Plant Soil (2013) 367:313–326
DOI 10.1007/s11104-012-1464-4

REGULAR ARTICLE

Insights into root growth, function, and mycorrhizal abundance from chemical and isotopic data across root orders

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Received: 18 January 2012 / Accepted: 14 September 2012 / Published online: 3 October 2012

Abstract
Background and aims Detailed analyses of root chemistry by branching order may provide insights into root function, root lifespan and the abundance of root-associated mycorrhizal fungi in forest ecosystems.
Methods We examined the nitrogen and carbon stable isotopes ($\delta^{15}N$ and $\delta^{13}C$) and concentration (%N and %C) in the fine roots of an arbuscular mycorrhizal tree, *Fraxinus mandshurica*, and an ectomycorrhizal tree, *Larix gmelinii*, over depth, time, and across five root branching orders.
Results and conclusions *Larix* $\delta^{15}N$ increased by 2.3‰ from 4th order to 1st order roots, reflecting the increased presence of ^{15}N-enriched ECM fungi on the lower root orders. In contrast, arbuscular mycorrhizal *Fraxinus* only increased by 0.7‰ from 4th order to 1st order roots, reflecting the smaller ^{15}N enrichment and lower fungal mass on arbuscular mycorrhizal fine roots. Isotopic and anatomical mass balance calculations indicate that first, second, and third order roots in ectomycorrhizal *Larix* averaged 36 %, 23 %, and 8 % fungal tissue by mass, respectively. Using literature values of root production by root branching order, we estimate that about 25 % of fine root production in ECM species like *Larix* is actually of fungal sheaths. In contrast to %N, %C, and $\delta^{15}N$, $\delta^{13}C$ changed minimally across depth, time, and branching order. The homogeneity of $\delta^{13}C$ suggests root tissues are constructed from a large well-mixed reservoir of carbon, although compound specific $\delta^{13}C$ data is needed to fully interpret these patterns. The measurements developed here are an important step towards explicitly including mycorrhizal production in forest ecosystem carbon budgets.

Keywords Carbon · Isotope · Mycorrhizal fungi · Nitrogen · Root order

Abbreviations
ECM Ectomycorrhizal

Responsible Editor: Angela Hodge.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-012-1464-4) contains supplementary material, which is available to authorized users.

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AM	Arbuscular mycorrhizal
Larix	*Larix gmelinii*
Fraxinus	*Fraxinus mandshurica*
N	Nitrogen
C	Carbon
‰	Per mil

Introduction

Stable isotope measurements are widely reported in forest ecosystem studies, especially for aboveground components such as foliage and fungal fruiting bodies. Isotopic measurements of aboveground components have provided both valuable insights and relatively simple methods for assessing several ecosystem processes including soil nutrient availability, plant water use efficiency, and interactions with mycorrhizal fungi (Farquhar et al. 1989; Hobbie and Hobbie 2006; Högberg 1997). Carbon and nitrogen stable isotopes (expressed as $\delta^{13}C$ and $\delta^{15}N$) of belowground components such as fine roots should provide insights into the movement of carbon (C) and nitrogen (N) between above and belowground pools and between plants and mycorrhizal fungi. Further, isotopic data on fine roots may provide a relatively simple means for estimating ecosystem parameters that are very difficult to measure, such as the degree of reliance on mycorrhizal fungi for N acquisition and the belowground carbon investment by plants in their mycorrhizal fungal symbionts (Hobbie and Hobbie 2006).

While foliar and even fungal $\delta^{13}C$ and $\delta^{15}N$ have been studied extensively in forest ecosystems aboveground (Craine et al. 2009 and references therein; Hobbie and Hobbie et al. 2008 and references therein; Mayor et al. 2009), C and N isotopes of roots and mycorrhizal fungi belowground have been less well studied because of the relative difficulty in collection and identification. In fact, only a few studies have reported $\delta^{15}N$ of fine roots from forest ecosystems (e.g. Bauer et al. 2000; Gebauer and Schulze 1991; Högberg et al. 1996; Michelsen et al. 1998; Pardo et al. 2006; Templer et al. 2007), with fine roots classified as either less than 1 or less than 2 mm in diameter. This size classification can include several branching orders (1st through 5th orders), some of which are heavily colonized by mycorrhizal fungi. To our knowledge no studies report $\delta^{13}C$ and $\delta^{15}N$ isotopic patterns of roots by branching order.

Examining patterns in root chemistry and anatomy across branching order is important because recent work has shown that the relatively crude classification of "fine roots" using a 2 mm diameter threshold is not appropriate when trying to understand root function, lifespan, and chemistry (Guo et al. 2004; Pregitzer 2002, 2008). Classifying and examining roots by branching order is a more appropriate way to understand root function as well as nutrient dynamics (Fan and Jiang 2010; Guo et al. 2008a; King et al. 2002; Mei et al. 2010; Pregitzer et al. 2002), and recent studies have found that lower order (more distal) roots have higher nitrogen concentrations, higher maintenance respiration costs, and shorter lifespans than higher order roots (Eissenstat et al. 2000; Pregitzer et al. 1997; Jia et al. 2011).

Fine roots in forests are tightly coupled to mycorrhizal fungi. For example, in a study of anatomical features of roots by root order for 23 species of trees in China, Guo et al. (2008c) observed that 1st through 3rd order roots generally had little secondary development but high rates of mycorrhizal colonization, while 4th and 5th order roots generally lacked mycorrhizal colonization but showed distinct secondary development of xylem for transport. There is also evidence that mycorrhizal fungi colonizing root tips may increase root lifespan and alter root carbon demand (Guo et al. 2008b; King et al. 2002; Langley et al. 2006; Sun et al. 2010; Zadworny and Eissenstat 2011). Despite continued progress in our understanding of root–mycorrhizal fungal associations, ecosystem models of C and N have lacked adequate incorporation of belowground processes, especially of fine roots and mycorrhizal fungi. In fact, most estimates of belowground net primary production in forests do not explicitly include allocation to mycorrhizal fungi (Hobbie 2006; Litton and Giardina 2008; but see Orwin et al. 2011).

One reason for the omission of mycorrhizal fungi from ecosystem C and N models is that we lack data on how mycorrhizal fungi influence belowground C and N dynamics. Specifically, the degree of reliance on mycorrhizal fungi for N acquisition and the subsequent C allocation to mycorrhizal fungi to obtain soil N remains uncertain. $\delta^{15}N$ measurements (or the ratio of ^{15}N:^{14}N) across root branching order offer a promising approach to quantify C and N dynamics between plants and ectomycorrhizal fungi and may help to fill this data gap in belowground C and N

allocation. For example, because ectomycorrhizal (ECM) fungi discriminate against ^{15}N during the transfer of N to plants, host plants tend to have relatively more ^{14}N (they are depleted in ^{15}N), while ECM fungi are enriched in ^{15}N. This is evident in numerous aboveground field studies where ectomycorrhizal fruiting bodies are often enriched in ^{15}N compared to their host plant (Hobbie and Hobbie 2008; Taylor et al. 2003; Trudell et al. 2004). Using known fractionation factors and the $\delta^{15}N$ of plants and ECM fungi, the fraction of N retained in the ECM fungi can be calculated, and the amount of C supplied to ECM fungi can be stoichiometrically estimated using the N demand of plants and the fungal C: N ratio (Hobbie and Hobbie 2008). However, using aboveground ECM fungal fruiting bodies and foliage for these estimates can be problematic because $\delta^{15}N$ fractionation occurs during fungal fruiting body formation (Hobbie et al. 2012). Also it is difficult to compare aboveground $\delta^{15}N$ to soil available N because soil $\delta^{15}N$ varies strongly with depth (Hobbie and Ouimette 2009). Less is known about how to interpret N dynamics from $\delta^{15}N$ measurements in arbuscular mycorrhizal (AM) fungi symbioses. AM plants are often intermediate in $\delta^{15}N$ between ectomycorrhizal and nonmycorrhizal plants (Craine et al. 2009), but we lack isotopic data on AM sporocarps or AM hyphae.

The few previous studies that report isotopic data on roots support aboveground patterns in $\delta^{15}N$ between plants and ECM fungi, with ECM root tips being more enriched in ^{15}N than nonmycorrhizal plant components. For instance, Michelsen et al. (1998) compared the $\delta^{15}N$ of foliage to heavily colonized fungal root tips ($\Delta_{root\text{-}foliage}$) in two ectomycorrhizal species at two heath sites and found that the root tips were generally ^{15}N-enriched compared to foliage. However, this difference was much smaller in northern Sweden ($\Delta_{root\text{-}foliage}$=0.5–1.2‰) than in Greenland ($\Delta_{root\text{-}foliage}$=3.2–4.5‰), and was attributed to lower levels of mycorrhizal colonization at the Swedish site. Högberg et al. (1996) and Bauer et al. (2000) also found that ECM root tips were enriched in ^{15}N compared to nonmycorrhizal roots. Högberg et al. (1996) examined this pattern in more detail by separating the fungal sheath from the nonmycorrhizal core of *Fagus* roots at several sites. They found that the $\delta^{15}N$ of fungal material was 2.4–6.4‰ greater than nonmycorrhizal root parts. Similarly, Langley and Hungate (2003) found that mycorrhizal roots of *Pinus* less than 1 mm in diameter were about 4.5‰ higher in $\delta^{15}N$ than nonmycorrhizal roots.

In addition to colonization by mycorrhizal fungi, the $\delta^{15}N$ of roots may also be influenced by the $\delta^{15}N$ of the associated soil horizon. The few studies on $\delta^{15}N$ in roots show that root $\delta^{15}N$ increases with depth (as does soil), but differences between the $\delta^{15}N$ of roots and soil are generally larger in deeper soil horizons than in more shallow soil layers (Gebauer and Schulze 1991; Högberg et al. 1996). Still fewer studies have reported $\delta^{15}N$ (or $\delta^{13}C$) on belowground fungal components (Boström et al. 2007; Wallander et al. 2004), and generally suggest that $\delta^{15}N$ of ectomycorrhizal mycelia track the $\delta^{15}N$ of the soil layer in which they reside but, unlike roots, show only minor differences in $\delta^{15}N$ compared to soil $\delta^{15}N$.

Similarly, relatively few studies have assessed fine root $\delta^{13}C$ with depth or across size classes of fine roots, though there is some evidence of variability in fine root $\delta^{13}C$ by size. As an example, Polley et al. (1992) reported that fine roots 0.35–0.61 mm in diameter averaged 1.2‰ higher in $\delta^{13}C$ compared to fine roots 1.0–1.2 mm in diameter in *Prosopis glandulosa*. The controls on $\delta^{13}C$ of fine root material are not well understood, but they are likely influenced by the $\delta^{13}C$ of sucrose transported from foliage along with any fractionation during transport (Badeck et al. 2005; Cernusak et al. 2009; Hobbie and Werner 2004). The few studies on belowground fungal $\delta^{13}C$ also show that it does not vary with depth and is thought to track the carbon source supplied from host plants with a slight enrichment in ^{13}C (Boström et al. 2007; Wallander et al. 2004). Seasonal variability in the $\delta^{13}C$ of fixed carbon (Pate and Arthur 1998) could further influence the $\delta^{13}C$ of fine roots if roots are not evenly produced throughout the growing season. Seasonal changes in photosynthate $\delta^{13}C$ may be most pronounced in lower root orders which have shorter lifespans and potentially several flushes of growth throughout a growing season. For these reasons, we might expect higher variability in $\delta^{13}C$ of lower root orders compared to longer-lived coarser root orders.

Research on fine root anatomy, physiology, and ecology is now moving towards analysis by root orders rather than treating fine roots as a single size class. However, to date, there are no published studies describing $\delta^{13}C$ and $\delta^{15}N$ across root orders in fine

roots. This study examines the $\delta^{13}C$ and $\delta^{15}N$ in fine roots by branching order in both an arbuscular mycorrhizal (AM) and an ectomycorrhizal (ECM) tree species. The data presented here come from a temperate forest in China, in which roots from ECM larch (*Larix gmelinii*) and AM ash (*Fraxinus mandshurica*) were collected over two soil depths throughout the growing season. Our goal is to document these isotopic patterns across root branching order in both an ectomycorrhizal and an arbuscular mycorrhizal species, and to demonstrate how future isotopic measurements on roots by branching order can improve our knowledge of plant–fungal N dynamics and provide estimates of the partitioning of carbon allocation between fine roots and mycorrhizal fungi.

Methods

Study site

The study sites were located at the Maoershan research station (45°21′–45°25′N, 127°30′–127°34′E) of Northeast Forestry University, in temperate monsoonal Heilongjiang, China. Mean January, July and annual temperatures are −19.6 °C, 20.9 °C, and 2.8 °C, respectively, while the mean annual precipitation is 723 mm, with over 65 % of the precipitation falling from June to August (Zhou 1994). At the station, two plantations were chosen, a *Larix gmelinii* (*Larix*) and a *Fraxinus mandshurica* (*Fraxinus*), which were established in 1986 and sampled in 2003. Stand densities in 2002 were 2,267 and 2,111 individuals per hectare for Larix and Fraxinus respectively (or approximately 200 trees per 30×30 m plot). Soils at the two sites are well-drained Hap-Broic Luvisols with high organic matter content. Both study sites were part of a larger root anatomical study (Guo et al. 2008c).

Sample collection and analysis

Root samples were collected at two soil depths (0–10 cm and 10–20 cm) in May, July and September of 2003. For each of the two species, *Larix* and *Fraxinus*, three 30×30 m plots were selected for each species, and on each sampling date three 1×1 m subplots were randomly chosen within each plot (yielding 9 total subplots per species per sampling date). At each subplot, a 20×20 cm block of soil was collected and intact root networks consisting of more than five branch orders were sampled at two depths (0–10 and 10–20 cm). Within each soil block several root branches, likely from several individual trees, were collected and composited. Following collection, roots were briefly rinsed in tap water, placed in Ziploc bags and stored on ice for transportation back to the lab within 4 h. In the lab tree roots were dissected into the first five root orders following Pregitzer et al. (2002) and Guo et al. (2004). After root dissection, roots from each block were composited across three subplots at each soil depth by branching order, thus resulting in one composited root sample per order at each of the three plots for each soil depth and sampling date.

Soil samples were collected and composited in an identical manner as root collection from the same soil blocks on the July 2003 sampling date only. Foliage was sampled from three overstory trees per plot for each species and composited. Soils (sieved to 2 mm), roots, and foliage were oven dried at 60 °C before grinding in a ball mill prior to elemental and isotopic analysis. Isotopic and elemental analyses were done at the University of New Hampshire Stable Isotope Lab (http://www.isotope.unh.edu/). The natural abundance of carbon and nitrogen stable isotopes was measured using a Costech 4010 Elemental Analyzer coupled to a Finnigan Delta Plus XP isotope ratio mass spectrometer. The C and N isotope composition was expressed in standard delta notation ($\delta^{13}C$, $\delta^{15}N$) in per mil (‰) relative to V-PDB standard for C and atmospheric N_2 for N. Precision of duplicate analyses averaged less than .08‰ for $\delta^{13}C$ and less than 0.20‰ for $\delta^{15}N$. Duplicate analyses for carbon and nitrogen concentrations (%C, %N) varied less than 1.0 % and .05 % respectively.

All statistical analyses were performed using Jmp 9.0.0 statistical software. For each species differences in $\delta^{15}N$, $\delta^{13}C$, %N, and %C across depth for all root orders were analyzed using paired t-tests. Differences in $\delta^{15}N$, $\delta^{13}C$, %N, and %C across root order in each species were performed by least squares regressions using pooled data from both depths. Analysis of covariance (ANCOVA) tests for $\delta^{15}N$, $\delta^{13}C$, %N, and %C across root order for the two depths indicated that the use of pooled data from both depths for regression analyses was valid (no significant differences in the slope of $\delta^{15}N$, $\delta^{13}C$, %N, or %C versus root order between the depths for either species). Error estimates are reported as standard deviation unless otherwise

noted. The equality of variances by root order in %C and $\delta^{13}C$ was assessed using Levene's test of equality of variances.

To estimate the fraction of root N, C, and biomass that were fungal by root order both isotopic mass balance and anatomical calculations were used (see Discussion). The fraction of root N that is fungal was estimated using the following isotopic mass balance calculation:

$$\textit{Fraction of Root N as Fungal N} = \frac{\left(\delta^{15}N_{RootOrder(i)} - \delta^{15}N_{5thOrderRoot}\right)}{\left(\delta^{15}N_{FungalTissue} - \delta^{15}N_{5thOrderRoot}\right)} \quad (1)$$

where, $\delta^{15}N_{RootOrder(i)}$ equals the measured $\delta^{15}N$ of a root order i, $\delta^{15}N_{5thOrderRoot}$ is the measured $\delta^{15}N$ of 5th order (nonmycorrhizal) roots, and $\delta^{15}N_{FungalTissue}$ is the $\delta^{15}N$ of fungal tissue. In the present study, only the first three root orders of *Larix* were colonized by ECM fungi and we assumed that 5th order roots represent non-mycorrhizal tissue (which was found for all ECM species in Guo et al. 2008c).

To convert the proportion of root nitrogen that is fungal to a fraction of root carbon and root biomass that is fungal tissue it is necessary to use the %C and C/N ratio of fungal tissue as well as the %C and C/N ratio of plant tissue by root order. For each root order (*i*) the fraction of root C that is fungal C is calculated as:

$$\textit{Fraction of C as Fungal C}(i) = \frac{\left(f_{N\_Fungal} \times C/N_{Fungal}\right)}{\left[\left(f_{N\_Fungal} \times C/N_{Fungal}\right) + \left(f_{N\_Plant} \times C/N_{Plant}\right)\right]} \quad (2)$$

where, for root order i, f_{N_Fungal} is the fraction of root N as fungal (from Eq. 1), C/N_{Fungal} is the C/N ratio of pure fungal tissue, f_{N_Plant} is the fraction of root N as plant N for root order i (or $1-f_{N\_Fungal}$), and C/N_{Plant} is the C/N ratio of pure plant material of root order i. Finally, for each root order (*i*) the fraction of root biomass as fungal biomass is calculated as:

$$\textit{Fraction of Biomass as Fungal}(i) = \frac{\left(f_{C\_Fungal}/\%C_{Fungal}\right)}{\left[\left(f_{C\_Fungal}/\%C_{Fungal}\right) + \left(f_{C\_Plant}/\%C_{Plant}\right)\right]} \quad (3)$$

where, for root order i, f_{C_Fungal} is the fraction of root C as fungal (from Eq. 2), $\%C_{Fungal}$ is the %C of pure fungal tissue, f_{C_Plant} is the fraction of root C as plant C for root order i (or $1-f_{C\_Fungal}$), and $\%C_{Plant}$ is the %C of pure plant material of root order i.

Equations 1, 2 and 3 were used to derive point estimates of the fraction of root N, C, and biomass that were made up of fungal tissue, independently for each root order, based on "best guess" parameter values from literature and data. The input parameters needed in the isotopic calculations (Eqs. 1, 2 and 3) are the measured $\delta^{15}N$ of individual root orders, the %C and C/N of plant tissue by root order, and fungal %C, C/N, and $\delta^{15}N$. As an example, Table 1 lists the parameter values and sources used to derive the point estimates for first order *Larix* roots for the 0–10 cm soil depth. Since only the $\delta^{15}N$ of individual root orders was measured directly, Monte Carlo simulations were performed by varying input parameter values to quantify uncertainty in our point estimates. In these simulations measured root $\delta^{15}N$ was allowed to vary with a uniform distribution over the interval of plus or minus one standard deviation around measured root $\delta^{15}N$. Although the C/N of roots was measured by root order, fine roots are composed plant and fungal tissue and thus the C/N of plant tissue was not measured directly. For these simulations the C/N of plant tissue was varied uniformly over the range of the measured C/N plus a 10 unit range for each root order (e.g. C/N of 25–35). Fungal C/N was allowed to vary between 10 and 20 with a uniform distribution throughout the range. Finally, because fungal $\delta^{15}N$ typically closely tracks the $\delta^{15}N$ of soil (see Boström et al. 2007; Högberg et al. 1996; Wallander et al.

Table 1 "Best guess" parameter values used in Eqs. 1, 2 and 3 to generate point estimates of the fraction of root N, C, and biomass that is fungal for 1st order *Larix gmelinii* roots in the 0–10 cm soil horizon

Parameter	Value	Source
$\delta^{15}N_{RootOrder(i)}$	2.54	Measured
$\delta^{15}N_{FungalTissue}$	4.73	Measured soil $\delta^{15}N$ + 0.5‰
$\delta^{15}N_{5thOrderRoot}$	−0.01	Measured
C/N_{Fungal}	15.0	Literature values
C/N_{Plant}	30.0	Measured value of root order i + 5
$\%C_{Fungal}$	40.8 %	Literature values
$\%C_{Plant}$	48.5 %	Used measured value of 5th order roots

2004), for these simulations fungal $\delta^{15}N$ was allowed to vary with a uniform distribution over the interval of plus or minus 1.5‰ around measured soil $\delta^{15}N$.

Anatomical calculations of the fraction of root biomass that is fungal tissue were only performed for ECM *Larix* (AM fungi do not form a visible sheath covering roots). For the anatomical calculations, fungal sheath thickness, root diameter, and the fraction of roots colonized by root order are required as input parameters. For fungal sheath thickness, a literature search was performed to summarize typical ectomycorrhizal sheath thickness (Supplementary Table 1). Fungal sheath thickness was allowed to vary with a uniform distribution over the interval of plus or minus 1 standard deviation of the mean of 35 literature reported measurements (28±11 μm). Root diameter and the fraction of roots colonized by root order were measured for *Larix* by Guo et al. 2008c. For these simulations root diameter was allowed to vary between plus or minus 0.10 mm around the measured values in Guo et al. 2008c, while the fraction of fungal colonization was allowed to vary between plus or minus 0.10 around the measured values in Guo et al. 2008c. For both isotopic and anatomical calculations, the median values of ten thousand simulations were reported with error estimates reported as median absolute deviation.

For both isotopic and anatomical Monte Carlo simulations a sensitivity analysis was performed by allowing one parameter to vary over the range used in the full model simulations while holding all other parameters constant at their "best guess" values. Median absolute deviations of the fraction of root N, C, and biomass that are fungal are reported for the full model simulations, as well as for the sensitivity analysis for each individual parameter (Table 2a and b).

Potential contamination of roots with soil

Several authors have noted the difficulty in obtaining clean, soil-free fine roots for chemical and isotopic analysis, and have provided methods of correction for chemical data of fine roots contaminated with soil particles (Hunt et al. 1999; Janzen et al. 2002). Root carbon concentration can be used as a proxy for the extent of soil contamination (Janzen et al. 2002). Across both species and all dates, depths, and orders, root %C varied from 42.5 % to 50.1 %, which is within the range expected for soil-free root material, and only three of the 175 root samples had less than 44 % carbon.

The expected carbon concentration and the visual cleanliness of the root samples suggest little to no soil C or N contamination of root chemical data. A simple mass balance approach illustrates this point more clearly. Assuming "clean" root material has 48 % C and soil has 2 % C (typical of the horizons collected), then the root sample with the lowest carbon concentration, 42.5 % (potentially most soil C contamination) would have less than 1 % of its total C contributed by adhering soil particles. The same calculation for nitrogen reveals that at most, 1 % of total N could be

Table 2 Sensitivity analysis of Monte Carlo simulations for a) isotopic and b) anatomical estimates of the fraction of root N, C, and biomass that are fungal for 1st order *Larix gmelinii* roots in the 0–10 cm soil horizon. Sensitivity analyses were performed by allowing one parameter to vary over the range used in the full model simulations while holding all other parameters constant at their "best guess" values. Median absolute deviations of the fraction of root N, C, and biomass that are fungal are reported for the full model simulations, as well as for the sensitivity analysis of each individual parameter

A. Isotopic	Fraction of root N as fungal	Fraction of root C as fungal	Fraction of root biomass as fungal
Full model	0.09	0.09	0.10
$\delta^{15}N_{RootOrder(i)}$	0.07	0.07	0.07
$\delta^{15}N_{FungalTissue}$	0.08	0.07	0.08
$\delta^{15}N_{5thOrderRoot}$	0.02	0.02	0.02
C/N_{Fungal}	0.00	0.04	0.04
C/N_{Plant}	0.00	0.02	0.02
$\%C_{Fungal}$	0.00	0.00	0.00
$\%C_{Plant}$	0.00	0.00	0.00
B. Anatomical	Fraction of root biomass as fungal		
Full model	0.06		
Fungal sheath thickness	0.05		
Root diameter	0.05		
Fraction of fungal colonization	0.00		

derived from soil contamination on root tips. Therefore, isotopic and elemental corrections were not made for soil potentially adhering to root material in this study.

Results

Nitrogen

Clear patterns emerged in the $\delta^{15}N$ signatures of roots across species, depth, and branching order. Root $\delta^{15}N$ increased with depth in both species with differences in mean root $\delta^{15}N$ between the two soil depths of 1.1 ‰ ($p<0.0001$) in *Larix* and 2.1‰ ($p<0.0001$) in *Fraxinus* (Supplementary Table 2). Across both depths, the difference between soil $\delta^{15}N$ and mean root $\delta^{15}N$ was much smaller for ectomycorrhizal *Larix gmelinii* (3.5±1.2‰) than for arbuscular mycorrhizal *Fraxinus mandshurica* (5.7±0.8‰) ($p<0.0001$). For *Larix* the difference between soil $\delta^{15}N$ and mean root $\delta^{15}N$ was greater in the deeper soil (4.0±1.0‰) than in the shallow soil (3.1±1.2‰) ($p<0.0001$), similar to findings by Gebauer and Schulze (1991) and Högberg et al. (1996). The difference between *Fraxinus* mean root $\delta^{15}N$ and soil $\delta^{15}N$ varied only weakly with depth ($p=0.07$). Nitrogen concentration of roots (%N) did not vary by depth in either species ($p>0.30$ for both species) (Supplementary Table 2).

Across all depths and dates, $\delta^{15}N$ generally decreased with increasing root order in both species, with the decrease across the first five branching orders three times greater in *Larix*, (slope=−0.6‰/order, $r^2=0.50$, RMSE=0.86, $p<0.0001$), than in *Fraxinus* (slope=−0.2‰/order, $r^2=0.04$, RMSE=1.30, $p=0.03$). The difference in slopes was greatest in the 0–10 cm depth with $\delta^{15}N$ decreasing with increasing root order more in *Larix* (slope=−0.66‰/order, $r^2=0.65$, RMSE=0.68, $p<0.0001$) than in *Fraxinus* (slope=−0.09‰/order, $r^2=0.03$, RMSE=0.59, $p=0.14$) (Fig. 1b). %N also decreased with increasing root order, with an average 0.2 % decrease per order in *Larix*, and a 0.4 % decrease per order in *Fraxinus* (Fig. 1a).

For *Larix*, foliar $\delta^{15}N$ (1.0±0.5‰) was intermediate between the average $\delta^{15}N$ of 4th and 5th order roots (non-mycorrhizal root orders) from the shallow (0.1±0.9‰) and deep (1.4±0.3‰) soil horizons. In contrast, *Fraxinus* foliar $\delta^{15}N$ (1.2±0.1‰) was more similar to the average $\delta^{15}N$ of 4th and 5th order roots from the deep (0.7±0.3‰) rather than the shallow (−1.2±0.2‰) soil horizon.

Carbon

$\delta^{13}C$ of roots did not vary by depth in either species (Supplementary Table 2). *Fraxinus* averaged −27.4±0.3‰ at both depths, and *Larix* averaged −27.8±0.5‰ at both depths. In both *Larix* (slope=−0.13‰/order, $r^2=0.15$, RMSE=0.42, $p=0.0002$) and *Fraxinus* (slope=−0.13‰/order, $r^2=0.43$, RMSE=0.20, $p<0.0001$) $\delta^{13}C$ decreased slightly with increasing root order (Fig. 2). The temporal variability of $\delta^{13}C$ did not differ by root order in either species (Levene's test $F>0.11$ for *Fraxinus*, Levene's test $F>0.50$ for *Larix*).

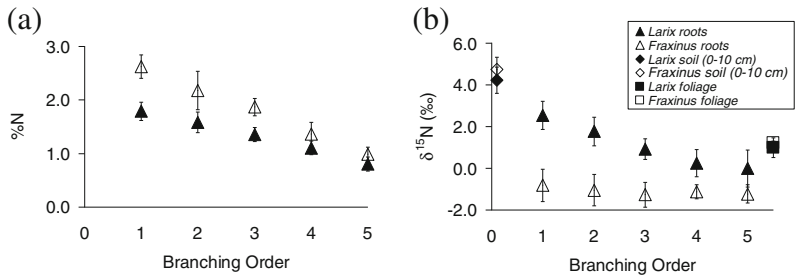

Fig. 1 **a** Nitrogen concentration (%N) by branching order in roots across all depths and dates of ectomycorrhizal *Larix gmelinii* (*solid triangles*) and arbuscular mycorrhizal *Fraxinus mandshurica* (*hollow triangles*). **b** Nitrogen stable isotopes ($\delta^{15}N$) across all dates in roots from 0 to 10 cm soil depth of ectomycorrhizal *Larix gmelinii* (*solid triangles*) and arbuscular mycorrhizal *Fraxinus mandshurica* (*hollow triangles*). $\delta^{15}N$ of soil from 0 to 10 cm (*diamonds*) and foliage (*squares*) are also shown. %N decreases with increasing root order in both species, with an average 0.2 % decrease per order in *Larix*, and a 0.4 % decrease per order in *Fraxinus*. $\delta^{15}N$ decreases with increasing root order more in *Larix* (slope=−0.66‰/order, $r^2=0.65$, RMSE=0.68, $p<0.0001$) than in *Fraxinus* (slope=−0.09‰/order, $r^2=0.03$, RMSE=0.59, $p=0.14$) consistent with higher proportion of ^{15}N-enriched fungal tissue on lower order roots of ectomycorrhizal species such as *Larix*

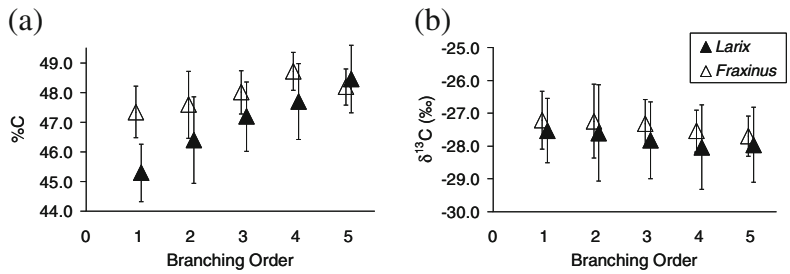

Fig. 2 **a** Carbon concentration (%C) by branching order in roots across all depths and dates of ectomycorrhizal *Larix gmelinii* (*solid triangles*) and arbuscular mycorrhizal *Fraxinus mandshurica* (*hollow triangles*). **b** Carbon stable isotopes ($\delta^{13}C$) composited across all dates and depths of ectomycorrhizal *Larix gmelinii* (*solid triangles*) and arbuscular mycorrhizal *Fraxinus mandshurica* (*hollow triangles*). %C increases with increasing root order, but more so in *Larix* (slope=0.87 %/order, r^2=0.49, RMSE=1.25, p<0.0001) than in *Fraxinus* (slope=0.29 %/order, r^2=0.19, RMSE=0.85, p<0.0001) consistent with higher proportion of low %C fungal tissue on lower order roots of ectomycorrhizal species such as *Larix*. In both *Larix* (slope=−0.13 ‰/order, r^2=0.15, RMSE=0.42, p=0.0002) and *Fraxinus* (slope=−0.13‰/order, r^2=0.43, RMSE=0.20, p<0.0001) $\delta^{13}C$ decreases slightly with increasing root order. Data for both species are for orders 1–5. Points are offset slightly to show error bars

Carbon concentration of roots ranged from 42.5 % to 50.1 % across both species and all dates, depths, and orders. *Fraxinus* varied less in C concentration (45.0 %–49.9 %) than did *Larix* (42.5 %–50.1 %, Levene's test F<0.0001). Across all depths and dates, %C increased with increasing root order, but more so in *Larix* (slope=0.87 %/order, r^2=0.49, RMSE=1.25, p<0.0001) than in *Fraxinus* (slope=0.29 %/order, r^2= 0.19, RMSE=0.85, p<0.0001) (Fig. 2).

Discussion

Nitrogen isotopes

Much of the variation in root $\delta^{15}N$ appears to be driven by the $\delta^{15}N$ of the soil horizon in which the roots reside and the type of mycorrhizal symbiosis. Root $\delta^{15}N$ increases with increasing soil depth in both species, and decreases with increasing root order more rapidly in ectomycorrhizal *Larix* than in arbuscular mycorrhizal *Fraxinus* roots (Fig. 1). $\delta^{15}N$ in plant tissues can record the $\delta^{15}N$ of the form of N used by plants (Robinson 2001), although soil $\delta^{15}N$ varies strongly with depth (Hobbie and Ouimette 2009). In this study, the $\delta^{15}N$ of roots increased as soil $\delta^{15}N$ increased but roots remained lower in $\delta^{15}N$ compared to soil in both species (Fig. 1). This ^{15}N depletion in roots compared to the surrounding soil can be explained by: 1) fractionation against ^{15}N during uptake, 2) a difference between the $\delta^{15}N$ of bulk soil and the $\delta^{15}N$ of available N, or 3) fractionation against ^{15}N during transfer of N from mycorrhizal fungi to plant hosts. All three mechanisms may contribute to the discrepancy between the $\delta^{15}N$ of soil and roots. Future studies measuring the $\delta^{15}N$ of available N should help elucidate these patterns.

Differences in $\delta^{15}N$ across root order appears to be largely a function of the variable proportion of fungal to plant biomass found across different root orders. The decrease in $\delta^{15}N$ with increasing root order (increasing diameter) across the first four orders was three times greater for ectomycorrhizal *Larix gmelinii* (−0.76‰ per order) than for arbuscular mycorrhizal *Fraxinus mandshurica* (−0.24‰ per order) (Fig. 1), reflecting greater fungal biomass on ectomycorrhizal than on arbuscular mycorrhizal roots. Data from seven ectomycorrhizal and ten arbuscular mycorrhizal temperate/boreal forest tree species from Kong et al. (manuscript in preparation) also support this trend, with an average change in $\delta^{15}N$ with increasing order of −0.72‰ per order in ECM species compared to −0.32‰ per order in AM species (across the first 4 orders).

The probable mechanism driving this pattern is the change from relatively large amounts of ^{15}N-enriched fungal tissue on ectomycorrhizal 1st order roots to the lack of fungal tissue on 4th order and higher roots. Typically, the proportion of fungal matter is higher on ectomycorrhizal roots than on arbuscular mycorrhizal roots (Hobbie 2006; Smith and Read 2008). ECM fungi are generally enriched in ^{15}N compared to their

plant hosts (Hobbie and Colpaert 2003; Taylor et al. 2003) and the $\delta^{15}N$ of fungal mycelia tend to closely track the $\delta^{15}N$ of the soil layer in which they are found (Wallander et al. 2004; Boström et al. 2007). Very few studies report the $\delta^{15}N$ of arbuscular mycorrhizal fungal material compared to plant hosts. Courty et al. (2011) reported that AM spores were 3‰ higher in $\delta^{15}N$ (although often not significantly) compared to AM roots. Additionally, although AM fungi generally lack macroscopic structures and evidence for ^{15}N patterns between AM fungi and plant hosts is scarce, the higher $\delta^{15}N$ in most AM plants relative to co-occurring ECM plants also suggests that ^{15}N fractionation by AM fungi is less than in ECM fungi (Craine et al. 2009).

Further support that ^{15}N-enriched ECM fungal tissue is driving $\delta^{15}N$ shifts across root orders is that mycorrhizal fungi only colonized the first three orders in the ectomycorrhizal *Larix* root samples (Guo et al. 2008c). With this colonization pattern, we would expect that the change in $\delta^{15}N$ with root order would be large across the first 4 orders (where the ratio of fungal to plant material is decreasing), and minimal between the 4th and 5th orders where there is no fungal colonization. Across all dates and depths the change in $\delta^{15}N$ over the first 4 orders of *Larix* is 0.76‰ per order, while between 4th and 5th order roots there is no change (0.03‰, $p=0.92$). The similarity in the $\delta^{15}N$ of 4th and 5th order roots suggests that intra-plant fractionation is minimal (at least across root orders), and supports findings of minimal intra-plant fractionation of nitrogen isotopes in nonmycorrhizal tissues observed elsewhere (Hobbie and Colpaert 2003; Hobbie et al. 2008). Since soil and root $\delta^{15}N$ vary with soil depth, then, this suggests that comparing an integrated plant N pool, such as foliar $\delta^{15}N$, to 4th and 5th order root $\delta^{15}N$ at various depths may provide information on the average depth of N assimilation.

Fungal biomass

Despite considerable progress in modeling ecosystem C and N dynamics, the lack of data on belowground fungal biomass and production has prevented models from explicitly including mycorrhizal fungi. Both anatomical and isotopic measurements of different root orders can be used to estimate the proportion of fungal biomass and productivity in ectomycorrhizal species. For example, in the present study, only the first three root orders of *Larix* were colonized by ECM fungi (Guo et al. 2008c). Assuming that 5th order roots represent non-mycorrhizal tissue (which was found for all ECM species in Guo et al. 2008c) we can use ^{15}N isotopic mass balance to estimate the proportion of N, C, and biomass that is fungal tissue on lower root orders using Eqs. 1, 2 and 3 (see Methods).

Since many of these parameters were not measured directly, Monte Carlo simulations were performed with a range of input parameter estimates (see Methods). The results of these isotopic mass balance simulations estimate that in ECM *Larix* about 54 % of root N and 39 % of root biomass is found in the fungal sheath for 1st order roots, with decreasing contributions of fungal material on higher root orders (Table 3, Fig. 3). Anatomical estimates of fungal sheath biomass independently confirm these estimates. We employed Monte Carlo simulations with a range of input parameter values (see Methods for details), and estimate that 33 % of root biomass is found in the fungal sheath for *Larix* 1st order roots, with decreasing contributions of fungal material on higher root orders (Table 3, Fig. 3). Across all root orders isotopic and anatomical measurements compare reasonably well (Fig. 3, Table 3) highlighting the contribution ECM fungi make to the chemistry and anatomy of the finest root orders. These estimates also compare well with data from a range of studies using anatomical estimates of fungal sheath biomass on root tips (average of 31 % fungal biomass on first-order roots, summarized in Supplementary Table 1).

Although fungal biomass on *Fraxinus* roots cannot be estimated anatomically (AM fungi do not form visible fungal sheaths on host roots), fungal biomass was estimated isotopically. Courty et al. (2011) reported that AM spores were 3‰ enriched in ^{15}N compared to host roots but other useful information is lacking. Here, we allowed AM fungal $\delta^{15}N$ to vary around measured soil $\delta^{15}N$ similar to ECM fungal $\delta^{15}N$. Under this assumption, isotopic mass balance calculations for arbuscular mycorrhizal *Fraxinus* estimate that AM fungal tissue contributes minimally to total root biomass (5 %, 2 %, 0 %, 0 %, 0 % for 1st through 5th orders, respectively).

In a sensitivity analysis of both isotopic and anatomical approaches, results are sensitive to parameters that are relatively easy to measure or estimate. For example, isotopic mass balance calculations are fairly insensitive to the C/N ratios of the fungal and plant components of root tissue (which would be difficult to

Table 3 Mean root $\delta^{15}N$, root diameter, fraction of fungal colonization and the fraction of total root nitrogen, carbon, and biomass that is fungal by root order as estimated by isotopic and anatomical methods across all dates in the 0–10 cm soil depth for *Larix gmelinii*. See Methods for details on input parameter values used in calculations

Order	Mean root $\delta^{15}N$ (‰)	Mean root diameter (mm)[a]	Fraction fungal colonization[a]	Fraction root N as fungal N[b,c]	Fraction root C as fungal C[b,c]	Fraction root as fungal biomass isotopic[b,c]	Fraction root as fungal biomass anatomical[b,d]
1	2.54	0.25	1.0	.54±.09	.35±.09	.39±.10	.33±.06
2	1.83	0.35	1.0	.37±.08	.18±.05	.21±.06	.26±.03
3	0.92	0.45	0.3	.20±.07	.07±.02	.09±.02	.07±.01
4	0.24	0.55	0.0	.05±.07	.01±.02	.01±.02	.004±.002
5	0.00	1.45	0.0	0.00	0.00	0.00	0.00

[a] From Guo et al. 2008c

[b] See Methods for values of input parameters used in simulations

[c] Calculated using isotopic mass balance

[d] Calculated using anatomical measurements

separate and measure) but instead are most sensitive to root order $\delta^{15}N$ (measured) and fungal $\delta^{15}N$, for which soil $\delta^{15}N$ is a good proxy (Table 2a). Anatomical estimates of fungal biomass on roots are most sensitive to root diameter (measured) and fungal sheath thickness, for which there are fairly well constrained literature value estimates (Table 2b).

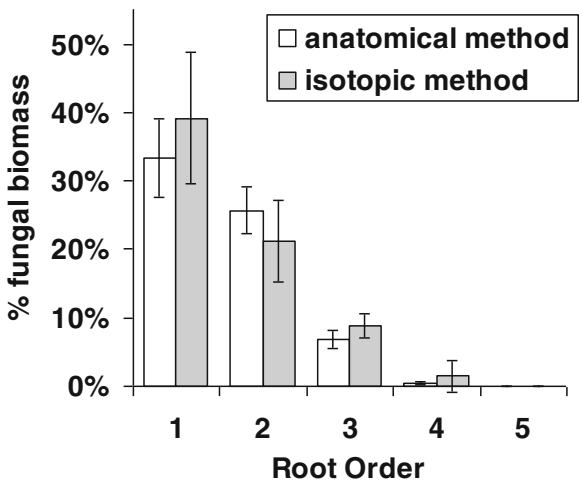

Fig. 3 The percent of total root biomass that is fungal by root order as estimated by anatomical and isotopic methods across all dates in the 0–10 cm soil depth for *Larix gmelinii*. *White bars* are estimates using anatomical methods while *gray bars* are using isotopic calculations. The point estimates using initial "best guess" parameter values are not shown but were nearly identical to estimates from Monte Carlo simulations (*top of the columns*). *Error bars* from Monte Carlo simulations are reported as median absolute deviation

In ECM *Larix*, further support that fungal sheaths drive root chemistry may come from patterns in %C across root order. The minimal change in %C across root order (Fig. 2) in arbuscular mycorrhizal *Fraxinus* (1st order=47.3 %, 5th order=48.2 %, $p=0.022$) contrasts with the larger change in %C across root order in ectomycorrhizal *Larix* (1st order=44.9 %, 5th order=48.5 %, $p<0.0001$). This agrees with data from Goebel et al. (2011) who found minimal change in %C of 1st + 2nd order roots compared to 3rd + 4th order roots in arbuscular mycorrhizal *Acer* (48.8 and 49.3 % C, respectively), and larger changes in two ectomycorrhizal gymnosperms (average 44.9 and 47.2 % C for 1st + 2nd order roots and 3rd + 4th order roots, respectively). Interestingly, an ECM angiosperm measured by Goebel et al. (2011) differed little in %C in 1st + 2nd order roots compared to 3rd + 4th order roots. This could be due to the presumably lower fungal colonization found in ECM angiosperms (Smith and Read 2008).

The smaller %C of lower root orders may be partially driven by the low %C of fungal tissue. Pure fungal hyphal tissue from ten species of cultured fungi averaged 40.8±3.6 %C ($n=36$; Data from Hobbie and Colpaert 2004), and pure fungal cap and stipe tissue from 139 species of field collected sporocarps averaged 41.8±2.6 % and 39.0±2.1 %, respectively ($n=339$; Hobbie et al. 2012). The low %C of fungal tissue compared to the higher %C of non-mycorrhizal root material should partially account for the smaller %C in lower root orders of *Larix*.

Assuming fungal tissue is 40.8 % C and plant tissue is similar to 5th order roots (48.5 % C), then mass balance calculations necessitate that about 50 % of 1st order roots in *Larix* (44.9 % C) be fungal carbon if patterns in %C are totally fungal driven. This estimate is higher than our isotopic and anatomical derived estimates of fungal biomass on first order roots of 33–39 %. A large part of the discrepancy in the proportion of fungal biomass estimated using %C data likely arises from assuming plant tissue of 1st and 5th order roots are chemically identical with similar C concentrations. Instead, in addition to the presence of fungal material, plant tissue itself likely changes chemically across root orders. For example, *Fraxinus* roots which have little to no fungal biomass, have similar 5th order root %C (48.2 % C) as *Larix* (48.5 %C), while 1st order roots have only 47.4 % C. If we assume plant tissue of 1st order *Larix* roots is similar to 1st order *Fraxinus* roots, our estimate of fungal biomass using %C data is 38 %, within the range predicted by isotopic and anatomical approaches.

Changes in %C by root order are probably driven by both shifts in the fungal contribution and shifts in root tissue chemistry. Lower order roots are more N-rich with higher concentrations of proteins (44–46 % C). Carbohydrates (e.g. glucose, cellulose, starch) also have relatively low C concentrations (40–44 %) while lignin (>60 % C) and lipids or waxes such as suberin (>70 % C) have relatively high carbon concentrations. The increase in %C of higher order roots to greater than 48 % C is therefore likely driven by an increase in the proportion of C-rich lipids, suberin, and lignin (Goebel et al. 2011) as well as the lack of C-poor fungal tissues.

With measurements of root biomass and root production for the first five branching orders we can appreciate the contribution of fungal tissues to fine root systems. We used the pattern of root biomass by root order in *Larix gmelinii* measured at these same sites by Wang et al. (2006), to estimate that fungal sheath tissue contributes nearly 17 % to total fine root biomass across the first four root orders. Scaling estimates of fungal sheath biomass to predictions of ECM fungal sheath production, however, requires data on the production of individual root orders, and only a limited number of studies contain data on the production of individual root orders. Xia et al. (2010) is one of the few studies that document root production by root order (in arbuscular mycorrhizal *Fraxinus mandshurica* at these sites). Although they report on an AM species, using their estimates of fine root production by root order and our estimates of the proportion of fungal biomass on lower root orders, we can estimate the amount of fungal production that typically is included as "fine root" production. According to Xia et al. (2010), 54 % of fine root production was in 1st order roots, 18 % in 2nd order, and 15 % in 3rd order roots. Using the distribution of root production across root order from Xia et al. (2010) and our estimates of the proportion of fungal biomass across root order we estimate that nearly 25 % of measured fine root production is actually fungal sheath production in ECM species such as *Larix* (although data on root production by branching order are needed for ECM species). At these sites fine root production (<1 mm, which includes the first four root orders) of *Larix* (20–165 g/m^2) varies with the method used (Mei et al. 2010) and is generally lower than fine root production of 310 g/m^2/year in temperate forests globally (summarized from Litton and Giardina 2008). Using the upper estimates of *Larix* fine root production at these sites, 41 g/m^2/year of the 165 g/m^2/year of root production would be fungal sheath production. This amount of fungal sheath production is similar in magnitude to the production of fungal mycelia in soil in forests (42–59 g/m^2/year) reported by Wallander et al. 2004. This means total fungal production (sheaths, mycelia, and reproductive structures) could be similar in magnitude to fine root production, although care must be taken when attempting to scale these type of fungal measurements to predictions of fungal ecosystem production (Hobbie 2006).

Root $\delta^{13}C$ dynamics

The small enrichment in ^{13}C with decreasing root order (Fig. 2b) followed established patterns that heterotrophic tissues more distal from foliage should be more ^{13}C-enriched (Badeck et al. 2005; Cernusak et al. 2009; Hobbie and Werner 2004). However, root $\delta^{13}C$ patterns across branching order are undoubtedly more complex and driven by changes in the proportion of different carbon compounds (suberin, cellulose, lignin, starch, etc.) over time and across different branching orders as well as progressive enrichment in ^{13}C along transport pathways.

Despite this potential complexity, because the $\delta^{13}C$ of photosynthate varies seasonally, we expected more variation in the $\delta^{13}C$ of short-lived lower order roots

than longer-lived higher order roots (Joslin et al. 2006; Majdi et al. 2001; Sah et al. 2011). Instead, $\delta^{13}C$ varied little across time and depth, and only minimally across root order. These results are in contrast to some recent findings. For example, Marron et al. (2009) demonstrated that $\delta^{13}C$ of soil respiration is influenced by recent climatic conditions, indicating rapid movement of photosynthate to belowground components. Carbon isotope labeling studies have also shown that recently fixed carbon is transported within a few days to growing root tips and mycorrhizal fungi (Högberg et al. 2008; Keel et al. 2006; Staddon et al. 2003). Still, other research has highlighted the importance of stored carbon reserves in the construction of heterotrophic plant parts such as roots (Gaudinski et al. 2001, 2009; Helle and Schleser 2004). The homogeneity of $\delta^{13}C$ across time, depth, and root order suggests that root construction is from a well mixed pool of plant C. Future work on the $\delta^{13}C$ of different compound classes in roots by root order are needed to fully understand controls of root $\delta^{13}C$ over time and across branching order.

Conclusion

Studies examining the dynamics of fine roots are moving away from the traditional designation of fine roots as all roots less than 2 mm in diameter. Studying fine roots by branching order should provide more accurate estimates of root production and expand our understanding of root function. This is the first study to report carbon and nitrogen isotope patterns across root order. In the present study, variation in $\delta^{15}N$ across root order is largely driven by mycorrhizal type and the presence or absence of fungal biomass on different root orders. The increase in root $\delta^{15}N$ with decreasing branch order in ectomycorrhizal *Larix* indicates that over 50 % of nitrogen and 36 % of the biomass of 1st order roots is found in the fungal sheath, with smaller contributions of fungal material on higher root orders. When scaling to estimates of ecosystem production, the proportion of fungal sheath material equates to nearly 25 % of fine root production and may be of similar magnitude to the production of fungal mycelium in soil. Despite recent studies showing a close connection between recent photosynthate and both root respiration and fungal fruiting, $\delta^{13}C$ data in this study imply that construction of fine roots is primarily from a large and well-mixed pool of plant carbon. The homogeneity in root $\delta^{13}C$ and variability typically seen in photosynthate and respired $\delta^{13}C$ may indicate that carbon supply for root construction and maintenance respiration are largely uncoupled. More measures of root production by branching order could help to explicitly partition belowground carbon allocation between roots and mycorrhizal fungi.

Acknowledgments Support for this project came from a grant awarded by the US Department of Energy (DOE). The manuscript was improved by critical reviews from Luke McCormack and Lucie Lepine.

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