



## Decomposition of labelled roots and root-C and -N allocation between soil fractions in mountain grasslands

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### ABSTRACT

Given the high turnover of fine roots in mountain grasslands, knowledge of their decomposition rates and the capacity of mountain grassland soils to stabilize root-derived C are central to understand the role of these ecosystems as potential C sinks. Here we studied the decomposition of fine roots in mountain grasslands and estimated the rates at which root-C and -N incorporated into protected pools at two soil depths. For this purpose, we incubated standard <sup>13</sup>C- and <sup>15</sup>N-labelled wheat roots mixed with unlabelled soil at 5 and 20 cm depth in two mountain grassland sites. Particle size fractionation allowed the quantification of the labelled wheat root-C and -N allocated to each size fraction (coarse sand, fine sand and silt plus clay sized) as well as their incorporation rates into the finest fraction. Between 62% and 78% root-C remained in the soil after one year of field incubation, faster decomposition being registered at the warmest site. In the following two years, roots decomposed much more slowly. In contrast to reports in the literature, our results indicate that decay rates during the first year were highest in the deep layer. The incorporation of wheat root-derived organic matter into the silt plus clay size fraction was also much greater during the first year of decomposition than in the following two years and also slightly higher in the deep soil than in topsoil. The incorporation rates of root-<sup>13</sup>C and root-<sup>15</sup>N into this fraction also suggest that the wheat-derived organic matter associated with this fraction was N-enriched and less recalcitrant (i.e., less resistant to acid hydrolysis) than that recovered from the coarser fractions. Furthermore, recalcitrant organic matter incorporated much more slowly than labile organic matter did. We conclude that the conditions of the subalpine grassland subsoil are more favourable for root decomposition than the topsoil and that the organic matter that incorporates into the protected pool is characterised by a high N content and low biochemical recalcitrance.

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### 1. Introduction

The stabilisation of the inputs of soil organic matter is an essential process for the sequestration of organic C in soils. While most of the soil organic matter inputs derive from the root systems (Rasse et al., 2005) through its turnover and exudation, the stabilisation processes result from an equilibrium between microbial processes and physical and chemical protection mechanisms. The root contribution to C storage in soils is particularly relevant in grassland ecosystems where root:shoot ratios (Mokany et al., 2006)

and root turnover (Gill and Jackson, 2000) are generally high. As a result of microbial and faunal activities, plant residues are fragmented and partially mineralised while a fraction is incorporated into SOM, where it is decomposed or stabilised.

Most studies on root decomposition use the litterbag technique (Silver and Miya, 2001) and address root mass loss over a period of time. However, the weight of the remaining roots may lead to overestimation of root decomposition because this approach does not take into consideration the amount of root-derived OM incorporated into SOM. Alternatively, the incubation of labelled roots has been proven to be a useful method to quantify the remaining root-C and -N (i.e., including the amount incorporated into the soil) and also to discern the effects of small changes in the decomposing environment caused by disturbances or land use changes, such as pasture abandonment (Casals et al., 2010).

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The amount of OM derived from root material that remains in soil depends not only on the environmental conditions but also on its degree of stabilisation. While it has been shown that leaf litter decomposition can be described by single-pool models whose decomposition rates change with time (Rovira and Rovira, 2010), the modelling of SOM decomposition in the mineral soil environment is far more complex as it needs to account for the adsorption and stabilisation through interaction of SOM with mineral surfaces. Thus, in a mineral soil, SOM is better represented as comprising various functional pools that differ in their decay rates, related to their physical accessibility to microbial communities and their biochemical quality. Several fractionation methods have been developed to obtain a number of functionally meaningful SOM fractions covering from the most active to the most stabilised pools (von Lützow et al., 2007). However, one of the challenges is to quantify the rates at which stabilisation mechanisms operate in soils (Smernik and Skjemstad, 2009). This will be crucial in order to make reliable predictions of the role of soils as C sinks or sources under environmental and land use changes.

One of the main mechanisms of SOM stabilisation relies on its protection by interaction with fine soil particles (Hassink, 1997; Rasse et al., 2005; von Lützow et al., 2006). Organo-mineral associations are considered the main mechanism for long term preservation of organic C (Kögel-Knabner et al., 2008). Indeed, there is strong evidence that OM in coarse fractions is more available for decomposers than that associated to the fine particles (Eusterhues et al., 2003; Hassink, 1997; Sørensen, 1975). Furthermore, it has been shown that OM in silt and clay is older (Eusterhues et al., 2003) and has longer turnover times (Balesdent, 1996) than that present in coarser fractions; and particle size fractionation has been used to obtain a number of fractions that differed in their turnover rates (Bruun et al., 2008; Leifeld and Kögel-Knabner, 2005; Rovira and Vallejo, 2002; among others).

The biochemical quality of SOM has also been recognised as a factor regulating its mineralisation. However, the prevalent view is that it plays a secondary role in SOM stabilisation (Marschner et al., 2008; Mikutta et al., 2006; Rovira et al., 2010). Hence SOM recalcitrance might reflect its vulnerability to be mineralised in the absence of any other stabilisation mechanism. However, these two protection mechanisms (biochemical recalcitrance and physical protection) occur simultaneously and are not necessarily independent. While the biochemical quality of unprotected SOM is central for determining its mineralisation rates, the relevance of the biochemical quality of physically protected SOM may depend on the susceptibility of these pools to mineralisation when they are desorbed from mineral surfaces. Thus the rate at which a given soil stabilises OM results from the net balance between the incorporation of new OM from unprotected pools, the desorption rates of protected pools (which are expected to be low), and the biochemical quality of the pools that are physically accessible to microorganisms.

In most soils, subsurface horizons contain older SOM than the uppermost layers (Eusterhues et al., 2003; Paul et al., 1997; Rumpel et al., 2002), thereby suggesting that the position within the soil profile where dead plant material is deposited is also relevant for SOM dynamics and stabilisation processes. One reason for this may be that SOM deposited in topsoil moves down to deeper soil (Bruun et al., 2007). Another reason may be that the proportion of soil organic C adsorbed to mineral surfaces is also greater in the subsoil than in the topsoil (Rumpel et al., 2002). A lower proportion of physically stabilised SOM in surface soils could, in part, be due to its high SOM content, which could make their protective capacity closer to saturation (Stewart et al., 2009). Also, the higher inputs of fresh OM in the upper horizons may cause higher accumulation of unprotected OM in topsoil. However, both physical stabilisation into the fine soil fractions and microbial activity are also likely driven by

the environmental conditions. Given the changes in microclimate and microbial activity and composition along the soil profile, we hypothesize that the incorporation rates of plant-derived OM into the physically protected pool will be higher in the subsoil due to its more suitable microenvironmental conditions for decomposers activity and litter decay. Our aim here is to determine fine root decomposition rates in mountain grasslands and to assess the importance of the depth where roots were deposited for their decomposition and for the physical stabilisation of root-derived OM. For that purpose, we used isotopically labelled standard wheat root material mixed with soil, which were incubated in mountain grasslands buried at two depths (5 and 20 cm). We assessed remaining root-C and -N after one and three years, quantified the root-derived C and N recovery at different soil size fractions and measured the rates at which root-C and -N incorporated at the fine (silt plus clay size) soil fraction.

## 2. Material and methods

### 2.1. Study sites

The study was carried out at two mountain grassland sites (Prat Llong and Alinyà) on the southern face of the eastern Pyrenees (NE Iberian Peninsula). Prat Llong (42°12'N, 1°31'E) is a subalpine grassland located 2140 m a.s.l. It has a mean annual temperature of 4.4 °C and an annual precipitation of 1155 mm (Digital Climatic Atlas of Catalonia, Ninyerola et al., 2000), which falls as snow between November and May. Vegetation is co-dominated by *Festuca rubra* L. ssp. *commutata* Gaud. and *Nardus stricta* L., with the presence of *Carex caryophylla* Latourr., *Poa alpina* L., *Thymus serpyllum* L. and *Euphorbia cyparissias* L. Soil is thin (40–45 cm), with a silty clay texture, and although it has developed on cretaceous limestones, it has a pH(H<sub>2</sub>O) of 4.8 (5–15 cm depth).

Alinyà (42°10'N, 1°28'E) is an altimontane grassland located 1848 m a.s.l. The climate is warmer (5.8 °C) than in Prat Llong, and it also has high precipitation (1062 mm) (Digital Climatic Atlas of Catalonia, Ninyerola et al., 2000), which falls as snow between December and April. Vegetation is dominated by *F. rubra* ssp. *commutata*, but *Phleum pratense* L., *P. alpina*, *Taraxacum dissectum* (Ledeb.) Ledeb., *Achillea millefolium* L. and *Medicago lupulina* L. are also abundant. Developed on limestones, the soil is thin (35–45 cm), with a silty clay loam texture and a pH(H<sub>2</sub>O) of 7.1 (5–15 cm depth).

### 2.2. Soil–root mixture preparation and field incubation

To prepare the mixture of soil and labelled roots, soil was collected from the 5–15 cm layer at each site, homogenised, sieved to 2 mm to remove gravels and roots and air-dried. The soils' organic C contents were  $30.5 \pm 1.37 \text{ mgC g}^{-1}$  ( $n = 5$ ) in Prat Llong and  $27.5 \pm 0.54 \text{ mgC g}^{-1}$  ( $n = 5$ ) in Alinyà. Labelled root material was produced at the CNRS (Montpellier, France) by growing wheat plants in growth chambers for 4 months under a <sup>13</sup>C<sub>2</sub>-labelled atmosphere and with a solution with low nutrient (<sup>15</sup>N, P and K) concentrations plus micronutrients (Bottner et al., 2000; Coûteaux et al., 2001). The resulting material consisted of fine roots (<1 mm) with an enrichment of 9.441 at% <sup>13</sup>C and 9.184 at% <sup>15</sup>N. These roots were oven-dried at 60 °C until constant weight and stored in the dark until use.

Previous to the mixture preparation, air-dried root material was cut into  $4 \pm 2$  mm pieces and homogenised. Labelled roots (0.300 g) were then mixed with 20.00 g of root-free soil and the mixture was placed in nylon mesh bags (6.5 × 6.5 cm, 100 μm mesh). These were in turn placed horizontally inside larger bags (8.5 × 8.5 cm, 100 μm mesh) containing 40.00 g of the same soil as that used in the mixtures without the labelled roots. This soil was placed below the

mixture bags and used to assess the particulate or leached labelled material that could move down.

At each grassland site, a homogeneous area of 1250 m<sup>2</sup> (25 × 50 m) was chosen and eight incubation points were regularly located within the area. At each point, two mixture bags were buried, one at a depth of 5 cm and the other at 20 cm. The distance between incubation points was always at least 10 m. To bury the samples in the field, a soil profile of about 30 cm depth was dug at each incubation point and, at the appropriate depth (5 and 20 cm), a hole of the right size to be able to introduce the bags horizontally was made using a knife. Then the profile was refilled. In this way, the disturbance of the soil above the bags was minimised. Incubation lasted one year (3rd June 2004–26th May 2005 in Prat Llong; 5th May 2004–27th April 2005 in Alinyà). Four additional samples at each depth were incubated for about 3 years (until 7th July 2007) in Prat Llong.

To assess the effect of sample manipulation, six additional bag couples (three per site) containing the same root–soil mixtures were prepared but not incubated in the field. These samples were stored under dark and dry conditions in the laboratory and then processed and analysed in a similar way to the incubated samples (see below) and taken as initial conditions.

### 2.3. Soil processing and analyses

After bag collection, we carefully removed remaining recognisable roots from each sample with tweezers in the lab. This material was then weighed, finely ground and analysed for total OC and N and for <sup>13</sup>C and <sup>15</sup>N isotopic signature. The remaining root–soil mixture of each sample was homogenized and a subsample was taken and finely ground for total C, N and isotope analyses. The remaining material of the samples was used for particle size physical fractionation and to assess the biochemical recalcitrance of each size fraction.

Physical fractionation followed the method used by Rovira and Vallejo (2002), obtaining three size fractions (coarse sand sized [CS]: 2000–200 μm, fine sand sized [FS]: 200–50 μm, silt plus clay sized [S + C]: <50 μm). About 50 ml of distilled water and a glass ball were added to 12 g of soil and shaken for 10 min with a rotatory shaker (40 rpm) to break large aggregates. The ball was then removed and the sample was dispersed with an ultrasonic sonifier (Branson S-250D, output power 100 W) for 5 min. The suspension obtained was passed through two sieves of 200-μm and 50-μm mesh in a sieve while a flow of distilled water was applied until the outflow water was completely clear. The fractions obtained in the sieves (CS and FS) were transferred to plastic flasks and oven-dried at 60 °C until constant weight. The resulting suspension (S + C; <50 μm) was collected in 1-litre jars and flocculated by adding 1 ml of a saturated aluminium potassium sulphate (AlK(SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O) solution. After that, most of the water was suctioned and the remaining suspension was transferred to smaller plastic flasks, which were centrifuged (2000 r.p.m., 5 min) and excess water was again carefully suctioned. Finally, the remaining material was oven-dried at 60 °C until constant weight. All three fractions were finely ground and a subsample of each was used to analyse total C and N and <sup>13</sup>C and <sup>15</sup>N isotopic signatures.

Biochemical recalcitrance analysis was carried out on the three size fractions previously obtained and also on initial wheat roots. Recalcitrance was assessed by acid hydrolysis with 6 N HCl (Rovira and Vallejo, 2000). Finely ground material (400 mg) was hydrolysed in sealed Pyrex tubes with 20 ml of 6 N HCl in a block digester at 105 °C for 18 h. The liquid was then decanted and two successive centrifugations with distilled water were done to remove the residual HCl. The unhydrolysed residue was finally dried at 60 °C until constant weight and analysed for total C and N and <sup>13</sup>C and <sup>15</sup>N isotopic signatures.

All the isotopic analyses were performed at the Utah State University Stable Isotope Laboratory by continuous-flow direct combustion and mass spectrometry using a Europa Scientific SL-2020 system.

### 2.4. Calculations

Remaining root-derived C (C%) at any time was calculated as the ratio between the amount of <sup>13</sup>C excess in the samples at a given time (t) over the amount of <sup>13</sup>C initially recovered from non-incubated samples (t<sub>0</sub>) as follows:

$$C\% = [C_t \times ({}^{13}\text{C}_{\text{exc}})_t / C_{t_0} \times ({}^{13}\text{C}_{\text{exc}})_{t_0}] \times 100$$

where C is the amount of C (mg) and <sup>13</sup>C<sub>exc</sub> is the <sup>13</sup>C enrichment (atom% excess). Remaining root-derived N was calculated similarly.

The proportion of root-C recovered in each physical fraction was calculated as the ratio between amount of root-<sup>13</sup>C (mg <sup>13</sup>C<sub>exc</sub>) in the fraction and the amount of <sup>13</sup>C (mg <sup>13</sup>C<sub>exc</sub>) initially supplied with the incubated roots. The recovery of root-N was calculated in a similar way, using the amount of root-<sup>15</sup>N in the fraction and the amount of <sup>15</sup>N supplied with the roots. The incorporation rates (R<sub>inc</sub>) of root-C into the S + C fraction in a given period were then calculated as follows:

$$R_{\text{inc}} = [(RecC_{S+C})_t - (RecC_{S+C})_{t+1}] / t$$

where RecC<sub>S+C</sub> is the proportion of the incubated root-C recovered in the S + C fraction at a given time, and t is the time of incubation (y). R<sub>inc</sub> is then expressed in mgC gC<sup>-1</sup> y<sup>-1</sup>. The incorporation rates of root-N into the S + C fraction were calculated similarly.

The proportion of C content in the S + C fraction (fC<sub>S+C</sub>) that derives from the incubated roots was calculated by mass balance, taking the enrichment of unlabelled soil samples (natural abundance) and the enrichment of labelled non-incubated roots as end member values, as follows:

$$fC_{S+C} = [({}^{13}\text{C}_{\text{exc}})_{S+C} - ({}^{13}\text{C}_{\text{exc}})_{\text{na}}] / [({}^{13}\text{C}_{\text{exc}})_r - ({}^{13}\text{C}_{\text{exc}})_{\text{na}}]$$

where (<sup>13</sup>C<sub>exc</sub>)<sub>S+C</sub> is the <sup>13</sup>C enrichment of the fraction, (<sup>13</sup>C<sub>exc</sub>)<sub>na</sub> is the natural abundance <sup>13</sup>C (from the unlabelled blanks) and (<sup>13</sup>C<sub>exc</sub>)<sub>r</sub> is the enrichment of the incubated roots (all expressed in atom% excess). Calculations for N were made similarly using <sup>15</sup>N enrichment.

Biochemical recalcitrance of the root-derived OM recovered in each size fraction was expressed by the recalcitrance index for C and N (RIC and RIN) used by Rovira and Vallejo (2000), which is the ratio between the remaining root-C (or N) after acid hydrolysis and the total organic root-C (or N) in the fraction. To calculate RIC and RIN of the root-derived C and N, we used the <sup>13</sup>C excess and <sup>15</sup>N excess of the hydrolysed and unhydrolysed samples:

$$RIC = [C_{\text{rec}} \times ({}^{13}\text{C}_{\text{exc}})_{\text{rec}}] / [C_{\text{total}} \times ({}^{13}\text{C}_{\text{exc}})_{\text{total}}] \times 100$$

where C<sub>rec</sub> is the amount (mgC g<sup>-1</sup>) of recalcitrant (remaining after hydrolysis) C in the fraction, C<sub>total</sub> is the amount (mgC g<sup>-1</sup>) of total organic C in the fraction, (<sup>13</sup>C<sub>exc</sub>)<sub>rec</sub> is the enrichment (atom%) of the unhydrolysed C and (<sup>13</sup>C<sub>exc</sub>)<sub>total</sub> is the enrichment of the total organic C in the fraction. Calculations for RIN were made similarly.

### 2.5. Statistical analysis

Prior to the analyses, the normality of all variables was tested by the non-parametric Kolmogorov–Smirnov test. The effect of depth of burial on the remaining C and N, root-C and -N content in the soil fractions, RIC and RIN, and C/N ratios was analysed by repeated measures analysis of variance (ANOVA), with site as between-subjects factor and depth (5 or 20 cm) as within-subjects factor.

The difference in the proportion of C and N recovered at each site, depth and fraction between the initial, the first and the third year of incubation was tested by one-way ANOVA and by the Duncan's multiple range test when the ANOVA was significant. The enrichment of the soil below the mixture bags was also tested by one-way ANOVA by comparing with control bags without enriched roots. All the analyses were performed with the SPSS v.17 statistical package.

### 3. Results

#### 3.1. Remaining root C and N

In the non-incubated bags (initial mixtures), wheat root-C and -N recoveries were  $102.0 \pm 2.6\%$  and  $86.7 \pm 2.1\%$  respectively in Prat Llong, and  $97.3 \pm 1.6\%$  and  $81.8 \pm 3.1\%$  in Alinyà.

After one year of field incubation, remaining root-C, as a percentage of the C initially recovered, was lower in Alinyà than in Prat Llong, but the amount of root-N that remained in the root–soil mixture bags did not differ between sites (Table 1). Less root-C and -N was recovered at a depth of 20 cm than at 5 cm at both sites (Table 1). The amount of C recovered in Prat Llong at the third year was lower ( $p = 0.009$ ) than the amount recovered in the first year. However, at year three, no significant differences between depths were found as the variability in the remaining C was high.

The soils located below the mixture bags were slightly  $^{15}\text{N}$ - and  $^{13}\text{C}$ -enriched compared to the unlabelled controls (Table 2). However, the amount of the root-C and -N recovered in this soil accounted for less than 2% of the incubated C and N, except for  $^{13}\text{C}$  in Prat Llong, which reached 3.8% (Table 2).

#### 3.2. Particle size fractionation

After particle size fractionation, the initial root–soil mixtures of both sites contained similar proportions of the three size fractions, with CS accounting for 2.2–4.8% of weight, FS 14–16% and S + C 81–82% (Table 3). In Prat Llong, the highest C concentrations were found in the CS and S + C fractions. In Alinyà, CS showed the highest C concentration, which was much higher than in Prat Llong (Table 3). At both sites, CS had the highest C/N ratios and S + C the lowest. The RIC and RIN were generally higher in Alinyà, except for the RIN in the FS fraction, which showed similar values at both sites (Table 3).

#### 3.3. Root-C and -N recovery in size fractions

After one year of field incubation, the amount of wheat root C obtained by summing the C recovered in all fractions, and C as

**Table 1**  
Remaining labelled root-C and -N derived from wheat roots in Prat Llong and Alinyà at depths of 5 and 20 cm. Remaining  $^{13}\text{C}$  and  $^{15}\text{N}$  are expressed as a percentage over the recovered root- $^{13}\text{C}$  and  $^{15}\text{N}$  in the initial non-incubated bags.

Site	Time	Depth	$^{13}\text{C}$	$^{15}\text{N}$
	d	cm	%	%
Prat Llong	357	5	$77.9 \pm 2.37$	$98.0 \pm 2.52$
		20	$68.7 \pm 1.99$	$91.3 \pm 3.03$
	1129	5	$67.5 \pm 6.02$	$93.7 \pm 5.17$
		20	$59.6 \pm 4.67$	$91.0 \pm 9.54$
Alinyà	352	5	$68.3 \pm 2.98$	$93.8 \pm 2.42$
		20	$61.9 \pm 2.27$	$87.9 \pm 2.69$
<i>p</i> -values (GLM repeated measures) <sup>a</sup>				
Site			0.003	0.239
Depth			0.009	0.012
Depth × Site			0.589	0.866

<sup>a</sup> The repeated measures analyses were only performed to remaining root-C and -N after 357 days of incubation at both sites.

**Table 2**

C and N enrichment (atom%) of incubated unlabelled control soil (natural abundance) and of the soil placed below mixture bags, and the portion of  $^{13}\text{C}$  and  $^{15}\text{N}$  recovered in the soil below the mixtures as percentage over the initial amount incubated as roots.

	Control	Soil below mixture bags	Sign.	Recovery (%)
atom% $^{13}\text{C}$				
Prat Llong	$1.0842 \pm 0.0000$	$1.0866 \pm 0.0009$	0.062	$3.8 \pm 1.72$
Alinyà	$1.0838 \pm 0.0000$	$1.0887 \pm 0.0045$	0.041	$1.4 \pm 1.20$
atom% $^{15}\text{N}$				
Prat Llong	$0.3689 \pm 0.0002$	$0.3722 \pm 0.0005$	0.002	$1.8 \pm 1.43$
Alinyà	$0.3683 \pm 0.0002$	$0.3736 \pm 0.0016$	0.007	$1.4 \pm 0.79$

visible roots was similar to the C recovered from the non-fractionated subsample. In contrast, about 10–20% of the incubated N was lost during the fractionation process. Samples incubated in the field for three years also lost a significant part of root-C (13–24%) and -N (14–33%) during fractionation.

Visible roots and the CS fraction accounted for most of root-derived  $^{13}\text{C}$  and  $^{15}\text{N}$  in the mixtures of non-incubated bags (initial mixtures) (Figs. 1 and 2). The amount of root- $^{13}\text{C}$  in FS and S + C accounted for 1% or less of the incubated C (Fig. 1). However, a significant amount (3.3%) of the incubated  $^{15}\text{N}$  was found in the S + C fraction of the initial mixtures (Fig. 2).

After one year of field incubation, most of the wheat root-derived  $^{13}\text{C}$  and  $^{15}\text{N}$  still remained in the coarse fractions (visible roots and CS), but a significant amount was already incorporated into S + C (Figs. 1 and 2). While the amount recovered in coarse fractions was higher in Prat Llong than in Alinyà, the recovery in the S + C fraction was significantly higher in the latter (Table 4, Figs. 1 and 2). Although incorporation rates of root- $^{13}\text{C}$  into S + C was not significantly different between depths, the total amount of  $^{13}\text{C}$  recovered in this fraction was slightly higher at a depth of 20 cm than at 5 cm ( $4.7 \pm 0.46\%$  and  $7.0 \pm 0.43\%$  in Prat Llong and Alinyà at 20 cm and  $3.5 \pm 0.48\%$  and  $6.5 \pm 0.59\%$  at 5 cm, respectively) ( $p < 0.1$ , Table 4). Similarly, higher amounts of  $^{15}\text{N}$  accumulated at a depth of 20 cm than at 5 cm ( $11.8 \pm 1.10\%$  and  $19.7 \pm 1.24\%$  in Prat Llong and Alinyà at 20 cm and  $9.0 \pm 1.11\%$  and  $18.2 \pm 1.42\%$  at 5 cm, respectively) ( $p < 0.1$ , Table 4). After three years of field incubation in Prat Llong,  $6.9 \pm 0.70\%$  of  $^{13}\text{C}$  and  $20.1 \pm 2.0\%$  of  $^{15}\text{N}$  were recovered in the S + C fraction, with no differences between depths.

At both sites, the percentage of the incubated wheat root- $^{15}\text{N}$  recovered in S + C was higher than the root- $^{13}\text{C}$  recovery (Figs. 1 and 2). Indeed, net N incorporation rates ( $\text{mgN gN}^{-1} \text{y}^{-1}$ ) into S + C were higher than those for C ( $\text{mgC gC}^{-1} \text{y}^{-1}$ ) (Table 5). These incorporation rates were consistently lower in Prat Llong than in Alinyà ( $p < 0.001$ , Table 5), with no significant differences between depths of burial. In the following two years in Prat Llong, root-C was incorporated into the S + C fraction at rates that were about 35–37% lower than did during the first year (Table 5). In contrast, the incorporation rates of root-N were reduced by only about 15–17% compared to the first year.

At the end of the first year of incubation,  $0.7 \pm 0.09\%$  and  $0.9 \pm 0.08\%$  of the C in the S + C fraction of the Prat Llong soil were derived from the wheat roots incubated at depths of 5 and 20 cm respectively. These values increased to  $1.1 \pm 0.15\%$  and  $1.3 \pm 0.16\%$  at the end of the third year of incubation. The faster incorporation of C in Alinyà also produced higher proportions of root-C in the S + C fraction in the first year of incubation ( $1.5 \pm 0.14\%$  and  $1.6 \pm 0.09\%$  at 5 and 20 cm). At that time, the two sites showed similar values for the proportion of root-N over total N in the S + C fraction.

**Table 3**

Initial characteristics of the three size fractions of the non-incubated labelled root–soil mixtures. Values indicate means ± standard error (*n* = 3). CS: Coarse sand sized fraction; FS: Fine sand sized fraction; S + C: Silt plus clay sized fraction.

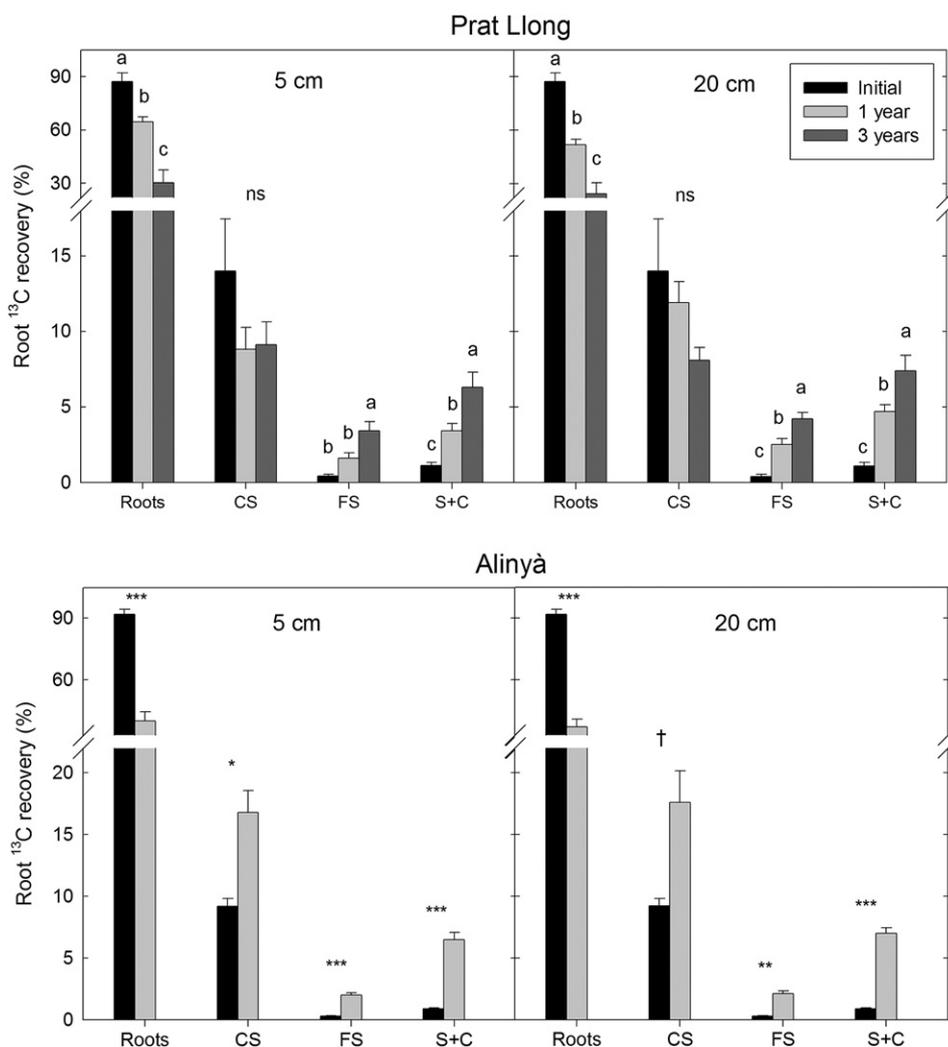
	Prat Llong			Alinyà		
	CS	FS	S + C	CS	FS	S + C
Weight (%)	4.8 ± 0.58	14.0 ± 0.05	81.2 ± 0.57	2.2 ± 0.13	15.8 ± 0.24	82.0 ± 0.34
OC (mg g <sup>-1</sup> )	42.4 ± 3.47	17.2 ± 0.62	40.1 ± 0.32	101.2 ± 4.91	25.1 ± 0.83	31.7 ± 0.36
N (mg g <sup>-1</sup> )	1.5 ± 0.13	1.2 ± 0.05	4.1 ± 0.02	3.9 ± 0.28	1.5 ± 0.09	3.3 ± 0.05
C/N	28.0 ± 0.82	14.1 ± 0.15	10.0 ± 0.03	26.1 ± 1.20	16.5 ± 0.46	9.7 ± 0.09
RIC (%)	30.1 ± 0.72	50.3 ± 4.21	38.6 ± 1.26	51.5 ± 1.00	61.3 ± 0.70	47.6 ± 1.53
RIN (%)	11.4 ± 0.48	23.4 ± 2.08	19.4 ± 0.66	30.2 ± 0.10	25.1 ± 0.53	25.0 ± 0.69
<sup>13</sup> C (atom%)	4.39 ± 0.442	1.16 ± 0.024	1.10 ± 0.003	3.13 ± 0.160	1.12 ± 0.006	1.10 ± 0.001
<sup>15</sup> N (atom%)	3.34 ± 0.487	0.41 ± 0.016	0.39 ± 0.004	2.09 ± 0.139	0.40 ± 0.004	0.39 ± 0.001

**3.4. Biochemical quality of root-C and -N incorporated into the S + C fraction**

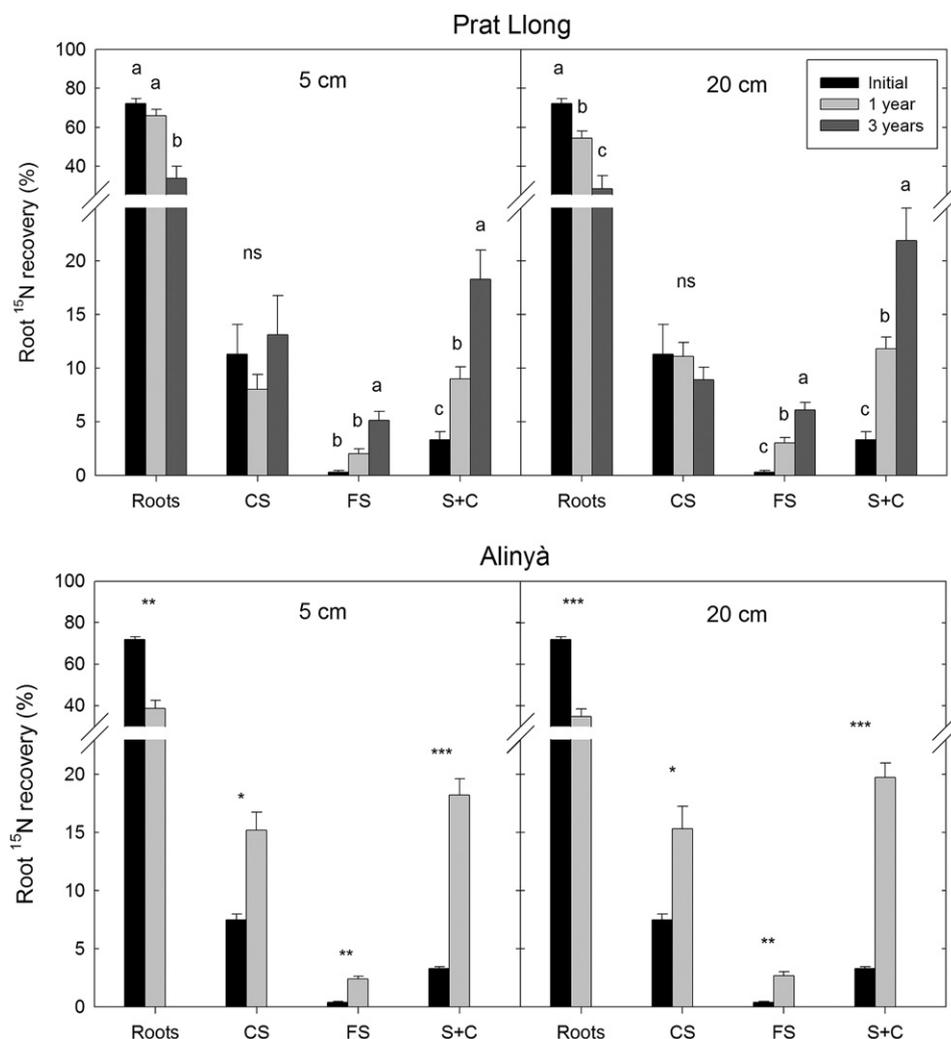
The roots used for field incubation had a C/N ratio of 29.4 ± 0.27, a RIC of 42.0 ± 0.62% and a RIN of 11.3 ± 1.32% (*n* = 4). After one year of incubation, root-derived OM associated with the S + C fraction had a lower C/N ratio (root-<sup>13</sup>C<sub>exc</sub>/root-<sup>15</sup>N<sub>exc</sub>, Table 6) and lower RIC and RIN than the original roots used in the mixtures and the root-derived OM included in coarser fractions (CS and FS) at both sites (Table 6). Both RIC and RIN of the S + C fraction were higher in

Prat Llong than in Alinyà. During the following two years of field incubation in Prat Llong, root-derived RIC in the S + C fraction remained constant, while RIN in the same fraction decreased (*p* < 0.001) (Table 6).

The C/N ratio and RIC of root-derived OM incorporated into S + C did not differ between incubation depths at either site. However, RIN in Prat Llong was lower at a depth of 20 cm than at 5 cm (Site × Depth, *p* = 0.011) after one year of incubation, and remained lower (*p* = 0.032) two years later (three years of incubation).



**Fig. 1.** Recovery of root-derived <sup>13</sup>C in remaining roots and soil fractions as a percentage of <sup>13</sup>C initially incubated as wheat roots after one year of field incubation, at two sites (Prat Llong and Alinyà) and depths (5 and 20 cm). Recovery after three years of incubation in Prat Llong is also indicated. Vertical bars show the standard error of mean.



**Fig. 2.** Recovery of root-derived <sup>15</sup>N in remaining roots and soil fractions as a percentage of <sup>15</sup>N initially incubated as wheat roots after one year of field incubation, at two sites (Prat Long and Alinyà) and depths (5 and 20 cm). Recovery after three years of incubation in Prat Long is also indicated. Vertical bars show the standard error of mean.

The incorporation of recalcitrant root-derived OM into the S + C fraction occurred at lower rates than total root-derived OM did (Table 5). After the first year, these rates in Prat Long, dropped to approximately 50% of the rates observed during the first year of incubation (Table 5).

#### 4. Discussion

The incubation of labelled wheat roots allowed us to measure the remaining wheat root-C and -N, including the amount incorporated into the soil, and to determine the allocation patterns in soil fractions. The slight enrichment of the soil below the mixture bags indicates that some of the root-derived C and N were lost by

leaching. However, the amount of labelled root material recovered in the underlying soil was low (<2%), thereby suggesting limited relevance of leaching in the estimation of remaining root-C and -N.

##### 4.1. Remaining root-derived C

After one year of field incubation in the Pyrenean mountain grasslands, 62–78% of incubated wheat-C remained in the soil. These values are in the same order of magnitude as those reported in other studies carried out in temperate mountain areas and other sites under mesic conditions. In the Pyrenees, Montané et al. (2010) recorded remaining mass values of grass roots in litterbags also buried at a depth of 5 cm that were similar (79% after 1 year) to our

**Table 4**  
Results (*p*-values) of repeated measures analyses showing the differences between sites (Alinyà and Prat Long) and depths of burial (5 and 20 cm) on the amount of C and N recovered from remaining roots and in the three size fractions after one year of field incubation.

	Carbon				Nitrogen			
	Roots	CS	FS	S + C	Roots	CS	FS	S + C
Site	<0.001	0.004	0.942	<0.001	<0.001	0.004	0.941	<0.001
Depth	0.034	0.336	0.169	0.077	0.034	0.355	0.185	0.078
Site × Depth	0.172	0.370	0.356	0.460	0.258	0.370	0.377	0.549

**Table 5**

Incorporation rates (mean  $\pm$  standard error) of total and recalcitrant C and N derived from the labelled roots into the S + C fraction. Rates are expressed as the proportion of total and recalcitrant C and N incorporated into the S + C fraction over the total and recalcitrant C and N that were initially supplied with the incubated roots, on a yearly basis.

		Total		Recalcitrant	
		Carbon (mgC gC <sup>-1</sup> y <sup>-1</sup> )	Nitrogen (mgN gN <sup>-1</sup> y <sup>-1</sup> )	Carbon (mgC gC <sup>-1</sup> y <sup>-1</sup> )	Nitrogen (mgN gN <sup>-1</sup> y <sup>-1</sup> )
Prat Llong:					
1st y	5 cm	27.3 $\pm$ 4.5	65.5 $\pm$ 10.5	20.0 $\pm$ 3.2	54.7 $\pm$ 6.4
	20 cm	37.1 $\pm$ 4.7	87.6 $\pm$ 11.2	25.2 $\pm$ 3.3	55.2 $\pm$ 7.7
2nd–3rd y	5 cm	16.4 $\pm$ 2.6	32.9 $\pm$ 13.2	9.2 $\pm$ 1.7	26.4 $\pm$ 4.5
	20 cm	17.3 $\pm$ 3.2	42.0 $\pm$ 9.1	8.9 $\pm$ 1.2	31.3 $\pm$ 5.6
Alinyà:					
1st y	5 cm	57.2 $\pm$ 6.0	152.8 $\pm$ 14.5	32.6 $\pm$ 3.4	73.3 $\pm$ 7.9
	20 cm	63.0 $\pm$ 4.4	168.2 $\pm$ 12.7	38.4 $\pm$ 2.6	85.4 $\pm$ 8.4

findings in Prat Llong (78%) and higher than the remaining C at the lower altitude site (Alinyà; 68%). The remaining root-C in Prat Llong after one year field incubation was also similar to the reported remaining mass of grass roots buried in litterbags (7 cm depth) after a single season incubation in cold Canadian grasslands (McLaren and Turkington, 2010), which in turn was higher than the remaining C in Alinyà. While most of the C loss occurred during the first year, a small portion was lost during the following two years. This observation indicates a decrease in decomposition rates over time, this decrease related to the reduction of the quality of the remaining roots during decay, as well as their physical stabilisation.

Decomposition rates are controlled mainly by climate, substrate quality (i.e., biochemical recalcitrance) and the nature of the decomposer community (Coûteaux et al., 1995). As we used the same substrate at both sites and depths, differences should mostly be attributable to variations in the microenvironment (i.e., soil temperature and moisture) and to the characteristics of the corresponding decomposer communities. The faster decomposition in the warmest and driest site (Alinyà) may highlight the prevalent role of temperature on decomposition processes at mesic sites. In addition, the acidity (pH 4.8) of Prat Llong soils as compared to Alinyà (pH 7.1) could also contribute to the slower decomposition at Prat Llong by changing microbial communities (i.e., increasing fungal-to-bacterial ratios, Rousk et al., 2009) or reducing nutrient availability (Kemmitt et al., 2005).

Deeper soils are commonly reported to have lower decomposition rates than the uppermost layers (Gill and Burke, 2002; Sanaullah et al., 2010), a decrease with depth that has been introduced into current models of soil-C cycling (Jenkinson and Coleman, 2008). Although lower decay rates in these horizons may occur in most ecosystems, it cannot be considered a common feature of all

soils as greater decomposition rates in the subsurface layers have been reported in both Mediterranean (Rovira and Vallejo, 1997) and alpine (Withington and Sanford, 2007) soils. However, it is difficult to distinguish the microclimate-driven effects of depth from those caused by the changes in the soil properties. As we used the same soil in the mixtures incubated at both depths, the effect of the changes in soil properties were minimised. Therefore, the faster decomposition in the subsoil (20 cm) than in the topsoil (5 cm) at the first steps of decomposition can be mostly attributed to the more suitable microclimatic conditions for decomposition in the subsoil. The greater and more constant moisture content and perhaps the lower frequency of strong freeze-thaw events may contribute to make activity of decomposer community more stable and continuous in the subsurface layer, thus allowing higher decay rates. Indeed, the higher grass root relative productivity (belowground production to biomass ratio) in subsurface horizons than in the uppermost soil at these sites has also in part been attributed to a better environmental conditions in the subsoil (Garcia-Pausas et al., 2011). By contrast, the slower decomposition in deep soils that is usually reported in the literature (Gill and Burke, 2002; Sanaullah et al., 2010) may be related to the prevalence of soil-related factors (e.g., nutrient availability, OM content, etc) limiting the decomposition in ecosystems where microclimate does not constrain the decomposer activity. However, note that depth appears not to be relevant at latter stages of decomposition, when most of labile root material has been mineralised or stabilised. Further work is needed to determine whether potential changes in the quality of deposited dead roots with depth could also determine decomposition rates along soil profiles.

#### 4.2. Stabilisation of C and N in fine soil particles

After one year of field incubation, most of the wheat root material remained in the coarse fractions but a significant amount was recovered in the fine fraction. The low amount of C and N recovered by adding all the fractions compared to the non-fractionated samples, particularly in the samples incubated for 3 years, indicated that a significant amount of soluble C and N were lost during fractionation. Bosshard et al. (2008) also found that a large proportion (37–55%) of amendment-derived N was lost during the dispersion of aggregates during fractionation. As they indicated, this loss may highlight the importance of aggregation in the protection of soluble OM. The highly-degraded root material after 3 years of field incubation may have caused the higher C and N loss compared to the first year.

SOM is stabilised by the sorption of OM onto mineral surfaces, mainly in fine-sized particles (von Lützow et al., 2006). Some studies report that protected OM in the fine particles predominantly come from microbial resynthesis (Rumpel et al., 2010; von Lützow et al., 2006) and dissolved OM (Kalbitz and Kaiser, 2008).

**Table 6**

Biochemical quality (C/N ratio, RIC and RIN) of root-C and -N among size fractions by site, incubation depth and time. RIC and RIN are the percentage of recalcitrant root-C and -N over total root-C and -N. RIC and RIN of remaining roots are not available. Time 0 refers to initial non-incubated root-soil mixtures. Values are mean  $\pm$  standard error. Different letters indicate significant differences between fractions (Duncan test). Initial values of the incubated roots were: C/N = 29.4  $\pm$  0.27, RIC = 42.0  $\pm$  0.62, RIN = 11.3  $\pm$  1.32 ( $n$  = 4).

Site	Time (d)	Depth (cm)	C/N				RIC			RIN		
			Roots	CS	FS	S + C	CS	FS	S + C	CS	FS	S + C
Prat Llong	0	–	30.6 $\pm$ 1.0a	31.5 $\pm$ 0.7a	26.0 $\pm$ 1.7b	8.4 $\pm$ 0.3c	21.7 $\pm$ 0.8a	21.2 $\pm$ 2.1a	16.2 $\pm$ 1.6a	10.9 $\pm$ 0.3a	17.1 $\pm$ 4.4a	17.9 $\pm$ 4.6a
	357	5	24.8 $\pm$ 0.3b	28.3 $\pm$ 0.3a	20.6 $\pm$ 0.5c	9.7 $\pm$ 0.2d	35.1 $\pm$ 1.2a	32.9 $\pm$ 1.4a	28.2 $\pm$ 0.9b	19.7 $\pm$ 0.9a	18.0 $\pm$ 0.7a	12.4 $\pm$ 1.1b
		20	24.2 $\pm$ 0.5b	27.4 $\pm$ 0.6a	21.3 $\pm$ 0.4c	10.1 $\pm$ 0.3d	34.4 $\pm$ 2.6a	32.1 $\pm$ 1.1ab	28.1 $\pm$ 0.9b	18.6 $\pm$ 1.8a	17.4 $\pm$ 0.7a	9.4 $\pm$ 0.6b
	1129	5	22.0 $\pm$ 1.5a	19.1 $\pm$ 2.2a	17.0 $\pm$ 0.6a	8.7 $\pm$ 0.3b	26.7 $\pm$ 2.1b	34.2 $\pm$ 2.1a	28.7 $\pm$ 1.1ab	9.2 $\pm$ 1.7b	15.0 $\pm$ 1.0a	8.7 $\pm$ 0.9b
20		21.3 $\pm$ 1.1a	23.5 $\pm$ 0.9a	17.4 $\pm$ 0.5b	8.6 $\pm$ 0.4c	34.8 $\pm$ 0.5a	32.3 $\pm$ 1.0b	26.7 $\pm$ 0.6c	14.3 $\pm$ 0.8a	14.8 $\pm$ 0.6a	7.3 $\pm$ 0.2b	
Alinyà	0	–	32.5 $\pm$ 1.5a	30.9 $\pm$ 0.1a	21.0 $\pm$ 2.1b	6.7 $\pm$ 0.2c	28.5 $\pm$ 1.0a	24.5 $\pm$ 3.0a	15.2 $\pm$ 0.7b	19.4 $\pm$ 0.3a	11.2 $\pm$ 1.3b	17.8 $\pm$ 0.9a
	352	5	26.4 $\pm$ 0.4b	28.0 $\pm$ 0.6a	20.8 $\pm$ 0.4c	9.0 $\pm$ 0.2d	37.9 $\pm$ 0.9a	34.1 $\pm$ 1.1b	24.9 $\pm$ 0.3c	26.5 $\pm$ 0.5a	17.9 $\pm$ 0.7b	7.4 $\pm$ 0.3c
		20	27.1 $\pm$ 0.9b	29.2 $\pm$ 0.7a	20.3 $\pm$ 0.2c	9.1 $\pm$ 0.1d	35.3 $\pm$ 0.9a	35.7 $\pm$ 1.3a	26.8 $\pm$ 0.6b	24.4 $\pm$ 0.5a	18.2 $\pm$ 0.7b	7.5 $\pm$ 0.4c

Hence, the OM derived from dead plant substrates may incorporate mostly into the S + C fraction during the first stages of decomposition, when most of the labile fraction of plant material is used by microbial decomposers. Indeed, net incorporation rates of root-derived C into the S + C fraction during the first year were faster than during following two years at both sites and depths. The higher root decay rates at a depth of 20 cm than at 5 cm also coincided with greater incorporation rates of root-derived C and N into the S + C fraction in this layer. Under natural conditions, the low OM/mineral soil ratio at the deep soil compared to topsoil suggests that deep horizons could have greater stabilisation capacity (Stewart et al., 2009). However, as we used the same soil at both depths, the higher incorporation of labelled material into the S + C fraction in the subsoil may be related to changes in the environmental conditions and the decomposition of the labelled roots.

The amount of wheat root-derived OM incorporated into the FS and S + C fractions increased over time. Its biochemical quality (RIC and RIN) in the FS fraction was mostly unchanged as compared to the CS fraction, thereby indicating that incorporation into this fraction was driven mainly by the physical comminution of roots into small debris and that this process does not imply significant chemical changes. In contrast, root-derived OM incorporated into the S + C fraction showed lower C/N ratios. This finding could in part be attributed to the longer time that N remains in soil compared to C (i.e., an amount of C is released to the atmosphere as CO<sub>2</sub>). However, labelled N incorporated into S + C fraction more than twice as fast as C did (Table 5), suggesting that root-derived N-rich OM (mainly peptides and proteins) is preferentially incorporated to S + C over other N-poor or N-devoid organic components, such as lignin. Our results are in line with previous findings that indicate that peptides adsorb strongly to the mineral surfaces, making them more stable and explaining the old age of these compounds in soil in spite of being easily degradable compounds (Knicker, 2011). Such N enrichment also suggests that it was not mostly composed of dissolved OM, whose high C/N ratios have been reported (Qualls et al., 2002; McDowell et al., 2004).

The low RIC and RIN of the root-C and -N in S + C fraction compared to the values in the other fractions also showed that the OM stabilised in S + C is mostly composed of the labile fraction of root-derived OM and that the chemically recalcitrant pools of OM remain physically unprotected, at least at the initial stages of decomposition. These observations are consistent with the lower rates at which recalcitrant root-C and -N were incorporated into S + C. In Mediterranean forest soils, Rovira and Vallejo (2007) also found that SOM associated with the clay fraction in B horizons had lower RIC than in other fractions. However, in our case, it is noteworthy that total C and N in the S + C fraction showed higher RIC and RIN than the C and N incorporated into this fraction from the labelled roots. This finding indicates that SOM associated with this fraction shows a greater degree of decomposition than the newly incorporated OM and suggests that the association of SOM with the S + C fraction does not completely prevent SOM decomposition. The lower rates at which recalcitrant root-C and -N is incorporated into S + C may also contribute to this discrepancy between the quality of labelled C and N incorporated into the fine fraction and the quality of the total C and N in the fraction.

## 5. Conclusions

Although mineralisation rates are generally assumed to decrease with depth, the environmental conditions of subsurface horizons in mountain grassland soils may favour decomposition rates as well as the incorporation rates of root-derived organic matter into the fine soil fractions.

OM derived from decomposing roots incorporates into the protected pool at higher rates during the initial stages of root decomposition than at later stages. The root material incorporated to the protected pool comprises mainly N-rich labile OM. Meanwhile recalcitrant OM remains physically unprotected for longer. Our results highlight the relevance of physical stabilisation for the protection of the labile OM pool in mountain grassland soils.

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