" pots (n=3 per fertility level) were considered as control samples and grown during the whole experiment in the other growth chamber with an unlabelled atmosphere (total of 30 pots). The design of the experiment is described in S1 and S2.

In the growth chamber with the ^{13}C-labelled atmosphere, a 50 ml syringe (SGE, Ringwood, Australia) and needle (model microlance 3, BD, Plymouth, UK) were filled each day and placed on a syringe pump which delivered 6ml h^{-1} of $^{13}CO_2$ (99.9%). This system allowed homogeneous labelling throughout the day. Calculations indicates that, given the CO_2 injected by the syringe pump and the volume of the growth chamber, the total concentration of CO_2 in the growth chamber without taking into account plant gas fluxes was equalled to 410 ppm. Based on IRGA measurements, $^{12}CO_2$ concentration varied from 300 to 500 ppm during the day depending on plant gas exchange activity (see S3). Diurnal isotopic composition of atmospheric CO_2 in the growth chamber reached 200 to 300 ‰ (see S3). No ^{13}C-CO_2 was injected at night.

Plant and soil sampling

After 81 days in the $^{13}CO_2$-enriched atmosphere, two harvests were performed 7 days apart in order to estimate ^{13}C flows and mass-balance ^{13}C of the soil-plant systems (t_i and t_f, see S2). In the growth chamber with the ^{13}C-labelled atmosphere, 8 replicates per species (n=4 per fertilization level) and 6 'bare soil' pots (n=3 per fertilization level) were harvested on 11/01/10 (t_i), and the remaining pots seven days later (i.e. 18/01/10, t_f). It is worth noting that species harvested at t_f stayed in enriched atmosphere during the seven days.

In the growth chamber with the unlabelled atmosphere, all pots (with and without plants) were sampled the same day on the 21/01/10 (see S2).

All pots with and without plant from ^{13}C-labelled and unlabelled atmospheres were processed following the same experimental procedure.

When plants were present, the whole plant was removed from the soil and was hand shaken in order to recover soil which remained on the roots. Roots and leaves were then separated.

In parallel, fresh soil was sieved at 2 mm in order to homogenize it and remove the remaining root fragments. These root fragments were pooled with their respective root samples. Leaf samples were dried (60°C for 48h), weighed and ground. Root samples were washed and separated into two pools: half of the biomass was immediately frozen and later used for total C content analysis, whereas the rest was used for measuring the root CO_2 efflux and its isotopic signature ($\delta^{13}C$) (see below). These analysis did not permit to estimate ^{13}C rhizodeposition (see section "Isotopic calculations"), but helped understand processes underlying rhizodeposition patterns between species and under both fertility regimes.

Soil analysis

Once sieved, root-free fresh soil samples were used for subsequent analysis. 18 g of dry-weight (DW) equivalent soil were used to estimate the amount and isotopic signature ($\delta^{13}C$) of SOM-derived CO_2-C efflux, following the same procedure described for roots (see below). Gravimetric soil water content (105 ºC for 5 h) and soil pH in water (1:5) were also determined in additional subsamples. Soil microbial and labile (i.e., extractable) C and N fractions were determined by the fumigation-extraction method (Vance *et al*., 1987). In brief, 8 g DW equivalent soil were mixed with 20 ml 0.5 M K_2SO_4, then shaken for 60 minutes, centrifuged and filtered. In parallel, equivalent soil subsamples were fumigated for 24 h with ethanol-free $CHCl_3$ inside a dark vacuum chamber, prior to a similar K_2SO_4-extraction. Soil labile C content and its isotopic composition of both the $CHCL_3$-fumigated and non-fumigated soil extract were estimated by injecting 1 ml of soil extracts into a HPLC coupled to an IRMS (Delta V Advantage, Thermo-Finnigan, Germany). This is a Surveyor MS HPLC Pump plus (Thermo-Finnigan) coupled to a Delta V Advantage isotope ratio mass spectrometry (Thermo-Finnigan) via an LC Isolink interface (Thermo-Finnigan). The mobile phase used was a degased MilliQ water constantly set to 400 µl/min. The quantitative chemical oxidation of compounds was performed in the LC Isolink interface with sodium peroxodisulfate ($Na_2S_2O_8$) solution (100 g/l) and orthophosphoric acid (H_3PO_4) (1.5M) within an oxidation reactor kept at 99.9ºC.

Ammonia (NH_4^+) and nitrate (NO_3^-) contents were analysed by spectrophotometry using 1 to 3 ml of extract, following the protocols described in Baethgen and Alley (1989) and Cataldo et al. (1975) respectively. Soil microbial C and N fractions were subsequently calculated as the difference between fumigated and non-fumigated extractions (microbial values not corrected for extraction efficiency, Vance et al. 1987). Total N immobilized in soil microbial biomass was calculated as the difference of NH_4^+ and NO_3^- soil content between t_i

and t_f.

CO_2 gas exchange and isotopic composition measurements

During sampling days (t_i and t_f), empty 0,4L-Perspex™ dark chambers were previously flushed for 30 min with CO_2-free air passing through the soda-lime column of the IRGA (model Li-6200, LI-COR, Inc., Lincoln, NE, USA). Each root and soil sample was then enclosed in one of the chambers. Root and SOM derived CO_2-C effluxes were estimated every minute for 10–15 min Air temperature (i.e. 20°C) and relative air humidity were recorded during all measurements. Dark chamber was then closed hermetically and CO_2 was allowed to accumulate up to a concentration of 600-1000 ppm after which air samples were collected using the above mentioned 50 ml syringe and needle (Nogués et al. 2004). The gas samples were passed through a magnesium perchlorate column (water vapour trap) and then immediately injected into a 10 ml vacutainer (BD vacutainer, Plymouth, UK). To avoid contamination by the air present in the syringe and needle, both were flushed with N_2 before taking each sample. The vacutainers were also overpressurized with N_2, with the pressure inside above atmospheric pressure.

Isotopic composition of root and SOM derived CO_2-C effluxes was analysed through a gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) as previously described by Nogués et al. (2008). After gas sampling, roots were then dried at 60°C for 48h and weighed. Samples were analysed to determine the carbon isotope composition using an Elemental Analyser Flash 1112 (Carlo Erba, Milan) coupled to isotope ratio mass spectrometry IRMS Delta C through a Conflo III Interface (Thermo-Finnigan, Germany).

Isotopic calculations

To estimate ^{13}C enrichment in leaf, root and soil samples, %Atom (*i.e.*, ^{13}C proportion) for ^{13}C was calculated using the following equation:

$$\%Atom = \frac{\delta + 1000}{\delta + 1000 + \frac{1000}{R_{standard}}}$$

where δ is the isotopic signature of C content in leaf, root and soil samples. $R_{standard}$ is the international standard reference (i.e. $^{13}C/^{12}C$, PeeDee Belemnite).

%Atom excess was then calculated as the %Atom ^{13}C differences between labelled and unlabelled leaf, root and soil samples (from control pots in unlabelled atmosphere, see S1 and S2):

$$\%Atom\ excess = Atom\%_{labelled} - Atom\%_{unlabelled}$$

The labelling-derived ^{13}C content ($\gamma^{13}C$, in µg ^{13}C) in leaf, root and soil samples was calculated as follows:

230 $$\gamma^{13}C = \%Atom\ excess \cdot \%C_{sample} \cdot mass_{sample}$$

231 where %C is the percentage of carbon in the sample.

232 Root and SOM derived $^{13}CO_2$-C effluxes ($\gamma^{13}C$, in µg ^{13}C g^{-1} DW) were calculated as follows:

233 $$\gamma^{13}C = \frac{\%Atom\ excess \cdot R_{sample}}{mass_{sample}}$$

234 where $mass_{sample}$ is the mass of the sample considered (DW), R_{sample} is CO_2-C effluxes and %Atom excess is the

235 ^{13}C atom excess in CO_2.

236 **Mass-balance ^{13}C budget calculations**

237 Cumulative rhizodeposited ^{13}C was calculated as the sum of (1) the cumulative labelling-derived ^{13}C content in

238 labile soil C between t_i and t_f, (2) the cumulative labelling-derived ^{13}C content immobilized in microbial biomass

239 between t_i and t_f, and (3) the cumulative labelling-derived ^{13}C respired by microbial biomass between t_i and t_f.

240 Because air temperature during soil CO_2 efflux measurements was strictly similar to temperature inside the

241 growth chamber (i.e. 20°C), cumulative labelling-derived ^{13}C respired by microbial biomass was merely

242 estimated by integrating soil CO_2 efflux measurements over seven days.

243 Hence, cumulative rhizodeposited ^{13}C (µg^{13}C) was equalled to :

244 $$\int_{t_i}^{t_f} {}^{13}C_{rhizodeposited} = \int_{t_i}^{t_f} \gamma^{13}C_{labile\ soil} + \int_{t_i}^{t_f} {}^{13}\gamma^{13}C_{microbial\ \ biomass} + \int_{t_i}^{t_f} \gamma^{13}C_{respired}$$

245 Rhizodeposited ^{13}C (µg^{13}C d^{-1}) was calculated as cumulative rhizodeposited ^{13}C divided by the number of days

246 between t_i and t_f (i.e. : seven days). Lastly, specific rhizodeposited ^{13}C (µg^{13}C g^{-1} root DW d^{-1}) was calculated as

247 rhizodeposited ^{13}C divided by root biomass (DW).

248

249 **Statistical analyses**

250 For all data, a three way ANOVA was performed in order to test for the effects of N fertility, species and time (t_i

251 and t_f) effects. Analyses which related rhizodeposition parameters to plant and root biomass and plant traits (i.e.,

252 leaf and root nitrogen concentration) used regressions. A Tukey's studentized range (HSD - Honest Significant

253 Differences) test was used to examine a posteriori differences among species. All analyses were performed with

254 Jump software (SAS Institute Inc., Cary, NC, USA).

255

Results

Plant characteristics

Total biomass, leaf and root biomass differed significantly between species. The observed ranking, DG>LP>AO=BM, mainly reflected variations in root biomass (Table 1 and 2). LP exhibited a root/shoot ratio significantly higher than the others species (1.53 *vs.* 0.88 on average for the other three species). Leaf and root N concentration displayed slight but significant variations between species (Table 1 and 2), BM being characterized by the highest values.

N fertilization led to a general increase of 22% in total biomass, 12% of which was due to leaf biomass increase. In contrast, N fertilization had no consistent effect on leaf and root N concentration (Table 1 and 2). Finally, root biomass was highly and negatively related to root N concentration (data not shown, R^2=0.62, F=99.7, P<0.0001). This was not the case between leaf biomass and leaf N concentration (data not shown).

The species × fertility interaction was significant for all biomass parameters (P<0.001) (Table 2). Indeed, in contrast to DG and AO, BM experienced a reduction in leaf and root biomass in response to N fertilization. LP displayed an increase of leaf biomass but a decrease in root biomass in response to N fertilization. However, root:shoot ratio did not change in response to fertilization (data not shown) but only differed between species with highest ratio for DG and LP in comparison to AO and BM (data not shown, F=9.23, P<0.001).

Steady-state ^{13}C labelling

Results demonstrate that, although isotopic composition of leaves displayed significant differences between t_i and t_f, isotopic composition of roots was similar when comparing these two times (see S4). Root isotopic composition differed significantly between species (see S.4) with a lower global value for AO (in average 238‰) compared to the others species (in average 276‰) (data not shown). However, this trend was not reflected in leaf isotopic composition data (see S4 for statistical analysis, mean global value for LP, BM and DG: 322‰ compared to 324‰ for AO, data not shown).

Microbial biomass might directly fix ^{13}C through PEP-Carboxylation. However in our study no direct ^{13}C carboxylation by microbial biomass was observed (see S5) as the isotopic composition of extractable soil C and microbial biomass in “bare soil” pots was similar to those in growth chambers with labelled and unlabelled atmosphere. Only microbial biomass from pots with plants displayed enriched ^{13}C.

Finally, $^{13}CO_2$ respired by the microorganisms largely correlates with ^{13}C microbial immobilization as show statistical results (R^2=0.55, F=36.28, P<0.0001, see S6).

Estimation of total rhizodeposited ^{13}C

Based on the entire dataset (i.e. species and fertility treatments taken together), cumulative labelling-derived ^{13}C content in labile soil C was very low at t_i and t_f, and globally stable over the period (i.e., -0.0068 ± 0.0012 $\mu g^{13}C$ over the period). In contrast, the average of total labelling-derived ^{13}C immobilized in microbial biomass was high and equal to 48.1 ± 8.0 $\mu g^{13}C$. Only BM pots, under fertilized treatment, displayed a reduction in cumulative labelling-derived ^{13}C content over the period. The mean value of CO_2 respired by microbial biomass was 0.58 ± 3.31 $ngC.g^{-1}$ soil DW min^{-1} over the period whereas the mean value $^{13}CO_2$ repired by microbial biomass efflux was equal to 0.014 ± 0.001 $ng^{13}C.g^{-1}$ soil DW min^{-1}**.** Mean cumulative labelling-derived ^{13}C respired by microbial biomass over the period was equal to 30.0 ± 3.7 $\mu g^{13}C$.

Effect of species and fertility on rhizodeposited ^{13}C

Total rhizodeposited ^{13}C varies between 5 and 18 $\mu g^{13}C\ d^{-1}$ depending on species and fertilization (Fig. 1a). More than 65% of rhizodeposited ^{13}C was immobilized in microbial biomass, the rest being respired by microbial biomass (Fig. 2a and 2b). The immobilization of ^{13}C in labile soil C represented less than 0.001% of total rhizodeposited ^{13}C (data not shown).

Total rhizodeposited ^{13}C and specific rhizodeposited ^{13}C differed significantly between species (Table 3, Fig. 1a and 1b). DG had significantly higher total rhizodeposited ^{13}C than LP and AO followed by BM. In contrast, when rhizodeposition flux was expressed per g of root DW, specific rhizodeposited ^{13}C decreased from AO to BM then followed by DG and LP (Table 3, Fig. 1a and 1b). In both cases, total variation was always less than a factor of 3 between the highest and the lowest values.

Whereas fertilization did not consistently affect specific rhizodeposited ^{13}C, its effect on total rhizodeposited ^{13}C was very significant (Table 3, Fig. 1a and 1b). Averaged over the four species, total rhizodeposited ^{13}C increased by 13% in response to N fertilization. This global effect was mainly explained by an increase for both DG and AO in ^{13}C microbial immobilization and in ^{13}C respired by microbial biomass (Fig. 2a and 2b).

The species × fertility interaction was significant for both total rhizodeposited ^{13}C and specific rhizodeposited ^{13}C (Table 3). Whereas total ^{13}C rhizodeposited by DG and AO was higher with fertilization, the opposite was observed in the case of LP and BM. Beside, DG, LP and BM showed lower specific ^{13}C rhizodeposited with fertilization while AO produced a higher value.

Total rhizodeposited ^{13}C correlated significantly to leaf biomass and leaf N concentration (Fig. 3a, b) and to root biomass and root N concentration (Fig. 3c, d). A significant relationship was observed between specific rhizodeposited ^{13}C and root biomass (Fig. 4c). However, specific rhizodeposited ^{13}C did not significantly correlate to leaf biomass (Fig. 4a) neither to leaf N concentration (Fig.4b) or root N concentration (Fig. 4d).

Impact of species and fertility on soil properties

Contents of soil NH_4^+ and immobilized NH_4^+ in microbial biomass were highly sensitive to plant species, fertilization and time effects (Table 4 and 5). Soil NH_4^+ content was significantly higher for LP than for AO, DG and BM. In contrast, LP soil had lower immobilized NH_4^+ in microbial biomass compared to the other species. Whereas soil NH_4^+ content was significantly lower with fertilization (apart from under BM), the opposite trend was observed for immobilized NH_4^+ in microbial biomass (Table 4 and 5). Finally, soil NH_4^+ content and immobilized NH_4^+ in microbial biomass were systematically higher at t_i compared to t_f. Total C microbial biomass did not respond to species or fertilization (Table 4 and 5). Nevertheless, time effect was highly significant, with C microbial biomass being higher at t_f than at t_i.

Soil pH was significantly affected by species and fertilization (Table 5). Without plants, soil pH was from 0.30 to 0.50 units lower than with plants. Furthermore, soil pH was lower for BM, followed by AO, LP and DG. The variation between the lowest and the highest values was 0.12 units. Finally, fertilization significantly decreased soil pH by an average of 0.06 units.

Discussion

The aim of this study was to elucidate the impact of two fertility levels on rhizodeposited C by grassland species characterized by different nutrient acquisition strategies. Conclusions provided by previous studies are conditioned by technical difficulties that limit the comparison of rhizodeposited C between species or under different treatments such as fertility levels. Techniques based on a pulse-labelling approach were rarely sufficient to uniformly label plant inputs into soil. Consequently, only the contribution of recently labelled assimilates could be estimated (Paterson et al. 2005). The experimental design developed in this study avoided the different biases generally reported in the literature (Paterson et al. 2005; 2009). The fact that in our study the isotopic composition of roots remained constant from t_i to t_f for each species confirmed that a steady-state was reached. No direct ^{13}C carboxylation by microbial biomass was observed in this experiment as the isotopic composition of extractable labile soil C and microbial biomass in "bare soil" pots was similar in growth chambers with a labelled and an unlabelled ^{13}C atmosphere. Hence, these several controls demonstrate that most of the biases related to the nature of experiment (i.e. steady-state long-term labelling) were avoided. Although soil and root respiration were not carried out *in situ*, measurement of microbial and root CO_2 effluxes were similar to those reported in the literature (Tjoelker *et al.*, 2005; Baptist *et al.*, 2009). Hence, although it was not possible to estimate absolute rhizodeposited ^{13}C fluxes in real conditions, this experiment allowed for the interpretation of the data in a comparative manner, that is between species and under both fertility regimes.

Total rhizodeposited ^{13}C differed significantly across the four species as DG displayed significantly higher total rhizodeposited ^{13}C than LP and AO followed by BM. Large variations were also observed when rhizodeposition flux was expressed per g of root DW with higher values for AO, followed by BM and then DG and LP. These results differ from our expectations as conservative species display higher rhizodeposition fluxes per g of root than the two exploitative species. Fertilization consistently affected total rhizodeposited ^{13}C but not specific rhizodeposited ^{13}C. The rate of total rhizodeposited ^{13}C was higher in DG and AO with fertilization whereas the opposite was observed in the case of LP and BM. Hence, the two exploitative species studied, as well as the two conservative species, showed respectively an opposite response to fertilization. This finding suggests that plant C rhizodeposition responses to the fertilization were not in line with nutrient acquisition strategies as we expected but species dependent. Hence, rhizodeposition patterns could not be associated with the leaf economic spectrum (Freschet et al. 2010). Various underlying processes can explain these results including differences in sink strength applied by rhizospheric microbial biomass (Jones et al., 2009; Bahn et al., 2013) or differences in plant features such as root biomass and morphology (Van der Krift *et al*., 2001; Warembourg et al., 2003, Darwent et

al. 2003).

Our results suggest that rhizospheric microbial biomass was not the main driver of rhizodeposition process in contrast to several studies (Jones et al. 2009; Bahn et al. 2013). Indeed, in accordance with the study of Henry et al. (2005) no differences in soil C microbial biomass were found between plant species and fertility treatment whereas changes in rhizodeposition patterns were observed. Furthermore, no correlation was found between total or specific rhizodeposited ^{13}C and soil microbial biomass variations between t_i and t_f (data not shown). This C microbial biomass stability could be explained by the fact that microorganisms were carbon replete whatever the plant species and fertility levels (Paterson et al. 2009) and might suffer from another limitation such as nitrate deficiency. Indeed, no nitrate was detected in soils whatever species and fertility treatments. Following this hypothesis, microbial community may have suffered either from nitrogen competition with plant species (Legay et al. 2013) or from exudated plant C compounds inhibiting nitrification (Subbarao et al. 2009) leading consequently to a reduction in microbial community growth. Although microbial biomass did not change, we cannot exclude a change in community composition in response to fertilization which could favour development of bacteria at the expense of fungal community (Denef et al. 2009). Such change could modify the strength of the C sink applied by rhizospheric microbial biomass (Jones et al., 2009; Bahn et al., 2013) and also the plant rhizodeposition rates. Further analyses of structure of microbial community, under different N level for each species, would be needed to resolve this issue

The fact that microbial biomass did not change in response to different rhizodeposition patterns of plant species support the hypothesis that in our experimental conditions, microbial biomass was not a driving force for rhizodeposition rate. In contrast, strong correlations between total rhizodeposited ^{13}C and leaf or root biomass were observed supporting previous studies which assume that exudation patterns were affected by species identity and particularly root biomass (Van der Krift et al. 2001; Blagodatskaya et al. 2014). All together, these results suggest that changes of rhizodeposition rate following fertilization were not dependent of soil microbial community but rather support a substantial influence of plant features (Farrar et al., 2003; Warembourg et al., 2003; Kuzyakov and Cheng, 2004; Badri and Vivanco, 2009) on rhizodeposition processes as already demonstrated by previous studies on grassland (Van der Krift *et al*., 2001; Pausch *et al*., 2013) and forest species (Bowden *et al*., 2004).

An interesting finding is that total rhizodeposited ^{13}C and specific rhizodeposited ^{13}C were both correlated to total root biomass but in a contrasted manner. The first one was positively correlated to root biomass, as so for leaf biomass. Hence, the more the plant grows the more the plant rhizodeposits. However,

specific rhizodeposited ^{13}C, that is to say, ^{13}C rhizodeposited per g of root was negatively correlated to root biomass. Hence, the more the plant allocates biomass to roots, the less the plant rhizodeposits per g of root underlying a loss in rhizodeposition efficiency when belowground biomass increases (Henry et al. 2005). Such results were not observed between specific rhizodeposited ^{13}C and leaf biomass, or N leaf and root concentration. This loss of rhizodeposition efficiency was in accordance with trends observed in two grasses with contrasting nutrient acquisition strategy, *L. perenne* (exploitative) and *Festuca rubra* (conservative) (Paterson and Sim, 1999, 2000). These two species displayed a decrease in rhizodeposition rate in response to fertilisation, despite larger root biomass. This was mainly due to larger specific root length at low N. These authors suggest that high specific root length (SRL) would increase soil exploration and also exudation efficiency through a finest root system, both allowing higher nutrient acquisition and stimulation of microbial mineralization processes. Moreover, it is acknowledge that a high SRLis associated with high numbers of root tips (Arredondo and Johnson 1999) which have the greatest rhizodeposition rate compared with other area of the root system (Darwent et al. 2003). Taken together, these findings suggest that changes in the amount of rhizodeposited C per unit of root dry weigh were mainly driven by belowground biomass and morphology rather than traits associated to plant resource acquisition strategies.

Conclusions

The new continuous labelling method developed in this study permitted the separation of plant-derived CO_2–C from microbial-derived CO_2–C and avoided most of the biases related to the nature of this type of experiment (i.e. steady state long term labelling). It provides a useful approach in order to assess mechanisms involved in different patterns of plant rhizodeposition under different fertility levels. The results of this study suggest that changes in the amount of rhizodeposited C under different levels of nutrient availability were driven mainly by plant biomass rather than soil microbes. Finally, these results underline the potential importance of plant features (i.e. plant biomass) as opposed to traits associated with the plant resource acquisition strategies in predicting total C rhizodeposition. However, it is necessary that further studies with more species be performed in order to validate these findings.

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Table 1. Responses of plant traits to fertilization treatments without (-N) or with nitrogen addition (+N). As time effect was not significant (see Table 2), data from all pots per treatment at t_i and t_f were amalgamated to give mean values (n=8) followed by standard error (SE).

Species	**Abbr.**	**Total biomass (g DW)**		**Leaf biomass (g DW)**		**Root biomass (g DW)**		**Leaf N concentration (% DW)**		**Root N concentration (% DW)**	
Fertility level		-N	+N	-N	+N	-N	+N	-N	+N	-N	+N
Dactylis glomerata	DG	0.92 (0.07)	1.16 (0.07)	0.56 (0.05)	0.82 (0.04)	0.47 (0.06)	0.89 (0.09)	1.63 (0.14)	1.51 (0.04)	0.76 (0.03)	0.62 (0.03)
Lolium perenne	LP	0.60 (0.03)	0.84 (0.03)	0.29 (0.02)	0.41 (0.04)	0.56 (0.04)	0.47 (0.07)	1.70 (0.05)	2.00 (0.10)	0.65 (0.03)	0.78 (0.03)
Anthoxantum odoratum	AO	0.54 (0.04)	0.83 (0.10)	0.15 (0.02)	0.40 (0.03)	0.16 (0.02)	0.31 (0.03)	1.71 (0.17)	1.51 (0.20)	0.86 (0.04)	0.80 (0.03)
Briza media	BM	0.54 (0.05)	0.49 (0.12)	0.42 (0.04)	0.33 (0.08)	0.27 (0.05)	0.19 (0.05)	1.71 (0.12)	2.35 (0.25)	0.92 (0.09)	1.21 (0.18)

Table 2. Effects of species, fertilization and time on plant biomass and plant functional traits. Values are results of ANOVAs (F).

Variable	Effect	F (dl)	P
Total biomass (g DW)	*Species*	37.47 (3,47)	**<0.0001**
	Time	2.00 (1,47)	0.16
	Fertility	16.16 (1,47)	**0.0002**
	Species × Time	1.64 (3,47)	0.19
	Species × Fertility	5.88 (3,47)	**0.001**
	Time × Fertility	0.10 (1,47)	0.77
Leaf biomass (g DW)	*Species*	34.15 (3,47)	**<0.0001**
	Time	1.01 (1,47)	0.32
	Fertility	17.38 (1,47)	**<0.0001**
	Species × Time	0.97 (3,47)	0.41
	Species × Fertility	6.28 (3,47)	**0.001**
	Time × Fertility	0.002 (1,47)	0.96
Root biomass (g DW)	*Species*	32.82 (3,47)	**<0.0001**
	Time	2.60 (1,47)	0.11
	Fertility	7.04 (1,47)	**0.01**
	Species × Time	2.16 (3,47)	0.10
	Species × Fertility	9.77 (3,47)	**<0.0001**
	Time × Fertility	0.20 (1,47)	0.65
Leaf N concentration (% DW)	*Species*	3.32 (3,44)	**0.03**
	Time	0.56 (1,44)	0.45
	Fertility	1.96 (1,44)	0.16
	Species × Time	0.18 (3,44)	0.90
	Species × Fertility	2.66 (3,44)	0.06
	Time × Fertility	0.12 (1,44)	0.73
Root N concentration (% DW)	*Species*	16.93 (3,59)	**<0.0001**
	Time	1.07 (1,59)	0.30
	Fertility	3.65 (1,59)	0.06
	Species × Time	2.11 (3,59)	0.11
	Species × Fertility	6.94 (3,59)	**0.0006**
	Time × Fertility	1.54 (1,59)	0.22

Table 3. Species and fertilization effects on total rhizodeposited ^{13}C, specific rhizodeposited ^{13}C, ^{13}C microbial immobilization, $^{13}CO_2$ respired by microbial biomass and on labile soil ^{13}C. Values are results of ANOVAs (F).

Variable	Effect	F (dl)	P
Total rhizodeposited ^{13}C (µg ^{13}C d^{-1})	*Species*	66.4 (3,22)	**<0.0001**
	Fertility	10.9 (1,22)	**0.0032**
	Species × Fertility	83.2 (3,22)	**<0.0001**
Specific rhizodeposited ^{13}C (µg ^{13}C g^{-1} root DW d^{-1})	*Species*	55.3 (3,22)	**<0.0001**
	Fertility	0.14 (1,22)	0.70
	Species × Fertility	27.1 (3,22)	**<0.0001**
^{13}C microbial immobilization (µg ^{13}C d^{-1})	*Species*	66.0 (3,22)	**<0.0001**
	Fertility	15.9 (1,22)	**0.006**
	Species × Fertility	150.1 (3,22)	**<0.0001**
$^{13}CO_2$ respired by microbial biomass (µg ^{13}C d^{-1})	*Species*	12.7 (3,22)	**<0.0001**
	Fertility	1.38 (1,22)	0.25
	Species × Fertility	17.1 (3,22)	**<0.0001**
Labile soil ^{13}C (µg ^{13}C)	*Species*	2.55 (3,22)	0.08
	Fertility	5.72 (1,22)	**0.03**
	Species × Fertility	1.58 (3,22)	0.22

Table 4. Soil characteristics for plots without (-N) or with nitrogen addition (+N). Soil nitrate content was very low in soil samples, for this reason, results were not presented in the table. As time effect was significant for all the variables (see Table 5), mean values from all pots per treatment at t_i and at t_f were presented (n=4) followed by standard error (SE). For bare soil plots, n=3 followed by standard error (SE). NA = not acquired.

Species / Bare soil plots	**Time**	**Soil NH_4^+ (µg N-NH_4^+ kg^{-1} soil DW)**		**Immobilized NH_4^+ in microbial biomass (µg N-$NH_4^+$$kg^{-1}$ soil DW)**		**C microbial biomass (µg C g^{-1} soil DW)**		**pH**	
Fertility level	-	-N	+N	-N	+N	-N	+N	-N	+N
Dactylis glomerata	t_i	0.56 (0.08)	0.46 (0.001)	3.05 (0.06)	3.44 (0.12)	312.6 (125.5)	115.0 (6.6)	6.87 (0.04)	6.83 (0.04)
	t_f	0.38 (0.02)	0.40 (0.03)	2.44 (0.21)	3.05 (0.27)	399.9 (72.2)	539.7 (65.5)	6.88 (0.02)	6.80 (0.02)
Lolium perenne	t_i	0.73 (0.04)	0.67 (0.03)	2.68 (0.09)	2.87 (0.11)	330.9 (156.5)	141.0 (67.8)	6.82 (0.01)	6.79 (0.06)
	t_f	0.75 (0.04)	0.63 (0.04)	2.46 (0.12)	2.52 (0.02)	462.0 (119.7)	382.7 (142.7)	6.85 (0.02)	6.77 (0.03)
Anthoxantum odoratum	t_i	0.63 (0.001)	0.63 (0.03)	2.95 (0.09)	3.67 (0.16)	158.0 (37.1)	58.4 (25.4)	6.78 (0.02)	6.75 (0.03)
	t_f	0.63 (0.07)	0.34 (0.06)	2.85 (0.13)	2.71 (0.10)	329.6 (30.0)	520.8 (47.2)	6.80 (0.01)	6.75 (0.01)
Briza media	t_i	0.46 (0.05)	1.92 (0.80)	2.94 (0.06)	3.17 (0.05)	73.5 (40.1)	68.9 (20.4)	6.76 (0.04)	6.69 (0.03)
	t_f	0.30 (0.05)	10.21 (5.70)	2.84 (0.13)	2.84 (0.11)	434.0 (84.1)	479.4 (46.6)	6.76 (0.02)	6.69 (0.03)
Bare soil	-	0.46 (0.02)	0.42 (0.001)	1.80 (0.09)	1.98 (0.35)	NA	NA	6.54 (0.04)	6.29 (0.01)

Table 5. Effects of species, fertilization and time on soil properties and microbial biomass. Values are results of ANOVAs (F).

Variable	Effect	F (dl)	P
Soil NH_4^+ (µg N-NH_4^+ kg^{-1} soil DW)	*Species*	41.4 (3,46)	**<0.0001**
	Time	22.8 (1,46)	**<0.0001**
	Fertility	12.7 (1,46)	**0.0009**
	Species × *Time*	2.16 (3,46)	0.10
	Species × *Fertility*	0.96 (3,46)	0.41
	Time × *Fertility*	1.50 (1,46)	0.23
Immobilized NH_4^+ in microbial biomass (µg N-$NH_4^+$$kg^{-1}$ soil DW)	*Species*	8.37 (3,46)	**0.0002**
	Time	34.6 (1,46)	**<0.0001**
	Fertility	15.5 (1,46)	**0.0003**
	Species × *Time*	1.44 (3,46)	0.24
	Species × *Fertility*	1.91 (3,46)	0.14
	Time × *Fertility*	3.65 (1,46)	0.06
C microbial biomass (µg C g^{-1} soil DW)	*Species*	0.98 (3,45)	0.41
	Time	48.3 (1,45)	**<0.0001**
	Fertility	0.34 (1,45)	0.56
	Species × *Time*	1.05 (3,45)	0.38
	Species × *Fertility*	0.98 (3,45)	0.41
	Time × *Fertility*	5.73 (1,45)	**0.02**
pH	*Species*	11.8 (3,47)	**<0.0001**
	Time	0.03 (1,47)	0.86
	Fertility	14.9 (1,47)	**0.0004**
	Species × *Time*	0.07 (3,47)	0.97
	Species × *Fertility*	0.24 (3,47)	0.86
	Time × *Fertility*	1.03 (1,47)	0.31

Figure legends

Figure 1. (a) Total (µg ^{13}C d^{-1}) and (b) specific rhizodeposited ^{13}C (µg ^{13}C g root DW d^{-1}) under both species and fertilization treatments. Legends: *Dactylis glomerata* (DG), *Lolium perenne* (LP), *Anthoxantum odoratum* (AO), *Briza media* (BM), nutrient solution without (-N) or with nitrogen addition (+N). Significant differences between species (for all treatment confounded) are indicated by different letters ($p < 0.05$). Error bars indicate standard error.

Figure 2. (a) ^{13}C microbial immobilization (µg ^{13}C d^{-1}) and (b) $^{13}CO_2$ respired by microbial biomass (µg ^{13}C d^{-1}) under both species and fertilization treatments. Legends: *Dactylis glomerata* (DG), *Lolium perenne* (LP), *Anthoxantum odoratum* (AO), *Briza media* (BM), nutrient solution without (-N) or with nitrogen addition (+N). Significant differences between species (for all treatment confounded) are indicated by different letters ($p < 0.05$). Error bars indicate standard error.

Figure 3. Relationships between total rhizodeposited ^{13}C (µg ^{13}C d^{-1}) and (a) leaf biomass (g DW), (b) leaf N concentration (% g DW), (c) root biomass (g DW) and (d) root N concentration (% g DW). Legends: *Dactylis glomerata* (DG), *Lolium perenne* (LP), *Anthoxantum odoratum* (AO), *Briza media* (BM), nutrient solution without (-N) or with nitrogen addition (+N).

Figure 4. Relationships between specific rhizodeposited ^{13}C (µg ^{13}C d^{-1}) and (a) leaf biomass (g DW), (b) leaf N concentration (% g DW), (c) root biomass (g DW) and (d) root N concentration (% g DW). Legends: *Dactylis glomerata* (DG), *Lolium perenne* (LP), *Anthoxantum odoratum* (AO), *Briza media* (BM), nutrient solution without (-N) or with nitrogen addition (+N).

Figure 1

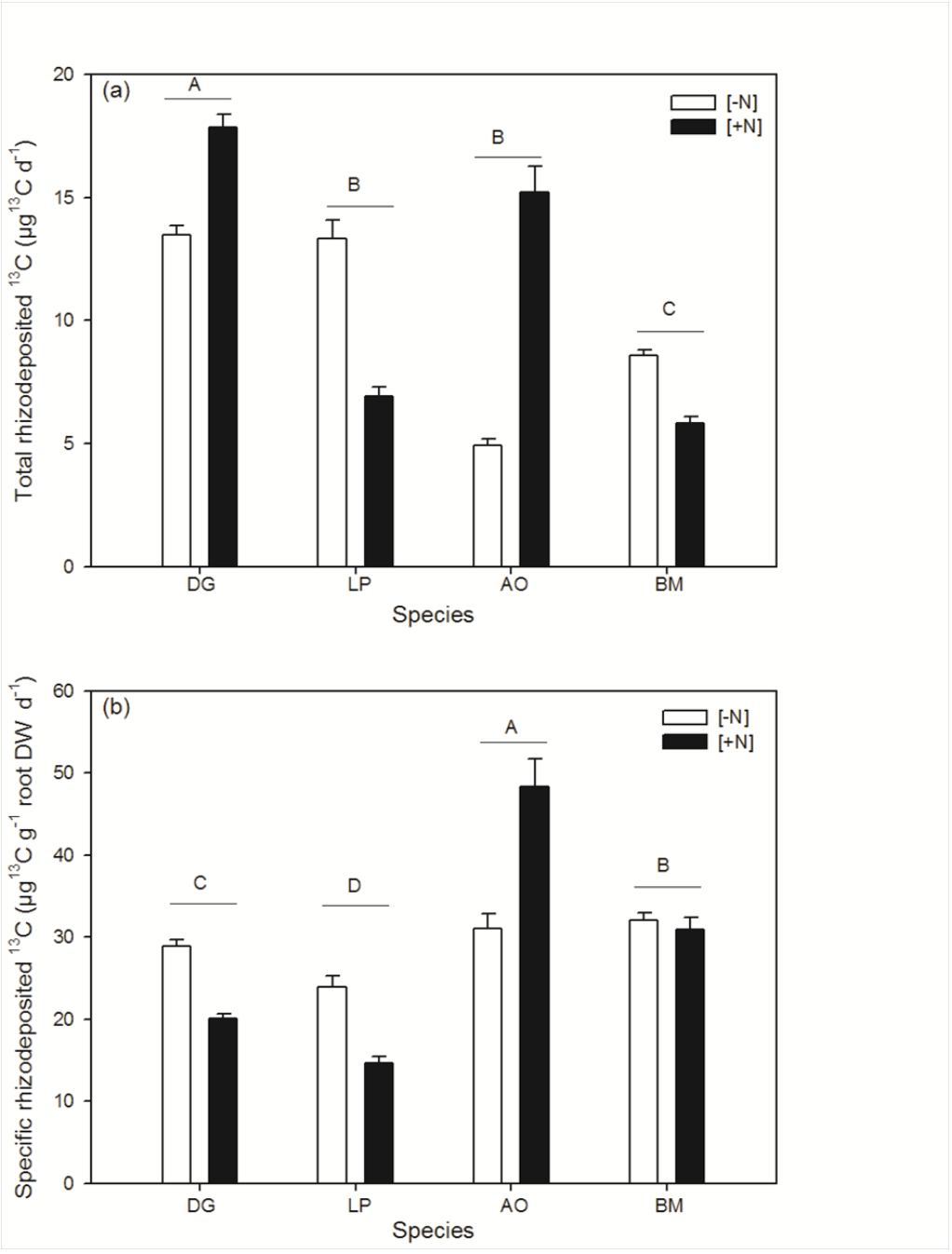

(a)
A
B
B
C
[-N]
[+N]
Total rhizodeposited ^{13}C (µg ^{13}C d^{-1})
0
5
10
15
20
DG
LP
AO
BM
Species
(b)
C
D
A
B
[-N]
[+N]
Specific rhizodeposited ^{13}C (µg ^{13}C g^{-1} root DW d^{-1})
0
10
20
30
40
50
60
DG
LP
AO
BM
Species

Figure 2

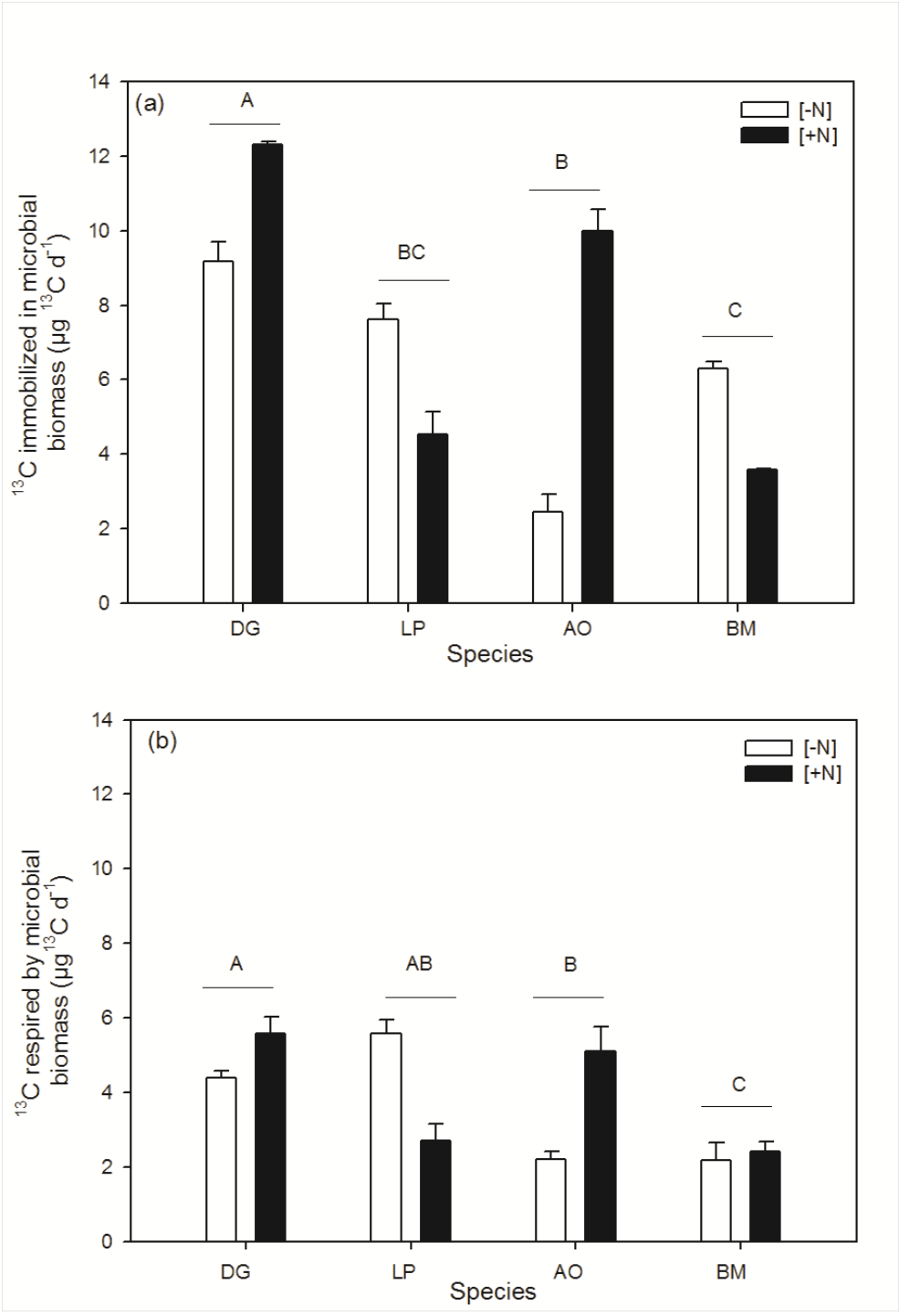

(a)
A
B
BC
C
[-N]
[+N]
^{13}C immobilized in microbial biomass (µg ^{13}C d^{-1})
0
2
4
6
8
10
12
14
DG
LP
AO
BM
Species
(b)
A
AB
B
C
[-N]
[+N]
^{13}C respired by microbial biomass (µg ^{13}C d^{-1})
0
2
4
6
8
10
12
14
DG
LP
AO
BM
Species

Figure 3

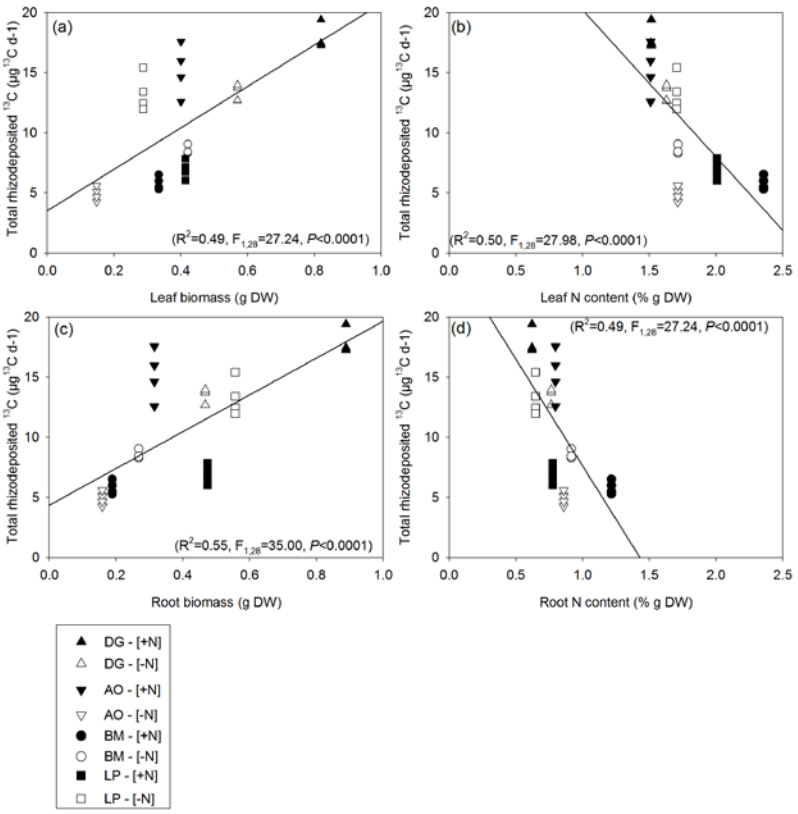
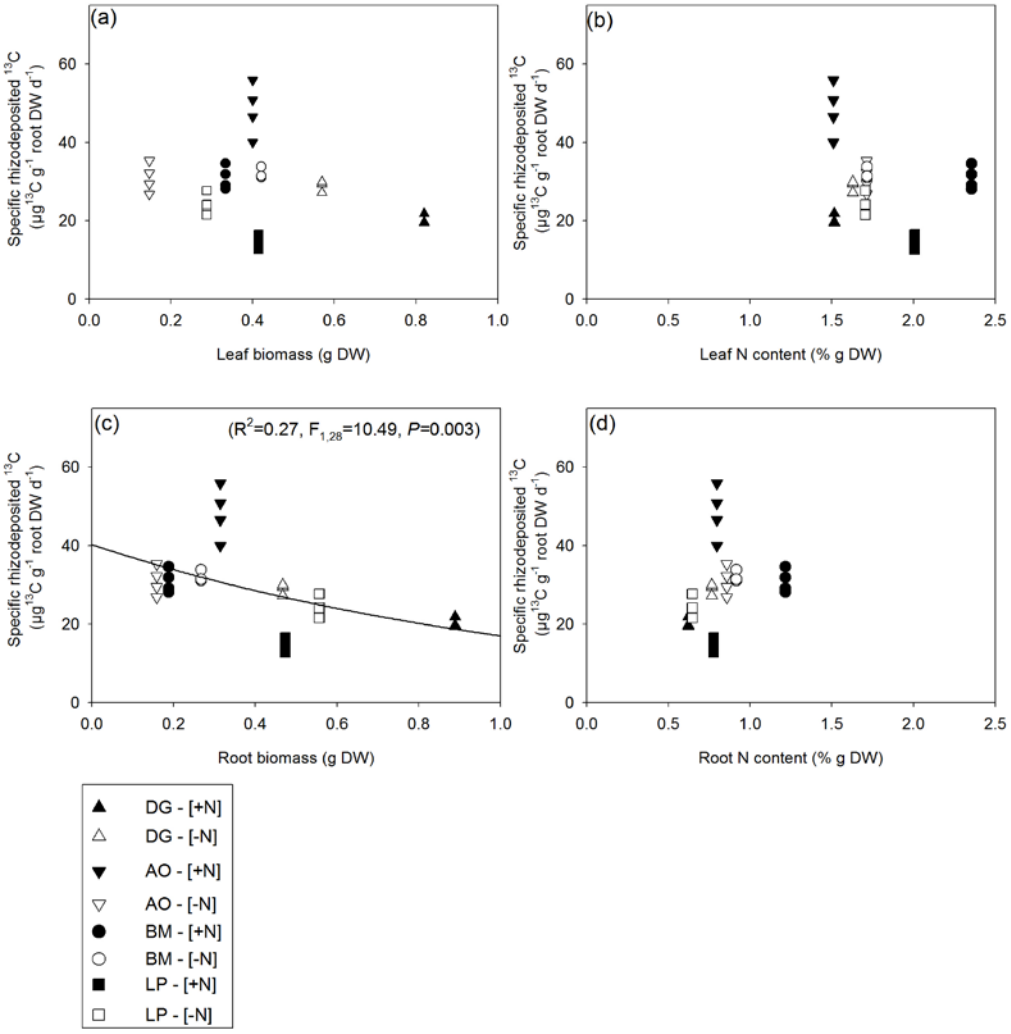

(a)
Total rhizodeposited ^{13}C (µg ^{13}C d-1)
(R²=0.49, F1,28=27.24, P<0.0001)
Leaf biomass (g DW)
(b)
(R²=0.50, F1,28=27.98, P<0.0001)
Leaf N content (% g DW)
(c)
(R²=0.55, F1,28=35.00, P<0.0001)
Root biomass (g DW)
(d)
(R²=0.49, F1,28=27.24, P<0.0001)
Root N content (% g DW)
DG - [+N]
DG - [-N]
AO - [+N]
AO - [-N]
BM - [+N]
BM - [-N]
LP - [+N]
LP - [-N]

Figure 4


(a)
(b)
(c)
(d)
(R²=0.27, F1,28=10.49, P=0.003)
Specific rhizodeposited ¹³C (µg ¹³C g⁻¹ root DW d⁻¹)
Leaf biomass (g DW)
Leaf N content (% g DW)
Root biomass (g DW)
Root N content (% g DW)
DG - [+N]
DG - [-N]
AO - [+N]
AO - [-N]
BM - [+N]
BM - [-N]
LP - [+N]
LP - [-N]