The size and the age of the metabolically active carbon in tree roots

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Abstract
Little is known about the sources and age of C respired by tree roots. Previous research in stems identified two functional pools of non-structural carbohydrates (NSC): an “active” pool supplied directly from canopy photo-assimilates supporting metabolism and a “stored” pool used when fresh C supplies are limited. We compared the C isotope composition of water-soluble NSC and respired CO2 for aspen roots (Populus tremula hybrids) cut off from fresh C supply after stem-girdling or prolonged incubation of excised roots. We used bomb radiocarbon to estimate the time elapsed since C fixation for respired CO2, water-soluble NSC and structural α-cellulose. While freshly excised roots (mostly <2.9 mm in diameter) respired CO2 fixed <1 year previously, the age increased to 1.6–2.9 years after root excision. Freshly excised roots from trees girdled ~3 months ago had respiration rates and NSC stocks similar to un-girdled trees but respired older C (~1.2 year). We estimate that over 3 months NSC in girdled roots must be replaced 5–7 times by reserves remobilized from root-external sources. Using a mixing model and observed correlations between Δ14C of water-soluble C and α-cellulose, we estimate ~30% of C is “active” (~5 mg C g−1).

KEYWORDS
fine roots, girdling, isotopic fractionation, nonstructural carbohydrates, phosphoenolpyruvate carboxylase (PEPC), radiocarbon (14C), respiration, storage dynamics, tree carbon dynamics, δ13C

INTRODUCTION
Fine root (≤ 2 mm) biomass is estimated at 40.8 Pg C globally, about 7% of total vegetation carbon (C) (Ciais et al., 2014; Jackson, Mooney, & Schulze, 1997). The proportion of C allocated belowground for growth, respiration, exudation, root connection and mycorrhizal associations are larger, 25–63% of assimilated C in forest ecosystems (Litton, Raich, & Ryan, 2007). These functions are concentrated in fine (typically <1 mm diameter) roots that respire the highest C per unit mass (Pregitzer, Laskowski, Burton, Lessard, & Zak, 1998), conduct root exudation and mycorrhizal interactions and have high mass turnover rates (Gaudinski et al., 2010). Nonstructural carbohydrates (NSC, mostly soluble sugars and starch) are known to fuel these functions with C and energy. Stored NSC reserves can support cellular functions when current C supply is insufficient (Chapin III, Schulze, & Mooney, 1990; Dietze et al., 2014).

The “bomb-radiocarbon” approach permits quantification of respiration sources and NSC dynamics in mature trees by tracing excess 14C created during atmospheric nuclear weapons tests that nearly doubled background values of radiocarbon signature (Δ14C) of...
atmospheric CO$_2$ ($\Delta^{14}$C$_{atm}$) in the early 1960s (Trumbore, Sierra, & Pries, 2016). Since then, decline in $\Delta^{14}$C$_{atm}$ reflect uptake of bomb- radiocarbon into the oceans and terrestrial biosphere, and dilution by radiocarbon-free CO$_2$ originating from fossil fuel emissions (Levin & Heshshaimer, 2016). Since 1964, an annual unique $\Delta^{14}$C$_{atm}$ signature is transferred to biomass C via photosynthesis. A comparison of the $\Delta^{14}$C signature of any C pool in plants to the current year’s $\Delta^{14}$C$_{atm}$ thus provides a means to estimate the mean time elapsed since the C was fixed.

Plants use soluble sugars both to transport C (predominantly sucrose) and as primary substrates in cellular metabolism (e.g. glucose and fructose). Starch is insoluble and thus cannot be translocated but can serve as local storage. These different properties are often mistakenly implied to reflect differences in turnover time, with sugars are assumed to comprise a fast-cycling pool and starch a slow-cycling pool (Dietze et al., 2014). However, in tree stems and large roots no systematic differences in radiocarbon signatures have been observed between the water-soluble (representing sugars) and insoluble (starch) C fractions, suggesting fast C exchange between the two pools (Richardson et al., 2013; Richardson et al., 2015; Trumbore, Czimczik, Sierra, Muhr, & Xu, 2015).

Nevertheless, NSC pools are not uniform in age. The $\Delta^{14}$C of stem respired CO$_2$ ($\Delta^{14}$C$_{resp}$) was lower than the water-soluble $\Delta^{14}$C ($\Delta^{14}$C$_{sw}$) in the outermost 2 cm of the stem, reflecting age differences of ~10 year (Carbone et al., 2013). Combined with evidence from $^{13}$C-labelling of fresh photo-assimilates from the canopy, it is clear that some young sugars support respiration, while other, older, sugars are stored intact (Epron et al., 2011). Accordingly, C allocation models usually distinguish two functional NSC-sub pools: a “fast” pool of recently fixed C (assumed age < 1 year) supporting metabolism and respiration, and a “slow” NSC pool for storage (Herrera-Ramirez et al., 2020; Richardson et al., 2015). However, we are still missing ways to quantify and differentiate between these two pools.

Measuring $\delta^{13}$C can provide information about the substrate and the metabolic pathways in respiration. Variations in the $\delta^{13}$C signature of produced sugars are primarily controlled by photosynthetic fractionation at the leaf level (Farquhar, Oleary, & Berry, 1982). “Post-photosynthetic fractionations” during transport, biosynthesis, and respiration further modify $\delta^{13}$C signatures (Badeck, Tcherkez, Nogues, Piel, & Ghashghaie, 2005; Ghashghaie et al., 2003; Werner & Gessler, 2011). Starch is usually slightly enriched (~1‰) while lipids are more depleted (~5‰) in $^{13}$C compared to sugars (Bowling, Pataki, & Randerson, 2008), and are expected to accordingly affect the $\delta^{13}$C signature of the respired CO$_2$ ($\delta^{13}$C$_{resp}$) when decarboxylated. The “apparent isotopic fractionation” of respiration ($\Delta_R$) is defined as the difference in $\delta^{13}$C between the putative respiratory substrate (usually sugars) and $\delta^{13}$C$_{resp}$ (Ghashghaie et al., 2003). For roots of woody C$_3$ plants mainly negative $\Delta_R$ values ($\delta^{13}$C$_{substrate} < \delta^{13}$C$_{resp}$) have been reported (Ghashghaie & Badeck, 2014). The $^{13}$C enrichment in respired CO$_2$ is commonly explained by the dominance of CO$_2$ emitted from decarboxylation of pyruvate via pyruvate dehydrogenase (PDH) over CO$_2$ emitted from the tricarboxylic acid (TCA) cycle reactions (Tcherkez et al., 2003). The activity of phosphoenolpyruvate carboxylase (PEPC), which re-fixes CO$_2$ to replenish TCA cycle intermediates, was suggested to decrease respired CO$_2$ enrichment and increase $\Delta_R$ (Gessler et al., 2007; Ghashghaie & Badeck, 2014). To our knowledge, only one study to date has evaluated $\Delta_R$ in roots of mature trees (Gessler et al., 2007).

Here, we investigated the sources of respiration for fine and coarse roots of mature temperate aspen trees (Populus tremula hybrids). We performed a stem-girdling experiment (complete circumferential removal of bark, cambium, and phloem), terminating below-ground transport of fresh photo-assimilates and forcing mobilization of storage NSCs for survival beneath the girdling. Girdled Amazonian tree stems quickly shifted from using current year photosynthetic products as respiratory substrates in un-girdled trees to C fixed ~5 year previously a month after girdling (Muhr, Trumbore, Higuchi, & Kunert, 2018). We expected increases in $\Delta^{14}$C$_{resp}$ for roots of girdled trees, but with potentially smaller magnitude, since trees from the genus Populus often have root connections that enable C transfer from healthy trees (Gaspar & DesRochers, 2020; Pregitzer & Friend, 1996). To characterize the usage of NSC reserves in the roots themselves we conducted additional experiments where the age of C respired from detached roots was followed for a week. This experiment is also the base for our first approach to estimate the size and isotopic signature of the fast-cycling “active” C pool that feeds respiration and metabolism. The relationship between $\Delta^{14}$C$_{atm}$ and the $\Delta^{14}$C signature of the $\alpha$-cellulose ($\Delta^{14}$C$_{cell}$ representing structural C) allowed us to develop the second approach to estimate the size and isotopic signatures of the “active” C, as well as the slow-cycling “stored” C pool.

2 | MATERIALS AND METHODS

2.1 | Study site and experimental design

We sampled 12 Eurasian aspen trees (Populus tremula hybrids) with estimated age of 60–70 year growing in a forest stand located on a slope of the Großer Hermannsberg Mountain (867 m a.s.l), Germany (50°42’50” N, 10°36’13” E, site elevation 616 m a.s.l). Soils at the site are developed on volcanic parent rock, mean annual temperature is ~7°C and annual rainfall is 800–1,200 mm (Bouriaud, Marin, Bouriaud, Hessenmoller, & Schulze, 2016). During our field campaign in the summer of 2018, an extreme “hot drought” occurred in central Europe (Bastos et al., 2020), also observed in a nearby weather station situated 812 m a.s.l (https://www.bgc-jena.mpg.de/freiland/index.php/Sites/Hermannsberg). During the 2018 growing season (May and October), mean monthly temperatures were warmer by 1.5–3.5°C than those for the years 2010–2017, while total monthly rainfall was 260 mm, compared to the average of 323 mm in the years 2010–2017 (Figure 1).

The size of the forest stand where our study took place is ~40 x 40 m$^2$ with ca. 20 mature and 50 younger trees. Control and girdled trees were all mature with diameter at breast height of 87–129 cm, and interspersed. Distances between girdled trees and the
Mean monthly rainfall (mm, bars) and air temperature (°C, circles) during 2018 growing season, and in the previous 8 years (2010–2017) in the study site (Großer Hermannsberg, Germany). Error bars for the 2010–2017 data represent one standard deviation from the mean closest un-girdled trees, including unstudied trees, were 1.2–5.8 m. After sampling all 12 trees on 26 June and 4 July, six of the trees were girdled on July 4th by removing a ~4 cm wide band of bark, cambium and phloem from the stem 1.5 m above the ground. During subsequent samplings (25 September and 2 October), we differentiated “Girdling” and “Control” trees.

To ensure we sampled roots specific to the chosen trees, we tracked their connections back to the main root or stem. Roots were collected from the top 10 cm of the mineral soil. We aimed at finding two root clusters for each tree with total mass of several grams. In most trees, we harvested individual roots growing directly from large lateral roots to gain the desired mass. Hence, a sample from a single tree usually combined roots from several spatial locations. The samples were put on ice immediately after harvest until analysis. On the subsequent day, the roots were thoroughly washed to remove any remaining soil particles and then separated into two size classes: coarse roots (> 2 mm, mean 2.9 mm, max 6 mm) and fine roots (≤ 2 mm, almost all were suberized); each size fraction consisted of roots from different orders, and not necessarily from the same root cluster. Each size class was split for (a) NSC and α-cellulose extractions, and (b) respiration incubations (details below).

Incubations of excised roots lasted for 2 days and integrated CO₂ respired between the start of incubation (time 0) to day 2. To test roots’ ability to utilize their own C reserves for metabolism during C starvation, we made additional incubations that sampled integrated respiration between days 6 and 8. We report these using the mean incubation times (e.g., days 1 and 7). In addition, we performed short-term incubations (up to 1.5 hr) on days 0, 2 and 6 to measure the respiratory quotient (RQ), that is, the ratio CO₂ efflux/O₂ influx. The RQ is mainly defined by the respiratory substrate, which in plants is assumed to be carbohydrates with RQ = 1. Compounds more oxidized than carbohydrates like organic acids are expected to yield RQ > 1, whereas amino acids and lipids yield RQ values of 0.9 and 0.7, respectively (Masiello, Gallagher, Randerson, Deco, & Chadwick, 2008). Thus, RQ measurements can indicate the substrate used for respiration. For the correction of CO₂ efflux rates measured at laboratory temperatures (22°C) to field temperatures, we conducted short-term incubations at different temperatures to calculate Q₁₀, the factor by which CO₂ efflux increases with 10°C warming.

Because regional fossil fuel emissions have the potential to affect Δ¹⁴Cres from the girdled and control trees. Two stem cores from nearby tree were extracted in 2019 using a 5.15 mm increment borer. We visually identified the annual rings for the last 9 years and sampled their outermost halves, which presumably are dominated by latewood produced during midsummer-autumn mainly from C fixed in the current growing season, thus approximating the current year’s Δ¹⁴CO₂ atm (Kudsk et al., 2018; Pilcher, 1995). Samples from identical rings from both stem cores were pooled for α-cellulose extraction and Δ¹⁴Ccell analysis (Hopper, McCormac, Hogg, Higham, & Head, 2016). In addition, assuming leaves respire C fixed recently, we analysed Δ¹⁴CleafCO₂ atm with direct measurements of atmospheric air in different sites in Europe that are part of ICOS (the Integrated Carbon Observation System, see Table S1 for PID numbers).

### 2.2 NSC analysis

Aliquots of 50 mg oven-dried root tissue (±2 d at 60°C), were analysed for (a) sugars and starch concentrations, (b) Δ¹⁴Cres, (c) δ¹³Cws, and (d) Δ¹⁴Ccell. Since Δ¹⁴Cws is usually correlated with the age of the containing tissue (Furze et al., 2020; Richardson et al., 2015; Trumbore et al., 2015), for correct interpretation of Δ¹⁴Cres variance it is important to account to the root’s mean C age estimated using Δ¹⁴Ccell. Therefore, we coarsely cut rather than milled the roots since in the α-cellulose extraction the sample is rinsed with reagents through 16–40 µm mesh that might allow fine material to pass and clog the system’s tubing (Steinhof, Altenburg, & Machts, 2017). We decided only after the pre-girdling campaign to measure starch concentration, hence data are available only for roots from the girdled and control trees.

The methods for NSC, sugar and starch extractions are based on protocols S1 and S2 from Landhausser et al. (2018) with some modifications necessary for minimizing extraneous C additions that would affect the 14C measurement. We avoided plastic vials that contain C and used only glass vials that were pre-baked at 550°C to eliminate any C residuals. For sugar extraction we used water as a solvent instead of ethanol, which is slightly more efficient (Landhausser et al., 2018), to avoid possible C addition from the ethanol. To reduce starch dissolution in water, the extraction temperature was lowered from 90°C to 65°C. Extraction of water-soluble C from the 50-mg samples was carried out by shaking the samples in 5 ml deionized water for 10 min (65°C), in three repetitions. After each repetition the vials were cooled, centrifuged and the supernatant was transferred to
a glass vial kept on ice (to slow-down microbial degradation). A sub-sample of 2 ml from the total 15 ml was used for quantification of the sugars sucrose, glucose and fructose. For the Δ¹³C and Δ¹⁴C analysis, the rest of the solution was concentrated by freeze-drying and pipetted into tin capsules for Δ¹³C analysis, and into pre-baked silver capsules for Δ¹⁴C analysis. The starch in the pellet from the water extraction was converted by α-amylase (Sigma cat. no. A4551) into water-soluble glucans and then to glucose by amyloglucosidase (Sigma cat. no. ROAMYGLL) (Landhausser et al., 2018). The remaining pellet was used for Δ¹⁴Ccell analysis (Steinhof et al., 2017).

To measure the concentrations of the soluble sugars and the glucose hydrolysate from the starch digestion we used high-performance anion-exchange chromatography with pulsed amperometric detection (HPLC-PAD) device equipped with autosampler (Dionex® IC 3000, Thermo Fisher GmbH, Idstein, Germany) (Raessler, Wissuwa, Breul, Unger, & Grimm, 2010). The starch and sugar concentrations are calculated as glucose-equivalent weight per sample dry weight (mg glucose g⁻¹) (Landhausser et al., 2018), and further multiplied by 0.4 to express as C mass per dry weight (mg C g⁻¹).

2.3 | Respiration measurements

2.3.1 | Two-day incubations

Root samples (0.3–1.2 g dry weight) were incubated in gas-tight Plexiglas cylinders equipped with fittings (12.7 mm Swagelok Ultra-Torr) on each side for attaching sampling flasks (115 ml) equipped with a Louwers™ O-ring high-vacuum valve (Louwers-Hanique, Hapert, Netherlands) (Muhr et al., 2018). Total system volume was 269 ± 10 ml. Before incubation, the headspace containing the washed roots was flushed with synthetic air (0% CO₂, 20% O₂) for 3 min in order to remove any inherited CO₂. Then the set-up was closed quickly by connecting two flasks (pre-filled with the same synthetic air) at both ends of the chamber. Incubations were conducted at room temperature (22°C) in the dark and ended by closing the flask valves. Leaf incubations aimed to estimate the local Δ¹⁴CO₂stem used the same setup and lasted 1 day.

Incubations were conducted at room temperature that was higher than average field soil temperatures. While direct effects on substrate identity and thus age (Δ¹⁴Cresp) are not expected initially, higher temperatures could lead to faster depletion of "fast" sources and thus ageing of the substrate pools, expected to affect both Δ¹⁴Cresp and Δ¹³Cresp (Tcherkez et al., 2003). In addition, the temperature-induced metabolic change can affect Δ¹³Cresp directly reflecting temperature sensitivity of the processes emitting CO₂ (Kodama et al., 2008). Thus, the Δ¹³Cresp values should be regarded as room-temperature acclimated values and not extrapolatable to field values.

2.3.2 | Short-term incubations

Short-term incubations were done in a solid aluminium chamber equipped with an NDIR CO₂ sensor (COZIR 0%-1% CO₂ Sensor, CO₂Meter, Inc., Ormond Beach, FL) and a quenching-based O₂ sensor (LuminOx, Coatebridge, UK). CO₂ and O₂ fluxes were calculated by linear fit of the concentration change with time, where the slope is equivalent to the term ΔCO₂/i in Equation (2). Incubations with R² < 0.9 in any of the gases were discarded.

Flask-measured CO₂ efflux rates (measured at room temperature) were corrected to field (in situ) temperatures using Q₁₀ values estimated using incubations of roots collected in the September–October at two or three different temperatures (ranging between 5°C and 22°C). We used the R package respirometry that fits the measured CO₂ efflux (R) at a given temperature (T) with the Equation \( R = a \times e^{b \times T} \) and calculates Q₁₀ = \( e^{10 \times b} \). We assume the computed Q₁₀ value is also valid for the June–July campaign following Burton and Pregitzer (2003) who observed little to no seasonal temperature acclimation in fine roots. Soil temperature in the top 10 cm where the roots were collected is coupled to changes in air temperature with some lag time (Brown, Pregitzer, Reed, & Burton, 2000). To estimate air temperature at the site we added 1.6°C to the measured temperature at the weather station due to altitude difference (196 m) and assumed mean lapse rate of 0.8°C every 100 m. Soil temperature (0–10 cm) was estimated as the average air temperature over the previous 7 days.

2.4 | Radiocarbon analysis

For radiocarbon analysis, respired CO₂ from the flasks or CO₂ from combusted solid samples was cryogenically purified and graphitized on iron in the presence of H₂ at 550°C (Muhr et al., 2018; Steinhof et al., 2017). The graphitized samples were analysed by accelerator mass spectrometry (AMS; Micadas, Ionplus, Switzerland) in the radiocarbon laboratory in Jena, Germany (Steinhof et al., 2017). Radiocarbon data are expressed as Δ¹³C‰ and calculated according to Trumbore et al. (2016):

\[
Δ^{14}C = \frac{R_{25}}{0.95 \times R_{oxalic-19} \times e^{2.5 \times 1980/8267}} \times 1,000, \tag{1}
\]

where \( R_{25} \) is the sample's \(^{14}C/^{12}C\) ratio corrected for mass dependent fractionation by normalizing the sample's \(^{13}C\) to a \(^{13}C\) of −25‰. \( R_{oxalic-19} \) is the \(^{14}C/^{12}C\) ratio in the standard, oxalic acid, normalized to \(^{13}C\) of −19‰, and the 0.95 term converts to the absolute radiocarbon standard (1890 wood) activity in 1950. The exponent corrects for decay of \(^{14}C\) in the standard between 1950 and the year of measurement (x), to provide the absolute amount of \(^{14}C\) in our samples. For individual tree rings \( x \) matched the estimated year.

The measurement precision for each AMS measurement of \(^{14}C\) was 2–3‰. The estimated mean age of measured C is calculated from the difference between its \(^{14}C\) value and the \(^{14}C_{atm}\) during the 2018 growing season, divided by the mean annual decline in \(^{14}C_{atm}\) (see Equation [5] in Results).

Our samples were assumed to have \(^{14}C\) ≤ \(^{14}C_{atm}\); therefore, samples with \(^{14}C\) < \(^{14}C_{atm}\) were considered contaminated with extraneous fossil C. One batch of nine samples was discarded due to many results with highly negative values.
2.5 | CO₂ efflux rate and δ¹³C analysis

The CO₂ concentration and δ¹³C in the two-day incubations were determined from a sub-sample of air from one flask analysed by Isotope Ratio Mass Spectrometer (IRMS; Delta+ XL; Thermo Fisher Scientific) coupled to a modified gas bench with Conflow III and GC (Thermo Fisher Scientific, Bremen, Germany). Air from the flask was expanded into a pre-evacuated 12-ml Labco extainer (Labco Ltd, Lampeter, UK) equipped with a septum cap. Argon gas (Ar) was added to the extainer to create a small over pressure to enable passive sampling by the auto-sampler (CTC Combi-PAL autosampler, CTC-Analyt- ics, Zwingen, Switzerland). The air pressure values in the extainer before and after the Ar addition were recorded for pressure correction. Each extainer was sampled twice (30 or 50 µl per aliquot) by the auto-sampler and the aliquots were injected into the IRMS. Samples were analysed against a laboratory air standard on the Vienna Pee Dee Belemnite (VPDB) scale [Jena Reference Air Set-06 (JRA- 06), (Wendeberg, Richter, Rothe, & Brand, 2013)]. For estimating the CO₂ concentration the m/z peaks 44, 45 and 46 were integrated and calibrated against samples of standard gas with a known concentration of 2,895 ppm. δ¹⁳C resp and CO₂ concentration were determined from the mean of two sequential injections. For δ¹³C resp the mean standard deviation (SD) of the duplicate samples was 0.04‰. For CO₂ concentration the SD for the duplicate samples was 14 ppm, regardless of the samples’ concentration.

We tested the CO₂ concentration evaluation using the calibrated IRMS peak area by analysing flasks filled with known CO₂ concentrations using the same IRMS measurement protocol and found the IRMS overestimated CO₂ concentration by 4% at 10,000 ppm and 18% at 50,000 ppm. Empirical corrections based on a polynomial fit were used to correct reported sample concentrations. After correction, differences between known to corrected CO₂ concentrations were 0% on average and for 90% of the measurements the difference was < 2% of the measured concentration. In the same test, we found that the δ¹³C was stable when varying the injection volume up to 200 µl and in flask air pressures between 800 and 1,000 hPa, suggesting there are no isotopic fractionations in the sub-sampling procedure.

The mean CO₂ efflux during the incubation period normalized to the sample dry weight per day (mg C g⁻¹ day⁻¹) was calculated using Equation (2):

\[
\text{CO}_2 \text{ Efflux} = \frac{\Delta \text{CO}_2}{t} \times \frac{V_{\text{Hg}} \times \text{BP} \times M_C}{T \times M_{\text{dm}} \times R},
\]

where \(\Delta \text{CO}_2\) is the net change in CO₂ concentration (ppm/10⁶) during the incubation (equal to the measured CO₂ concentration), \(t\) is the incubation time (days), \(V_{\text{Hg}}\) is the volume of the headspace (269 ml), \(\text{BP}\) is the local barometric pressure (hPa), \(M_C\) is the molar mass of C (12 mg mole⁻¹), \(T\) is the temperature of incubation (295 K), \(M_{\text{dm}}\) is the dry mass of roots (g) and \(R\) is the ideal gas constant (83.14 ml hPa⁻¹ K⁻¹ mmol⁻¹). The error in the CO₂ efflux due to propagated uncertainties in \(\Delta \text{CO}_2\), \(V_{\text{Hg}}\), \(\text{BP}\) and \(M_{\text{dm}}\) is estimated to be 4% of the reported value.

To measure δ¹³Cws, the capsule containing water-soluble C was combusted using an elemental analyser (NA 1110, CE Instruments, Milan, Italy) coupled to a Delta+ XL IRMS (Thermo Finnigan, Bremen, Germany) via a ConFlow III. Samples were analysed against laboratory standards on the VPDB scale.

The apparent isotopic fractionation (\(\Delta_R\), ‰) in respiration was estimated with the equation (neglecting the denominator):

\[
\Delta_R = \delta^{13}C_{\text{ws}} - \delta^{13}C_{\text{resp}}.
\]

2.6 | Estimations of C pool sizes and age

The first approach (approach 1, Table 1) is based on the view that storage C age follows “last in, first out” dynamics (Lacointe, Kajji, Daudet, Archer, & Frossard, 1993), in which the most recent C transported to the root is the most accessible for respiration (Carbone et al., 2013). Accordingly, two pools were defined: (a) an “active” pool that supports metabolism and respiration, derived from recently transported C to the roots (in intact roots under no C limitation, recently fixed C < 1 year), and (b) a “stored” pool that only becomes active when the supply of transported C is reduced (Figure 2) (Herrera-Ramirez et al., 2020; Richardson et al., 2013). We measured respired C as a proxy to estimate the age and size of the metabolically “active” pool, with δ¹⁴C resp representing its age. To estimate the amount of storage C < 1 year old (the active pool size in intact roots) we used the repeated respiration measurements. First, C with δ¹⁴C signature of 1 year was defined as the 2018 δ¹⁴C dem + the mean yearly δ¹⁴C dem change (see Equation [5] in Results). Assuming excised roots will access increasingly older (higher δ¹⁴C) C, we estimated when the CO₂ respired by the roots increased beyond this 1 year threshold (\(t_{\text{depletion}}\)). Integration of the fitted CO₂ efflux versus time curve between time \(t = 0\) until \(t_{\text{depletion}}\) yielded an estimate of the size

| Table 1 | Methods for C pool size and age estimation |
|---------|-------------------------------------------|
| Stored C fraction (Fstored) | Slope |
| Active C fraction (Factive) | 1 – slope |
| \(\Delta^{14}C_{\text{stored}}\) | \(\Delta^{14}C_{\text{cell}}\) |
| \(\Delta^{14}C_{\text{active}}\) | \(\Delta^{14}C_{\text{resp}}\) |
| Size of active pool | Amount of respired CO₂ that is < 1 year |
| Size of stored pool | Total sugars \(\times F_{\text{passive}}\) |

Note: Approach 1 is based on the \(\Delta^{14}C\) signature of root-respired CO₂ \(\langle\Delta^{14}C_{\text{resp}}\rangle\) and approach 2 is based on the intercept and slope estimates of the fitted linear line between the \(\Delta^{14}C\) signatures of the water-soluble C \(\langle\Delta^{14}C_{\text{ws}}\rangle\) and \(\alpha\)-cellulose C \(\langle\Delta^{14}C_{\text{cell}}\rangle\) extracted from roots. Those C extractions represent soluble sugars and structural C, respectively.
Conceptual model indicates the carbon (C) dynamics in a root, represented by the box. Carbon transported into the root is the most accessible to metabolism and respiration, while a portion is allocated to the stored pool. The $\Delta^{14}C$ signature of respired CO$_2$ is assumed to be equal to the transported C signature. When photosynthesis is not suppressed the transported C has $\Delta^{14}C$ signature of current atmospheric CO$_2$. Respired CO$_2$ older than 1 year might originate from stored pool C, or from transported C translocated from root-external old storage. The $\Delta^{14}C$ of the total sugars, approximated by $\Delta^{14}C_{ws}$, is the weighted age of the young active pool and the older stored pool [Colour figure can be viewed at Wileyonlinelibrary.com]

Our second approach (approach 2, Table 1) is based on the strong correlation between $\Delta^{14}C_{ws}$ and tissue age and the observation that $\Delta^{14}C_{ws}$ is often less than (younger) than $\Delta^{14}C_{cell}$ (Furze et al., 2020; Richardson et al., 2015; Trumbore et al., 2015).

For a root that has lived for multiple years, we expect that $\Delta^{14}C_{cell}$ will reflect the $^{14}C$ of substrates used for growth that can vary from year to year, while the $\Delta^{14}C_{ws}$ will reflect mixing of C that may date from the year of root formation with soluble C translocated from other parts of the plant. The mean $\Delta^{14}C_{ws}$ signature of a root thus comprises a spectrum of $\Delta^{14}C$ values, ranging from recently assimilated C in young tissues to older storage C in the innermost ring. Hence, in simplified view, the measured $\Delta^{14}C_{ws}$ is a weighted mean of the two functional C fractions (Figure 2): a proportion of active C, $F_{active}$, with radiocarbon signature $\Delta^{14}C_{active}$ and the stored fraction C, $F_{stored} (= 1 - F_{active})$, with radiocarbon signature $\Delta^{14}C_{stored}$. The mass balance is described by (Equation [4]):

$$\Delta^{14}C_{ws} = F_{active} \times \Delta^{14}C_{active} + F_{stored} \times \Delta^{14}C_{stored}. \tag{4}$$

We assume that the stored pool C consists of C deposited as storage reserves concurrent with root growth, thus $\Delta^{14}C_{stored}$ can be approximated by the measured $\Delta^{14}C_{cell}$. Even if old C with high $\Delta^{14}C$ signature is allocated to a root, the formed structural C and storage C are expected to share the high isotopic signature and the assumed approximation will still hold. Using the linear regression estimates for the relation between the measured $\Delta^{14}C_{ws}$ with $\Delta^{14}C_{cell}$ enables us to solve the other variables of Equation (4): The slope of the linear equation is equal to $F_{stored}$, $F_{active}$ equals $1 - F_{stored}$ and the intercept equals to $F_{active} \times \Delta^{14}C_{active}$, thus $\Delta^{14}C_{active} = \text{intercept} / F_{active}$. Using this approach, we do not assume an age for the active C (e.g., < 1 year); instead, we estimate the $\Delta^{14}C$ of $F_{active}$ from the $\Delta^{14}C_{ws}$ value that is not explained by $\Delta^{14}C_{cell}$. The model estimates are based on roots from different trees hence they represent averaged values for several trees ($n = 5 - 12$).

It is important to note that the water-soluble fraction contains sugars, but also many other soluble compounds like tannins and amino and organic acids. Trumbore et al. (2015) estimated that ~50% of the C in the soluble fraction extracted from wood (they used methanol: water mixture as a solvent) originates from the sugars sucrose, glucose and fructose. Here, we assume that on timescales of $> 1$ year exchange of C between cellular metabolites means that the $\Delta^{14}C_{ws}$ signature approximates that of sugars.

### 2.7 | Statistical analysis

All data were analysed using R (R Core Team, 2019). We tested effects of treatment (Pre-girdling, girdling and control) and root class (coarse, fine) on the different measures. Normality was tested with the Shapiro–Wilks test and equality of variance with Levene’s test. When both assumptions were not violated we proceed with one or two-way ANOVA followed by Tukey’s HSD post-hoc test. When the assumptions of normality and/or homogeneity were violated, we used the non-parametric Kruskal–Wallis rank-sum test, followed by pairwise Wilcoxon Rank Sum Tests. For the linear models, we used the lm function.

### 3 | RESULTS

#### 3.1 | NSC concentrations

Total sugar concentrations did not vary by treatment ($p = .91$, Kruskal-Wallis) or root size class ($p = .91$, ANOVA) (Figure 3a). The overall mean ± SE ($n = 47$) was $42.9 ± 3.1$ mg glucose g$^{-1}$ dry root, equivalent to $17.1 ± 1.2$ mg C g$^{-1}$ (Table S2). The mean composition of the C (in glucose equivalents) was: sucrose 69.4%, glucose 16.6% and fructose 14.0%. Starch measured in girdling and control treatments was significantly higher ($p = .04$, Kruskal-Wallis) in the coarse roots than in the fine roots, with mean values of 4.8 ± 1.2 and 1.5 ± 0.3 mg C g$^{-1}$, respectively ($n = 12$).

#### 3.2 | CO$_2$ efflux rate

The CO$_2$ efflux rate in the two-day incubations was significantly higher in the fine roots than in the coarse roots ($p < .01$, Kruskal-Wallis) with mean ± SE values of $3.0 ± 0.3$ and $2.0 ± 0.2$ mg C g$^{-1}$ d$^{-1}$, respectively (Figure 3b). The mean $Q_{10}$ measured in September–October campaigns was $2.3 ± 0.1$ ($n = 11$) with no effect of root class or treatment. Correcting the CO$_2$ efflux rates measured in the laboratory to soil temperature decreased the values, but the root size effect remained significant ($p < .01$, Kruskal-Wallis), with mean values of $1.2 ± 0.1$ and $0.8 ± 0.1$ mg C g$^{-1}$ d$^{-1}$ were assessed for the fine and
coarse roots, respectively (Figure 3b). We used the mean efflux rates corrected to the daily mean of soil temperature to estimate the total amount of C respired by fine and coarse roots during the 82 days between the girdling and the first post-girdling campaign; these were 150 ± 19 and 100 ± 12 mg C g⁻¹, respectively (uncertainty was calculated by varying the mean efflux rates and Q₁₀ with their uncertainties). Accordingly, the mean daily C respired in the fine roots is 1.8 ± 0.2 mg C g⁻¹ and 1.2 ± 0.1 mg C g⁻¹ in the coarse roots.

### 3.3 Radiocarbon

Combining measures of Δ¹⁴Cₜₐₐₚ in tree-ring late wood, leaf Δ¹⁴Cₑₑₑ and the atmospheric record in the region, the annual decline of Δ¹⁴Cₐₐₚ averaged 4.7‰ per year during the last two decades (Figure 4). Our estimate for the atmospheric Δ¹⁴Cₑₑₑ during the 2018 growing season was +2.3‰, the mean Δ¹⁴Cₑₑₑ of the roots of the control trees (Table 2). This value is within the 0.6–4.4‰ range of Δ¹⁴Cₐₐₚ mean values measured during the 2018 growing season at several ICOS stations (Table S1). Thus, we calculated the mean age (year) of a C pool with radiocarbon signature Δ¹⁴C using Equation (5):

\[
\text{Mean age} = \frac{\Delta^{14}C - 2.3\%}{4.7\%/\text{year}}
\]

Root respired Δ¹⁴Cₑₑₑ values were mostly similar to the Δ¹⁴Cₐₐₚ in the year of collection, with overall mean ± SE of 4.8 ± 0.9 (0.5 ± 0.2 year; n = 36) and range of −5.2‰ to +23.7‰ (0–4.6 years). The mean Δ¹⁴Cₑₑₑ when both root classes are pooled together was higher in the girdled trees (7.9 ± 1.9; n = 12) than the pre-girdling (4.2 ± 0.8; n = 12) and the control (2.3 ± 1.8; n = 12) (Figure 5). Differences between the treatments were not significant (p = .14–.20, Wilcoxon). However, the mean Δ¹⁴Cₑₑₑ of the girdled trees was significantly higher than the control trees (represent current atmosphere) when compared exclusively (p = .04, Wilcoxon). The mean age of the respired Δ¹⁴Cₑₑₑ in the girdled trees was 1.2 ± 0.4 year, compared to 0 ± 0.4 year in the control roots.

The Δ¹⁴Cₑₑₑ values were higher than Δ¹⁴Cₑₑₑ for the same root samples, ranging between 0.8 and 90.7‰ (0–18.8 year). The linear regression model equations for the Δ¹⁴Cₑₑₑ versus Δ¹⁴Cₑₑₑ relationship, its statistical information and approaches 1 and 2 predictions are presented in Table 2 and Figures 5 and 6. The variability in Δ¹⁴Cₑₑₑ explained the majority of the variability in Δ¹⁴Cₑₑₑ (r² > 0.8 in most groups; Table 2; Figure 6). The slope estimates based on the linear fit equations were highly significant in most subgroups (p < .01), except in the fine roots of the girdled trees due to one outlier with much higher Δ¹⁴Cₑₑₑ than Δ¹⁴Cₑₑₑ (Figure 6). According to approach 2, Fₐₐₚ
### Table 2: A summary of approaches 1 and 2 results

| Treatment | Root class | Measured $\Delta^{14}C_{\text{resp}}$ and age (Δ$^{14}C_{\text{active}}$ estimation of approach 1) | Linear model equations$^b$ | $F_{\text{stored}}$ | $F_{\text{active}}$ | $\Delta^{14}C_{\text{active}}$ and age$^d$ (estimation of approach 2) |
|-----------|------------|-------------------------------------------------|-----------------------------|-----------------|----------------|-----------------------------------|
| Pre-       | Coarse     | $5.2 \pm 1.9\%$ (0.6 ± 0.4 year, $n = 6$) | $y = -4.4 (3.7) + 0.8 (0.1)$ $R^2 = 0.88, df = 9$ | 0.8, 0.2 | 0.7, 0.3 | $-23.0 \pm 22.8\%$ (−5.4 ± 4.9 year) $-11.1 \pm 8.8\%$ (−0.7 ± 1.9 year) |
|           | Fine       | $3.2 \pm 1.0\%$ (0.2 ± 0.2 year, $n = 6$) | $y = -3.8 (3.6) + 0.6 (0.1)$ $R^2 = 0.64, df = 10$ | 0.6, 0.4 | 0.9, 1.1 | $9.1 \pm 1.1\%$ (1.4 ± 1.9 year) |
| Girdling   | Coarse     | $8.25 \pm 3.2\%$ (1.3 ± 0.7 year, $n = 6$) | $y = 3.1 (2.7) + 0.7 (0.04)$ $R^2 = 0.99, df = 4$ | 0.7, 0.3 | 0.7, 0.3 | $11.0 \pm 9.6\%$ (1.9 ± 2 year) $17.5 \pm 15.8\%$ (3.2 ± 3.4 year) |
|           | Fine       | $7.5 \pm 2.3\%$ (1.1 ± 0.5 year, $n = 6$) | $y = 1.58 (18.1) + 0.4 (0.6)$ $R^2 = 0.1, df = 0$ | 0.4, 0.6 | 2.5, 2.5 | $25.2 \pm 36.8\%$ (4.9 ± 7.8 year) |
| Control    | Coarse     | $0.7 \pm 2.0\%$ (−0.3 ± 0.4 year, $n = 6$) | $y = 4.0 (1.0) + 0.5 (0.03)$ $R^2 = 0.99, df = 3$ | 0.55, 0.45 | 0.6, 0.4 | $9.0 \pm 2.2\%$ (1.4 ± 0.5 year) $11.3 \pm 3.5\%$ (1.9 ± 0.7 year) |
|           | Fine       | $3.8 \pm 1.0\%$ (0.3 ± 0.6 year, $n = 6$) | $y = 4.0 (2.9) + 0.6 (0.13)$ $R^2 = 0.86, df = 4$ | 0.7, 0.3 | 11.8, 11.8 | $9.9 \pm 2.1\%$ (2.0 ± 2.1 year) |
| All        |            | $4.8 \pm 0.9\%$ (0.5 ± 0.2 year) | $y = 1.7 (1.7) + 0.7 (0.05)$ $R^2 = 0.84, df = 44$ | 0.7, 0.3 |               | $5.7 \pm 5.9\%$ (0.7 ± 1.3 year) |

Note: The linear model equations are for the relationship between the $\Delta^{14}C$ signatures of the water-soluble C ($\Delta^{14}C_{\text{ws}}$) and α-cellulose C ($\Delta^{14}C_{\text{cell}}$) extracted from roots. Those C extractions represent soluble sugars and structural C, respectively. Approach 2 is based on the linear model and predicts the fractions of the stored ($F_{\text{stored}}$) and active ($F_{\text{active}}$) C pools from the total water-soluble C, and the $\Delta^{14}C$ signature of the active pool ($\Delta^{14}C_{\text{active}}$).

$^b$ Values are means ± SE.

$^d$ Bold numbers indicate significant ($p < 0.05$) estimate of the model. The standard errors of the coefficients are in parentheses.

$^e$ The error is the model’s slope SE, $F_{\text{stored}}$ equals the equation’s slope, $F_{\text{active}} = 1 - F_{\text{stored}}$.

$^f$ Calculated as the equation’s intercept/SE. $F_{\text{stored}}$ Errors are the propagated standard errors of the intercept and slope estimates.

### 3.4 Repeated Incubations

Temporal changes in all measured parameters were observed between day 1 and day 7 of the repeated incubations (Figure 7). Following approach 1, any carbon with a $\Delta^{14}C_{\text{cell}}$ of >3.4‰ was defined as older than 1 year. Using linear regression, we estimated that the 7-day change in $\Delta^{14}C_{\text{cell}}$ (2.3‰) and annual decline (4.7‰) corresponded to estimated ages of 2.9 ± 0.3 to 10.1 ± 0.9 years, respectively. Following approach 2, the total CO$_2$ efflux rate was 2.3 ± 0.1 and 2.1 ± 0.5 (Table 2), and the total CO$_2$ efflux rate was 1.4 ± 0.3 (Figure 7). Between time $= 0$ and time $= \tau_{\text{depletion}}$, the total CO$_2$ efflux rate was 2.5 ± 0.1. Using linear regression, we estimated that the 7-day change in $\Delta^{14}C_{\text{cell}}$ (2.3‰) corresponded to estimated ages of 2.9 ± 0.3 to 10.1 ± 0.9 years, respectively. Following approach 2, the total CO$_2$ efflux rate was 2.3 ± 0.1 and 2.1 ± 0.5 (Table 2), and the total CO$_2$ efflux rate was 1.4 ± 0.3 (Figure 7). Between time $= 0$ and time $= \tau_{\text{depletion}}$, the total CO$_2$ efflux rate was 2.5 ± 0.1. Using linear regression, we estimated that the 7-day change in $\Delta^{14}C_{\text{cell}}$ (2.3‰) corresponded to estimated ages of 2.9 ± 0.3 to 10.1 ± 0.9 years, respectively. Following approach 2, the total CO$_2$ efflux rate was 2.3 ± 0.1 and 2.1 ± 0.5 (Table 2), and the total CO$_2$ efflux rate was 1.4 ± 0.3 (Figure 7). Between time $= 0$ and time $= \tau_{\text{depletion}}$, the total CO$_2$ efflux rate was 2.5 ± 0.1. Using linear regression, we estimated that the 7-day change in $\Delta^{14}C_{\text{cell}}$ (2.3‰) corresponded to estimated ages of 2.9 ± 0.3 to 10.1 ± 0.9 years, respectively. Following approach 2, the total CO$_2$ efflux rate was 2.3 ± 0.1 and 2.1 ± 0.5 (Table 2), and the total CO$_2$ efflux rate was 1.4 ± 0.3 (Figure 7).
The Δ^{14}C signatures and mean age estimations of the active C pool according to two approaches; approach 1 assumes Δ^{14}C_{active} equals to the Δ^{14}C signature of respired CO₂ (Δ^{14}C_{resp}); approach 2 is based on the intercept and slope estimates of the fitted linear line between the Δ^{14}C signatures of the extracted water-soluble C (Δ^{14}C_{ws}) and α-cellulose C (Δ^{14}C_{cell}). According to this approach the fraction of the active C pool F_{active} = 1 - slope. Roots collected before girdling (Pre-girdling), ~3 months after girdling (Girdling) and ~3 months after girdling but in un-girdled trees (Control). One set of roots was used for respiration incubations (n = 12) and second set was used for the C extractions (n = 23, 12, 11, respectively). Error bars of approach 1 is the standard errors of the slope and intercept estimates [Colour figure can be viewed at wileyonlinelibrary.com]

size (p < .01), with no interaction effect (p = .833) (Figure 8a, Table S2). The post-hoc tests (Tukey's HSD) showed δ^{13}C_{ws} in the pre-girdling roots were significantly lower than the girdling and control roots that did not differ significantly. Water soluble C extracted from fine roots was 0.74% more enriched than the coarse root extracts. The treatment had significant effect also for δ^{13}C_{resp} (p < .001, Kruskal-Wallis), and the post-hoc test (Wilcoxon) indicates significant difference between all three campaigns: the pre-girdling roots had the lowest mean value, with girdled trees on average 1.15% higher and the control trees 2.64% higher than the pre-girdling (Figure 8b, Table S2). The coarse root δ^{13}C_{resp} was 0.80% more enriched than the fine roots (p = .052, Kruskal-Wallis). The mean Δ₀ value (δ^{13}C_{ws} – δ^{13}C_{resp}) for fine roots was 1.53% higher than for coarse roots (p < .01; one-way ANOVA; Figure 8c). The treatment had marginal effect (p = .053; Kruskal-Wallis), where the Δ₀ in the control roots was significantly lower (wilcoxon test) than in the pre-girdling and girdling (Figure 8c, Table S2).

| Root class          | All roots | Pre-girdling | Girdling | Control |
|---------------------|-----------|--------------|----------|---------|
| F_{stored}          |           | r² = 0.83    | r² = 0.65| r² = 0.99| r² = 0.86 |
| Coarse Fine roots   |           |              |          |         |
| F_{stored}          |           | r² = 0.99    | r² = 0.99| r² = 0.99|          |
| Fine roots          |           |              |          |         |
| F_{stored}          |           | r² = 0.99    | r² = 0.99|          |

The correlation between Δ^{14}C_{ws} and Δ^{14}C_{cell} (approach 2) suggests that the C stored over multiple years on average makes up 70 ± 5% (range of 55–80%) of the extracted, water-soluble C (Figure 6). Sugars make up only a fraction of the water-soluble C used for the Δ^{14}C measurement. Assuming sugars contribute in the same proportion to both active and stored pools found in water-soluble C, of the total average sugar concentration (17.1 ± 1.2 mg C g⁻¹; Table S2), 30% (5.1 ± 0.5 mg C g⁻¹) is found in the active pool and 70% (12.0 ± 0.5 mg C g⁻¹) in the stored pool (Table 1; approach 2). Based on the amount of C respired in repeated incubations (approach 1), we estimated the pool of C younger than 1 year as 5.1 mg C g⁻¹ for coarse roots and 5.3 mg C g⁻¹ for fine roots. Both approaches thus agree well in estimating the active pool size, suggesting that the < 1 year criterion provides a way to estimate the age and size of the active pool in intact roots. Both approaches (1 and 2) also agree with regard to the active pool age being younger than 1 year, that is, 0.5 ± 0.2 and 0.7 ± 1.3 year, respectively (Table 2). Our results in roots

4 | DISCUSSION

4.1 | Size and age estimates for active and stored C pools using two approaches

Our results provide unique estimates for the size and age of the functional sub-pools of NSC soluble in water using two distinct approaches.
corroborate those found by Richardson et al. (2013) for tree stems. When they constrained active (‘fast’) NSC pool age to < 1 year, they found this pool contributed 23–56% of the total NSC, compared with our estimates of 20–45% for roots (Table 2).

We also found disagreements between the two approaches, namely for individual subgroups (Figure 5, Table 2). There are apparently two reasons. The first reflects large uncertainties in the linear model’s prediction of the intercept value that approach 2 is based on, which leads to extreme and unrealistic \( \Delta^{14}C_{\text{active}} \) values, for example, equivalent to ~5.4 year for coarse roots during pre-girdling (Table 2). Still, considering the uncertainties, the two approaches to estimate \( \Delta^{14}C_{\text{active}} \) mostly agree (Figure 5). In addition, the tendency to use older C to fuel respiration (i.e., transferring older C to the active pool) in the girdled-trees roots that were predicted and observed in approach 1 (\( \Delta^{14}C_{\text{resp}} \)), was also observed in approach 2 (Figure 5).

The second source for the disagreement between the methods is potentially true difference, as apparent for the control roots, where the estimate from approach 2 for \( \Delta^{14}C_{\text{active}} \) (based on the \( \Delta^{14}C_{\text{cell}} \) that is not explained by variability in \( \Delta^{14}C_{\text{cell}} \)) is significantly higher than approach 1 considering the uncertainties (Figure 5, Table 2). A change in the age of the incoming C to the roots during the recent past might have impact on that discrepancy. Further understanding would require better estimation of the composition of water soluble components and the potential for their makeup and age to change with season.

4.2 The stored fraction should be also considered in driving \( \Delta_R \) values

Water deficit in the soil increased from midsummer to the end of the growing season because of the 2018 hot drought conditions (Figure 1). In control (un-girdled) trees, we observed enrichment in both \( \delta^{13}C_{\text{ws}} \) and \( \delta^{13}C_{\text{resp}} \) measured in Sept/Oct compared to the pre-girdling roots measured in June–July. These \( \delta^{13}C_{\text{resp}} \) increases are in line with expected water shortage effects on leaf-level fractionation (Figure 8) (Farquhar & Sharkey, 1982; Madhavan, Treichel, & Oleary, 1991; Pate & Arthur, 1998; Scartazza, Moscatello, Matteucci, Battistelli, & Brugnoli, 2015). Smaller enrichments in \( \delta^{13}C_{\text{ws}} \) could be explained by dilution with storage pools. A simple mass balance calculation using the relative enrichments in \( \delta^{13}C_{\text{ws}} \) (\( +1.12 \‰ \)) and \( \delta^{13}C_{\text{resp}} \) (\( +2.64 \‰ \)) between control and pre-girdling provides an estimate for \( F_{\text{active}}: \) \( 1.12/2.64 = 0.42 \), nearly equalling the 0.43 predicted by approach 2 for the control trees (Table 2). This suggests that \(^{13}C\)-enriched sugars produced in control-tree leaves are mixed into the active pool in the roots where they support respiration, while the stored water-soluble C integrates more depleted \( \delta^{13}C \) values inherited from previous years. Thus the apparent decrease in \( \Delta_R \) in the control roots most likely does not reflect a metabolic shift (e.g., reduced PEPC activity) in the roots. In contrast, the enrichment between pre-girdled and girdled roots is larger for \( \delta^{13}C_{\text{ws}} \) (\( +1.52 \‰ \)) than for \( \delta^{13}C_{\text{resp}} \) (\( +1.15 \‰ \)), perhaps reflecting differences in transported substrates.
4.3 The carbon balance in girdled-tree roots was maintained by translocation of stored NSC

The ability of roots to access older C for respiration when new C supply is cut off was demonstrated in the repeated incubations and in the girdling experiments. However, the supply of older C was not the same in both cases. Over the 7 days of the repeated incubations, the coarse and fine roots respired 7.1 and 13.4 mg C g⁻¹, respectively, roughly 35–70% of the 20.3 mg C g⁻¹ of NSC remained in the incubated roots (overall sugars and starch mean, Table S2). The estimated ages of the respired C increased from < 1 year to 1.6 and 2.9 year, for coarse and fine roots, respectively. This is consistent with roots respiration an initial active pool fed by freshly fixed C and switching to older substrates as this pool is exhausted (Herrera-Ramirez et al., 2020). In the case of excised roots, the source of the older C substrate was clearly in the roots themselves.

During the months that elapsed after girdling, root respiration rates did not decline as they did for excised roots (Figure 3b). During this period, assuming constant respiration rates, an estimated 100 and 150 mg C g⁻¹ were respired in the coarse and fine roots, respectively. This was roughly 5–7 times higher than the C stored in the NSC pools, which also did not decline following girdling (Figure 3a). Therefore, it is clear that the C being respired in the girdled roots must be replaced by C translocated from root-external sources. The C can originate from storage in below-girdling parts of the tree, and from neighbouring healthy trees. *Populus* trees are known to have root connections with shared root systems. *Populus* root systems can remain functional for at least 20 year after shoot removal, and can persist for millennia while shoots are repeatedly resprouting (Pregitzer & Friend, 1996). Thus, the root systems of our girdled trees may be older than the ~60–70 year stems and have disproportionately large NSC stocks with possible connection from girdled to healthy trees. Natural root grafting, the phenomena where two roots are pressed together to form vascular continuity that enables C transfer between trees, is also common in *Populus* trees and could be a source for the replenished C (Fraser, Lifers, & Landhausser, 2006; Gaspard & DesRochers, 2020; Mudge, Janick, Scofield, & Goldechmidt, 2009). Carbon transference from neighbouring trees can occur also via mycelial networks (Rog, Rosenstock, Kommer, & Klein, 2020). Indeed, the girdled trees in our study have survived for 2 years so far without visible signs of crown damage. Tree responses to girdling vary with species, stand age and experimental design that affect the size of the storage reserves and the connections between non-girdled and girdled trees (Levy-Varon, Schuster, & Griffin, 2012). The fact that our girdled trees were located among the un-girdled trees without physical or substantial spatial separation increase the chances for C sharing via roots. A way to reduce such effects in future is to girdle trees as a block of forest stand and isolate from root connections by trenching at the border.

Whatever the source of translocated C to girdled tree roots, our isotopic measurements indicate that it is not derived from fresh photo assimilates. Based on $\Delta^{13}$Cresp, the mean age of C respired from girdled roots was ~1.2 year older than C respired from control roots (Figure 5). This is younger than the respired C at the end of the repeated incubations (1.6–2.9 year). Difference in the $\delta^{13}$Cresp between the girdling and control roots further indicates they do not share the same respiratory C source (Figure 8b). The increase in $\delta^{13}$Cresp between pre-girdling and girdling can be explained by hydrolysis of starch, which tends to be more enriched than sugars (Brugnoli, Hubick, von Caemmerer, Wong, & Farquhar, 1988; Damesin & Lelarge, 2003; Gleixner, Danier, Werner, & Schmidt, 1993; Maunoury-Danger et al., 2010).

4.4 Hints for high PEPC activity in the fine roots

The $\Delta_R$ mean values by root class and treatment ranged between (−0.35‰) and (−4.38‰) (Figure 8, Table S2), a slightly wider range
than the (−0.7‰) to (−3.1‰) values measured in mature Eucalypt roots (Gessler et al., 2007). While some of this could be due to differences in temperature effects, these likely do not explain the range in ΔR values. As discussed earlier, the driver for the most negative ΔR values measured in the control trees probably reflects differences in δ13C between the active and the stored C pools (“mixing”), and not a change in respiration metabolism (Figure 8). In fact, it is difficult to resolve whether the main driver for a given ΔR value is mixing or metabolism, especially in perennial plants with old reserves. However, differences in metabolism might be inferred in some circumstances, for example when comparing ΔR in fine and coarse roots from the same trees where uniform mixing could be assumed. ΔR was higher for fine (−1.1‰) than coarse (−2.67‰) roots, a difference reflecting lower δ13Cresp (−0.80‰) and higher δ13Cwst (−0.74‰) in the fine roots (Figure 8). This pattern is in agreement with the expected net isotopic effect of refixation of internal CO2 by PEPC: a 13C depletion of the respired CO2 and a 13C enrichment of the products (Werner & Gessler, 2011).

Additional support for CO2 fixation with high PEPC activity in roots comes from the decline in the respiration quotient (RQ) from ~1 to ~0.6 during our repeated incubations (Figure 7d), since PEPC CO2 re-fixation would reduce CO2 efflux and thereby RQ (Hilman et al., 2019). The effect of PEPC on respiration grows as its activity as a fraction of total respiration increases (Badeck et al., 2005), as might occur with repeated incubations. Our results mirror those of Bathellier et al. (2009) who also observed a decrease in RQ from 1.1 to 0.8–0.9 while δ13Cresp remained stable in roots of French bean during 6 days of carbohydrate starvation by darkening. Alternative explanations for the change in RQ exist, including a shift from carbohydrates to lipids as the main respiration substrate (Figure 7d). However, we observed no simultaneous decline in δ13Cresp that would be expected to accompany such a substrate shift, given the depleted δ13C value of lipids (Fischer et al., 2015; Tcherkez et al., 2003). Other factors that could cause a decline in RQ include a relative increase of O2 uptake [e.g., through production of reactive oxygen species associated with cell death during the experiment (Chae & Lee, 2001)].

While not conclusive, our results suggest that the potential role of PEPC in tree roots deserves further exploration. PEPC has an important role in plant metabolism, synthesizing C3 organic acids from HCO3− and phosphoenolpyruvate (PEP). The carbon skeletons of the organic acids can be also used to generate amino acids. High activity of PEPC in roots of model plants is well documented and linked, among other factors, with exudation of organic and amino acids to the rhizosphere (Neumann & Römheld, 2007; O’Leary, Park, & Plaxton, 2011). The addition of ΔR and RQ analysis to the PEPC assessments toolbox that contains enzymatic and genetic assays can provide realistic estimations for the rate of PEPC activity, although this first requires resolving mixing effects on ΔR. Compound-specific analysis (using GC-C-IRMS) for δ13C of the putative respiratory substrates and metabolites might provide more accurate ΔR values and information about PEPC metabolism.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in ‘Zenodo’ at https://doi.org/10.5281/zenodo.4281013, and in the supplementary material of this article.

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SUPPORTING INFORMATION

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