1 **The size and the age of the metabolically active carbon in tree roots**
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11 **Summary statement**

12 Non-structural carbohydrates extracted from roots include an 'active' pool that supports

13 metabolism and a 'stored' pool used when C supplies are limited. Using radiocarbon we

14 estimate that about a third of NSC in aspen roots is active and demonstrate use of old

15 reserves under C limitation.

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20 Summary

21 Little is known about the sources and age of C respired from tree roots. Previous

22 research in tree stems has identified two functional pools of non-structural

23 carbohydrates (NSC): an 'active' pool supplied directly from canopy photo-assimilates

24 that supports metabolism and a 'stored' pool used when fresh C supplies are limited.

25 We compared the C isotope composition of water soluble NSC and respired CO_2 for

26 aspen roots (*Populus tremula* hybrids) that were cut off fresh C supply via stem-girdling

27 and prolonged incubation of excised roots. We used bomb radiocarbon to estimate the

28 time elapsed since C fixation for respired CO_2, water-soluble C, and structural α-

29 cellulose. While freshly excised roots respired CO_2 with mean age <1 yr, within a week

30 the age increased to 1.6-2.9 yr. Freshly excised roots from trees girdled ~3 months

31 previously had similar respiration rates and NSC stocks as un-girdled trees, but respired

32 older C (~1.2 yr). We estimate the NSC in girdled roots must be replaced 5-7 times by

33 reserves remobilized from root-external sources. Using a mixing model and observed

34 correlations between $\Delta^{14}C$ of water-soluble C and α-cellulose, we estimate ~30% of C is

35 'active' (~5 mg C g^{-1}).

36 *Key words*: fine roots, radiocarbon (^{14}C), girdling, nonstructural carbohydrates,

37 respiration, storage dynamics, tree carbon dynamics, $\delta^{13}C$, isotopic fractionation,

38 phosphoenolpyruvate carboxylase (PEPC)

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Introduction

Fine roots (≤ 2mm) contain an estimated 40.8 Pg C globally, about 7% of total vegetative C (Ciais et al., 2014; Jackson, Mooney, & Schulze, 1997). However, the annual carbon (C) mass allocated to fine roots is probably much greater. In forest ecosystems 25-63% of the total assimilated C is estimated to be allocated belowground, where C is used for root growth, respiration, exudation, root connections, and mycorrhizal associations (Litton, Raich, & Ryan, 2007). Most of these functions are concentrated in the fine roots that respire the highest amounts of C per unit mass among root class sizes (Pregitzer, Laskowski, Burton, Lessard, & Zak, 1998), conduct root exudation and mycorrhizal interactions, and have high mass turnover rates (Gaudinski et al., 2010). The C and energy that fuel these functions are thought to originate mainly in nonstructural carbohydrates (NSC, mostly soluble sugars and starch). Lipids might also be an important reserve compound, but relatively scarce measurements prevent present evaluation of their true role (Hartmann & Trumbore, 2016). However, NSCs are not merely a transient C pool supporting transport from leaves to roots, as NSC reserves in plants can support cellular functions when C supply from photo-assimilates is insufficient to support C sinks (Chapin III, Schulze, & Mooney, 1990; Dietze et al., 2014).

The 'bomb-radiocarbon' approach allows studying timescales of NSC dynamics in mature trees by tracing excess ^{14}C created during atmospheric nuclear weapons tests that nearly doubled background values of radiocarbon signature ($\Delta^{14}C$) of atmospheric CO_2 ($\Delta^{14}C_{atm}$) in the early 1960s (S. E. Trumbore, Sierra, & Pries, 2016). Since then, declines in $\Delta^{14}C_{atm}$ reflect uptake of bomb-radiocarbon into the oceans and terrestrial

86   biosphere, and dilution by radiocarbon-free CO_2 originating from fossil fuel emissions

87   (Levin & Hesshaimer, 2016). Since 1964, an annual unique $\Delta^{14}C_{atm}$ signature is

88   transferred to biomass C via photosynthesis. A comparison of the $\Delta^{14}C$ signature of any

89   C pool in plants to the current year's $\Delta^{14}C_{atm}$ thus provides a means to estimate the

90   mean time elapsed since the C was fixed.

91   Plants use soluble sugars to translocate C (predominantly sucrose) and as a primary

92   substrate in cellular metabolism (e.g. glucose and fructose). Starch on the other hand is

93   insoluble and thus cannot be translocated. These different properties are often

94   mistakenly implied to reflect differences in turnover time, where sugars are assumed to

95   comprise a fast-cycling pool and starch a slow-cycling pool (Dietze et al., 2014).

96   However, in tree stems and large roots no systematic differences in radiocarbon

97   signatures have been observed between the water-soluble (representing sugars) and

98   insoluble (starch) C fractions, suggesting fast C exchange between the two pools

99   (Richardson et al., 2015; Richardson et al., 2013; S. Trumbore, Czimczik, Sierra, Muhr,

100   & Xu, 2015).

101   Nevertheless, NSCs are not homogeneous in age. The radiocarbon signature of stem

102   respired CO_2 ($\Delta^{14}C_{resp}$) was much lower than the water-soluble $\Delta^{14}C$ ($\Delta^{14}C_{ws}$) in the

103   outermost 2 cm of the stem, reflecting age differences of ~10 yr (Carbone et al., 2013).

104   Combined with evidence from ^{13}C-labeling of fresh photo-assimilates from the canopy, it

105   is clear that some young sugars support respiration, while other, older, sugars are

106   stored intact (Epron et al., 2011). Accordingly, C allocation models usually distinguish

107   two functional NSC sub pools: a 'fast' pool of recently fixed C (assumed age <1 yr)

108   supporting metabolism and respiration, and a 'slow' NSC pool for storage (Herrera-

109   Ramirez et al., 2020; Richardson et al., 2015). However, we are still missing ways to

110   quantify and differentiate between these two pools.

111   While radiocarbon data provide information about age, $\delta^{13}C$ can provide information

112   about the substrate and the metabolic pathways in respiration. Variations in the $\delta^{13}C$

113   signature of produced sugars are primarily controlled by photosynthetic fractionation at

114   the leaf level (Farquhar, Oleary, & Berry, 1982). 'Post-photosynthetic fractionations'

115   during transport, biosynthesis, and respiration further modify $\delta^{13}C$ signatures (Badeck,

116   Tcherkez, Nogues, Piel, & Ghashghaie, 2005; Jaleh Ghashghaie et al., 2003; Werner &

117   Gessler, 2011). Starch is usually slightly enriched (~1‰) while lipids are more depleted

118   (~-5‰) in ^{13}C compared to sugars (Bowling, Pataki, & Randerson, 2008), and are

119   expected to accordingly affect the $\delta^{13}C$ signature of the respired CO_2 ($\delta^{13}C_{resp}$) when

120   decarboxylated. The 'apparent isotopic fractionation' of respiration (Δ_R) is defined as the

121   difference in $\delta^{13}C$ between the putative respiratory substrate (usually sugars) and

122     $\delta^{13}C_{resp}$ (Jaleh Ghashghaie et al., 2003). For roots of woody C_3 plants mainly negative Δ_R

123   values ($\delta^{13}C_{substrate} < \delta^{13}C_{resp}$) have been reported (J. Ghashghaie & Badeck, 2014). The

124   ^{13}C enrichment in respired CO_2 is commonly explained by the dominance of CO_2 emitted

125   from decarboxylation of pyruvate via pyruvate dehydrogenase (PDH) over CO_2 emitted

126   from the TCA cycle reactions (Tcherkez et al., 2003). The activity of

127   phosphoenolpyruvate carboxylase (PEPC), which re-fixes CO_2 to replenish TCA cycle

128   intermediates, was suggested to increase Δ_R (Gessler et al., 2007; J. Ghashghaie &

129   Badeck, 2014). To our knowledge, only one study to date has evaluated Δ_R in roots of

130   mature trees (Gessler et al., 2007).

131   Here, we investigated the sources of respiration for fine and coarse roots of mature

132   temperate aspen trees (*Populus tremula* hybrids). We performed a stem-girdling

133   experiment (complete circumferential removal of bark, cambium and phloem),

134   terminating belowground transport of fresh photo-assimilates, and forcing mobilization

135   of storage NSCs for survival beneath the girdling. Girdled Amazonian trees quickly

136   shifted from using current year photosynthetic products as respiratory substrate in un-

137   girdled trees to C fixed ~5 yr previously a month after girdling (Muhr, Trumbore, Higuchi,

138   & Kunert, 2018). We also expected increases in $\Delta^{14}C_{resp}$ for roots of girdled trees, but

139   with potentially smaller magnitude, since trees from the genus *Populus* often have root

140   connections that enable C transfer from healthy trees (Gaspard & DesRochers, 2020;

141   Pregitzer & Friend, 1996). To characterize the usage of NSC reserves in the roots

142   themselves we conducted additional experiments where the age of C respired from

143   detached roots was followed for a week. This experiment is also the base for our first

144   approach to estimate the size and isotopic signature of the fast-cycling 'active' C pool

145   that support respiration and metabolism. The relationship between $\Delta^{14}C_{ws}$ and the $\Delta^{14}C$

146   signature of the α-cellulose ($\Delta^{14}C_{cell}$, representing structural C) allowed us to develop the

147   second approach to estimate the size and isotopic signatures of the 'active' C, as well

148   as the slow-cycling 'stored' C pool.

149   ## Materials and Methods

150   ### Study site and Experimental design

151   We sampled 12 Eurasian aspen trees (*Populus tremula* hybrids) with estimated age of

152   60-70 yr growing in a forest stand located on a slope of the Großer Hermannsberg

153 Mountain (867 m a.s.l), Germany (50°42'50'' N, 10°36'13'' E, site elevation 616 m a.s.l).

154 Soils at the site are developed on volcanic parent rock, mean annual temperature is ~7°

155 C, and annual rainfall is 800-1200 mm (Bouriaud, Marin, Bouriaud, Hessenmoller, &

156 Schulze, 2016). During our field campaign in the summer of 2018, an extreme 'hot

157 drought' occurred in central Europe (Bastos et al., 2020), also observed in a nearby

158 weather station situated 812 m a.s.l

159 (https://www.bgc-jena.mpg.de/freiland/index.php/Sites/Hermannsberg). During the 2018

160 growing season (May and October), mean monthly temperatures were warmer by 1.5 -

161 3.5° C than those for the years 2010-2017, while total monthly rainfall was 260 mm,

162 compared to the average of 323 mm in the years 2010-2017 (Fig. 1).

163 After sampling all 12 trees on June 26th and July 4th, 6 of the trees were girdled on July

164 4th by removing a ~4 cm band of bark, cambium and phloem from the stem 1.5 m above

165 the ground. During subsequent samplings (September 25th and October 2nd) we

166 differentiated "Girdling" and "Control" trees. To ensure we sampled roots specific to the

167 chosen trees, we tracked their connections back to the main root or stem. Roots were

168 collected from the top 10 cm of the mineral soil. Root samples were put on ice

169 immediately after harvest until analysis. On the subsequent day, the roots were

170 thoroughly washed to remove any remaining soil particles and then separated into two

171 size classes: coarse roots (> 2 mm, mean 2.9 mm, max 6 mm) and fine roots (≤ 2 mm,

172 almost all were suberized); each size fraction consisted of roots from different orders,

173 and not necessarily from the same root cluster. Each size class was split for (1) NSC

174 and α-cellulose extractions, and (2) two-day respiration incubations. For details see

175 respective chapters below.

176   Additionally, we repeatedly measured excised roots over 8 days to test their ability to

177   utilize their own C reserves for metabolism during C starvation. We define t = 0 as the

178   time when the incubations were started. The first two-day incubations integrated CO_2

179   respired during days 1 and 2 and the second incubations integrated respiration during

180   days 7 and 8. For data analysis we used the mean incubation times, and the elapsed

181   days 1.0 and 7.0. In addition, we performed short-term incubations (up to 1.5 hr) on

182   days 0, 2, and 6 to measure the respiratory quotient (RQ), i.e. the ratio CO_2 efflux/O_2

183   influx. The RQ is mainly defined by the respiratory substrate, which in plants is assumed

184   to be carbohydrates with RQ =1. Compounds more oxidized than carbohydrates like

185   organic acids expected to yield RQ >1, whereas amino acids and lipids yield RQ values

186   of 0.9 and 0.7, respectively (Masiello, Gallagher, Randerson, Deco, & Chadwick, 2008).

187   Thus, RQ measurements can indicate the substrate used for respiration. For the

188   correction of CO_2 efflux rates measured at laboratory temperatures (22°C) to field

189   temperatures, we conducted short-term incubations at different temperatures to

190   calculate Q_{10}, the factor by which CO_2 efflux increases with 10°C warming, and used

191   this factor together with field measured temperatures.

192   Because regional fossil fuel emissions have the potential to affect $\Delta^{14}C_{atm}$, we collected

193   (in 2019) additional samples at the site to reconstruct the recent history of local $\Delta^{14}C_{atm}$

194   from tree rings. Two stem cores from nearby tree were extracted using 5.15 mm

195   increment borer. We visually identified the annual rings for the last 9 years and sampled

196   their outermost halves, which presumably are dominated by latewood produced during

197   midsummer-autumn mainly from C fixed in the current growing season, thus

198   approximating the current year's $\Delta^{14}CO_{atm}$ (Kudsk et al., 2018; Pilcher, 1995). Samples

199   from identical rings from both stem cores were pooled for α-cellulose extraction and

200    $\Delta^{14}C_{cell}$ analysis (Hoper, McCormac, Hogg, Higham, & Head, 2016). In addition,

201   assuming leaves respire C fixed recently, we analyzed $\Delta^{14}C_{resp}$ from aspen leaves as a

202   proxy for $\Delta^{14}C_{atm}$ during the 2019 growing season. We combined our data with the mean

203    $\Delta^{14}C_{atm}$ of the northern hemisphere zone 1 during the growing season (May-October) as

204   published by Hua, Barbetti, and Rakowski (2016). We further compared our estimation

205   for 2018 $\Delta^{14}C_{atm}$ with direct flask measurements of atmospheric air in different sites in

206   Europe that are part of ICOS (the Integrated Carbon Observation System, see

207   Supporting information Table S1 for PID numbers).

208   NSC analysis

209   The same root sample, containing 50 mg of oven-dried root tissues (60°C for at least

210   two days), was analyzed for the following: sugars and starch concentrations, $\Delta^{14}C_{ws}$,

211    $\delta^{13}C_{ws}$, and $\Delta^{14}C_{cell}$. Since $\Delta^{14}C_{ws}$ is usually correlated with the age of the containing

212   tissue (Furze et al., 2020; Richardson et al., 2015; S. Trumbore et al., 2015), for correct

213   interpretation of $\Delta^{14}C_{ws}$ variance it is important to account to the root's mean C age

214   estimated using $\Delta^{14}C_{cell}$. Therefore, we coarsely cut rather than milled the roots since in

215   the α-cellulose extraction the sample is rinsed with reagents through 16–40 µm mesh

216   that might allow fine material to pass and clog the system's tubing (Steinhof, Altenburg,

217   & Machts, 2017). We decided only after the pre-girdling campaign to measure starch

218   concentration, hence data is available solely for roots from the girdled and control trees.

219   The methods for NSC, sugar and starch extractions are based on protocols S1 and S2

220   from Landhausser et al. (2018) with some modifications necessary for minimizing

221 extraneous C additions that would affect the ^{14}C measurement. We avoided plastic vials

222 that contain C and used only glass vials that were pre-baked at 550°C to eliminate any

223 C residuals. For sugars extraction we used water as a solvent instead of the original

224 ethanol, which is slightly more efficient for the extraction (Landhausser et al., 2018), to

225 avoid possible C addition from the ethanol. To reduce dissolution of starch in the water

226 the extraction temperature was lowered from 90°C to 65°C. Extraction of water-soluble

227 C from the 50-mg samples was carried out by shaking the samples in 5 mL deionized

228 water for 10 minutes at 65°C, in three repetitions. After each repetition the vials were

229 cooled down, centrifuged, and the supernatant was transferred to a glass vial kept on

230 ice (to slow-down microbial degradation). A subsample of 2 mL from the total 15 mL

231 was used for quantification of the sugars sucrose, glucose, and fructose. For the δ^{13}C

232 and Δ^{14}C analysis the rest of the solution was concentrated by freeze drying and

233 pipetted into tin capsules for δ^{13}C analysis, and into pre-baked silver capsules for Δ^{14}C

234 analysis. The starch in the pellet from the water extraction was converted by α-amylase

235 (Sigma cat. no. A4551) into water soluble glucans and then to glucose by

236 amyloglucosidase (Sigma cat. no. ROAMYGLL) (Landhausser et al., 2018). The

237 remaining pellet was used for $\Delta^{14}C_{cell}$ analysis (Steinhof et al., 2017).

238 To measure the concentrations of the soluble sugars, and the glucose hydrolysate from

239 the starch digestion we used high-performance anion-exchange chromatography with

240 pulsed amperometric detection (HPLC-PAD) device equipped with autosampler

241 (Dionex® ICS 3000, Thermo Fisher GmbH, Idstein, Germany) (Raessler, Wissuwa,

242 Breul, Unger, & Grimm, 2010). The starch and sugar concentrations are calculated as

243 glucose-equivalent weight for sample dry weight (mg g^{-1}) (Landhausser et al., 2018),

244 and further multiplied by 0.4 to units of C mass for dry weight (mg C g^{-1}).

Respiration measurements

Two-day incubations

247 Root samples (0.3-1.2 g dry weight) were incubated in gas-tight Plexiglas cylinders

248 equipped with fittings (12.7 mm Swagelok Ultra-Torr) on each side for attaching

249 sampling flasks (115 ml) equipped with a Louwers^{TM} O-ring high-vacuum valve

250 (LouwersHanique, Hapert, Netherlands) (Muhr et al., 2018). Total system volume was

251 269 ±10 mL. Before incubation the headspace containing the washed roots was flushed

252 with synthetic air (0% CO_2, 20% O_2) for three minutes in order to remove any inherited

253 CO_2 and purely measure the roots' respired CO_2. Then the set-up was closed quickly by

254 plugging two flasks (pre-filled with the same synthetic air) into the connectors at both

255 ends of the chamber. Incubations were conducted at room temperature (22°C) in the

256 dark and ended by closing the flasks valves. Same set-up was used for the leaves

257 incubations aimed to estimate the local $\Delta^{14}CO_{atm}$, which lasted one day.

258 The incubations were conducted in room temperature that was higher than field

259 temperatures. The metabolic change induced by the temperature increase is not

260 expected to change the respiratory substrate and therefore no direct effect on $\Delta^{14}C_{resp}$ is

261 expected. However, indirect effect is faster change in the substrate age due to faster

262 depletion of the substrate pools, which is also expected to affect $\delta^{13}C_{resp}$ (Tcherkez et

263 al., 2003). The repeated incubations are expected to provide information about the rate

264 of this depletion effect. In addition, the temperature-induced metabolic change can

265 affect $\delta^{13}C_{resp}$ directly since the temperature sensitivity of the processes emitting CO_2 is

266 different (Kodama et al., 2008). Thus, the $\delta^{13}C_{resp}$ values should be regarded as room-

267 temperature acclimated values and not field values.

268 Short-term incubations

269 Short-term incubations were done in a custom-made solid aluminium chamber equipped

270 with an NDIR CO_2 sensor (COZIR 0%–1% CO_2 Sensor, CO_2Meter, Inc., Ormond

271 Beach FL, USA) and a quenching-based O_2 sensor (LuminOx, Coatbridge, UK). CO_2

272 and O_2 fluxes were calculated by linear fit of the concentration change with time, where

273 the fit's slope is equivalent to the term $\Delta CO_2/I_t$ in Eqn 2. Incubations with R^2 <0.9 in any

274 of the gases were discarded.

275 For the correction of the flask-measured CO_2 efflux rates (measured at room

276 temperature) to field (in situ) temperatures we determined Q_{10}. For that purpose

277 incubations with roots collected in the September-October campaigns were performed

278 at two or three different temperatures (ranging between 5°C and 22°C). For the

279 calculation we used the R package *respirometry* that fits the measured CO_2 efflux (R) in

280 given temperatures (T) with the equation $R = a \times e^{b \times T}$ and then calculates $Q_{10=} e^{10 \times b}$. We

281 assume the computed Q_{10} value is valid also for the June-July campaign following

282 Burton and Pregitzer (2003) who observed little to no seasonal temperature acclimation

283 in fine roots. Soil temperature in the top 10 cm where the roots were collected is

284 coupled to changes in air temperature with some lag time (Brown, Pregitzer, Reed, &

285 Burton, 2000). To estimate air temperature at the site we added 1.6° C to the measured

286 temperature at the weather station due to altitude difference (196 m) and assumed

287 mean lapse rate of 0.8°C every 100 m. Soil temperature was estimated as the average

288 air temperature over the previous 7 days.

289 Radiocarbon analysis

290 For radiocarbon analysis respired CO_2 from the flasks or CO_2 from combusted solid

291 samples were cryogenically purified and graphitized on iron in the presence of H_2 at

292 550°C (Muhr et al., 2018; Steinhof et al., 2017). The graphitized samples were analyzed

293 by accelerator mass spectrometry (AMS; Micadas, Ionplus, Switzerland) in the

294 radiocarbon laboratory in Jena, Germany (Steinhof et al., 2017). Radiocarbon data are

295 expressed as $\Delta^{14}C$ (‰) and calculated according to S. E. Trumbore et al. (2016):

296 $$\Delta^{14}C = \left[\frac{R_{-25} \times e^{\frac{1950-x}{8267}}}{0.95 \times R_{oxalic,-19}} \right] \times 1000 \qquad \text{Eqn 1}$$

297 Where R_{-25} is the $^{14}C/^{12}C$ ratio corrected to mass dependent fractionation that may occur

298 during flask sampling, CO_2 purification, and AMS analysis. For the correction the

299 sample's $\delta^{13}C$ is normalized to $\delta^{13}C$ of -25‰. The exponent corrects for decay of ^{14}C in

300 the sample from the year of growth 'x' to 1950. For individual tree rings x matched the

301 estimated year. $R_{oxalic,-19}$ is the $^{14}C/^{12}C$ ratio in the standard, oxalic acid, normalized to

302 $\delta^{13}C$ of -19‰, and the 0.95 term converts to the absolute radiocarbon standard (1890

303 wood) activity at 1950.

304 The measurement precision for each AMS measurement of ^{14}C was 2-3‰. To estimate

305 the mean age of measured C pool in this study we calculated the difference between its

306 $\Delta^{14}C$ value to $\Delta^{14}C_{atm}$ during 2018 growing season, divided by the mean annual decline

307 in $\Delta^{14}C_{atm}$ (see results).

308 Our samples assumed to contain bomb-radiocarbon with $\Delta^{14}C$ equal or above $\Delta^{14}C_{atm}$,

309 therefore any samples significantly below $\Delta^{14}C_{atm}$ have to be considered contaminated

310 with extraneous fossil C. One batch with 9 samples was discarded due to many results

311 with highly negative values.

312 CO$_2$ efflux rate and $\delta^{13}C$ analysis

313 The CO_2 concentration and $\delta^{13}C$ in the two-day incubations were determine from a sub-

314 sample of air from one flask analyzed in Isotope Ratio Mass Spectrometer (IRMS;

315 Delta+ XL; Thermo Fisher Scientific) coupled to a modified gas bench with Conflow III

316 and GC (Thermo Fisher Scientific, Bremen, Germany). Air from the flask was expanded

317 into a pre-evacuated 12-ml Labco extainer (Labco Ltd, Lampeter, UK) equipped with a

318 septum cap. Argon gas (Ar) was added to the exetainer to create a small over pressure

319 to enable passive sampling by the auto-sampler (CTC Combi-PAL autosampler, CTC-

320 Analytics, Zwingen, Switzerland). The air pressure values in the exetainer before and

321 after the Ar addition were recorded for pressure correction. Each exetainer was

322 sampled twice (30 or 50 μL per aliquot) by the auto-sampler and the aliquots were

323 injected into the IRMS. Samples were analyzed against a laboratory air standard on the

324 VPDB scale [Jena Reference Air Set-06 (JRAS-06), (Wendeberg, Richter, Rothe, &

325 Brand, 2013)]. For estimating the CO_2 concentration the m/z peaks 44, 45, and 46 were

326 integrated and calibrated against samples of standard gas with a known concentration

327 of 2895 ppm. $\delta^{13}C_{resp}$ and CO_2 concentration were determined by the mean values of the

328 duplicate injections. For $\delta^{13}C_{resp}$ the mean standard deviation (SD) of the duplicate

329 samples was 0.04‰. For CO_2 concentration the mean SD for the duplicate samples

330 was 14 ppm, regardless of the samples' concentration.

15

331 We further tested the CO_2 concentration evaluation using the calibrated IRMS peak

332 area by analyzing flasks filled with known CO_2 concentrations with the same

333 measurement IRMS protocol used for the samples. We observed overestimation of the

334 IRMS measurements ranging between 4% at 10,000 ppm and 18% at 50,000 ppm.

335 Empirical corrections based on a polynomial fit were used to correct this overestimation.

336 After correction, the differences between known to corrected CO_2 concentrations was

337 0% on average and for 90% of the measurements the difference was < 2% of the

338 measured concentration. In the same test we found that the $\delta^{13}C$ was stable when

339 varying the injection volume up to 200 μL and in flask air pressures between 800 and

340 1000 hPa. It suggests there are no isotopic fractionations in the sub-sampling

341 procedure.

342 The mean CO_2 efflux during the incubation period normalized to the sample dry weight

343 per day (mg C g^{-1} day^{-1}) was calculated using Eqn 2:

344 $$CO_2\,Efflux = \frac{\Delta CO_2}{I_t} \times \frac{V_{HS} \times BP \times M_C}{T \times M_{dm} \times R}$$ Eqn 2

345 where ΔCO_2 is the net change in CO_2 concentration (ppm/10^6) during the incubation

346 (equal to the measured CO_2 concentration), I_t is the incubation time (days), V_{HS} is the

347 volume of the headspace (269 mL), BP is the local barometric pressure (hPa), M_C is the

348 molar mass of C (12 mg $mmole^{-1}$), T is the temperature of incubation (295°k), M_{dm} is the

349 dry mass of roots (g), and R is the ideal gas constant (83.14 mL hPa k^{-1} $mmol^{-1}$). The

350 estimated error in the CO_2 efflux due to propagated uncertainties in ΔCO_2, V_{hs}, BP, and

351 M_{dm} is estimated at 4% of the reported value.

352 For $\delta^{13}C_{ws}$ measurement the capsuled water-soluble C was combusted into CO_2 in

353 elemental analyzer (NA 1110, CE Instruments, Milan, Italy) coupled to a Delta$^+$XL IRMS

354 (Thermo Finnigan, Bremen, Germany) via a ConFlow III. Samples were analyzed

355 against laboratory standards on the VPDB scale.

356 The apparent isotopic fractionation (Δ_R, ‰) in respiration was estimated with the

357 equation:

358 $$\Delta_R = \delta^{13}C_{ws} - \delta^{13}C_{resp} \hspace{4cm} \text{Eqn 3}$$

359 Estimations of C pool sizes and age

360 The first approach (approach 1, Table 1) is based on the view that storage C age

361 follows 'last in, first out' dynamics (Lacointe, Kajji, Daudet, Archer, & Frossard, 1993), in

362 which the most recent C transported to the root is the most accessible for respiration

363 (Carbone et al., 2013). Accordingly two pools were defined: (1) an 'active' pool that

364 supports metabolism and respiration, derived from recently transported C to the roots

365 (in intact roots under no C limitation, recently fixed C < 1 yr), and (2) a more passive

366 'stored' pool only becoming active when the supply of transported C is reduced (Fig. 2)

367 (Herrera-Ramirez et al., 2020; Richardson et al., 2013). We measured respired CO_2 as

368 a proxy to estimate the age and size of the metabolically 'active' pool, with $\Delta^{14}C_{resp}$

369 representing its age. To estimate the amount of storage C < 1 yr old (the active pool

370 size in intact roots) we used the repeated respiration measurements. First, C with $\Delta^{14}C$

371 signature of 1 yr was defined as the 2018 $\Delta^{14}C_{atm}$ + the mean yearly $\Delta^{14}C_{atm}$ change (see

372 Eqn 5 in Results). Assuming excised roots will access increasingly older (higher $\Delta^{14}C$)

373 C, we estimated when the CO_2 respired by the roots increased beyond this 1 yr

374 threshold ($t_{depletion}$). Integration of the fitted CO_2 efflux vs. time curve between time = 0

375 until $t_{depletion}$ yielded an estimate of the size of the 'active' pool of C younger than 1 yr in

376 intact roots. This approach is indifferent to the actual storage compound that supports

377 respiration.

378 We developed the second approach (approach 2, Table 1) on the basis of the strong

379 correlations between $\Delta^{14}C_{ws}$ and tissue age and the presence of sugars in old tissues

380 that were reported previously (Furze et al., 2020; Richardson et al., 2015; S. Trumbore

381 et al., 2015). Those reported findings can be explained by large fraction of water-soluble

382 C with the same age of the containing tissue (e.g. annual ring), which further imply only

383 a limited mixing of water-soluble compounds with other tissues. The mechanism that

384 prevents mixing of soluble compounds might be their deposition in vacuoles. Fine roots

385 contain annual rings similarly to stems (Solly et al., 2018), therefore a root sample can

386 be an analogue to a whole tree-stem sample. The $\Delta^{14}C_{ws}$ signature of a root is thus the

387 mean value of a spectrum of $\Delta^{14}C$ values, with fresh C recently transported to the root at

388 the young end, and C stored in the innermost ring in the old end. The measured $\Delta^{14}C_{ws}$

389 value can be further simplified to a weighted mean of the two functional C fractions (Fig.

390 2): a proportion of active C, F_{active}, with radiocarbon signature $\Delta^{14}C_{active}$ and the stored

391 fraction C, F_{stored} (= 1 − F_{active}), with radiocarbon signature $\Delta^{14}C_{stored}$. The mass balance is

392 described by (Eqn 4):

393 $\Delta^{14}C_{ws} = F_{active} \times \Delta^{14}C_{active} + F_{stored} \times \Delta^{14}C_{stored}$ \hspace{2cm} Eqn 4

394 We assume that the stored pool C consists of C deposited as storage reserves

395 concurrent with root growth, thus $\Delta^{14}C_{stored}$ can be approximated by the measured

18

396    $\Delta^{14}C_{cell}$. Even if old C with high $\Delta^{14}C$ signature is allocated to a root, the formed structural

397   C and storage C are expected to share the high isotopic signature and the assumption

398   will still hold. Using the linear regression estimates for the relation between the

399   measured $\Delta^{14}C_{ws}$ with $\Delta^{14}C_{cell}$ enables us to solve the other variables of Eqn 4; The slope

400   of the linear equation is equal to F_{stored}, F_{active} equals $1 - F_{stored}$, and the intercept equals

401   to $F_{active} \times \Delta^{14}C_{active}$, thus $\Delta^{14}C_{active}$ = intercept / F_{active}. Using this approach, we do not

402   assume an age for the active C (e.g. < 1 yr); instead we estimate the $\Delta^{14}C$ of F_{active} from

403   the $\Delta^{14}C_{ws}$ value that is not explained by $\Delta^{14}C_{cell}$. The model estimates are based on

404   roots from different trees hence they represent averaged values for several trees (n = 5

405   – 12).

406   It is important to note that the water-soluble fraction contains sugars, but also many

407   other soluble compounds like tannins and amino and organic acids. S. Trumbore et al.

408   (2015) estimated that ~50% of the C in the soluble fraction extracted from wood (they

409   used methanol: water mixture as a solvent) originates from the sugars sucrose, glucose,

410   and fructose. Here, we assume that on timescales of > 1yr exchange of C between

411   cellular metabolites means that the $\Delta^{14}C_{ws}$ signature approximates that of sugars.

412   Statistical analysis

413   All data were analyzed using *R (R Core Team, 2019)*. We tested effects of treatment

414   (Pre-girdling, girdling, and control) and root class (coarse, fine) on the different

415   measures. Normality was tested with the Shapiro-Wilk test and equality of variance with

416   Leven's test. When both assumptions were not violated we proceed with one or two-

417   way ANOVA followed by Tukey's HSD post-hoc test. When the assumptions of

418 normality and/or homogeneity were violated we used the non-parametric Kruskal–Wallis

419 rank sum test, followed by pairwise Wilcoxon Rank Sum Tests. For the linear models

420 we used the *lm* function.

Results

NSC concentrations

423 Total sugar concentrations did not vary by treatment (P= 0.91, Kruskal-Wallis) or root

424 size class (P= 0.91, ANOVA) (Fig. 3a). The overall mean ± SE (n = 47) was 42.9 ± 3.1

425 mg glucose g^{-1} dry root, equivalent to 17.2 ± 1.2 mg C g^{-1} (Table S2). The mean

426 composition of the C (in glucose equivalents) was: sucrose 69.4%, glucose 16.6%, and

427 fructose 14.0%. Starch measured in girdling and control treatments was significantly

428 higher (P= 0.04, Kruskal-Wallis) in the coarse roots than in the fine roots, with mean

429 values of 4.8 ± 1.2 and 1.5 ± 0.3 mg C g^{-1}, respectively (n = 12).

CO_2 efflux rate

431 The CO_2 efflux rate in the two-day incubations was significantly higher in the fine roots

432 than in the coarse roots (P< 0.01, Kruskal-Wallis) with mean ± SE values of 3.0 ± 0.3

433 and 2.0 ± 0.2 mg C $g^{-1}d^{-1}$, respectively (Fig. 3b). The mean Q_{10} measured in September-

434 October campaigns was 2.3 ± 0.1 (n = 11) with no effect of root class or treatment.

435 Correcting the CO_2 efflux rates measured in the laboratory to soil temperature

436 decreased the values, but the root size effect maintained significant (P< 0.01, Kruskal-

437 Wallis), where mean values of 1.2 ± 0.1 and 0.8 ± 0.1 mg C g^{-1} d^{-1} were assessed for the

438 fine and coarse roots, respectively (Fig. 3b). We used the mean efflux rates corrected to

439 the daily mean of soil temperature to estimate the total amount of C respired by fine and

440 coarse roots during the 82 days between the girdling and the first post-girdling

441 campaign; these were 150 ± 19 and 100 ± 12 mg C g^{-1}, respectively (uncertainty was

442 calculated by varying the mean efflux rates and Q_{10} with their uncertainties).Accordingly

443 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

444 the coarse roots.

445 Radiocarbon

446 Combining measures of $\Delta^{14}C_{cell}$ in tree-ring late wood, leaf $\Delta^{14}C_{resp}$, and the atmospheric

447 record in the region, the annual decline of $\Delta^{14}C_{atm}$ averaged 4.7‰ per year during the

448 last two decades (Fig. 4). Our estimate for the atmospheric $\Delta^{14}C_{resp}$ during the 2018

449 growing season was +2.3‰, the mean $\Delta^{14}C_{resp}$ of the roots of the control trees. This

450 value is within the 0.6 – 4.4‰ range of $\Delta^{14}C_{atm}$ mean values measured during the 2018

451 growing season at several ICOS stations (Table S1). Thus, we calculated the mean age

452 (yr) of a C pool with radiocarbon signature $\Delta^{14}C$ using Eqn. 5:

453 $$Mean\,age = \frac{\Delta^{14}C - 2.3‰}{4.7‰/yr}$$

454 Eqn. 5

455 Root respired $\Delta^{14}C_{resp}$ values were mostly similar to the $\Delta^{14}C_{atm}$ in the year of collection,

456 with overall mean ± SE of 4.8 ± 0.9 (0.5 ± 0.2 yr; n =36) and range of -5.2‰ to +23.7‰

457 (0 - 4.6 years). The mean $\Delta^{14}C_{resp}$ when both root classes are pooled together was

458 higher in the girdled trees (7.9 ± 1.9; n =12) than the pre-girdling (4.2 ± 0.8; n =12) and

459 the control (2.3 ± 1.8, n = 12) (Fig. 5). The differences between the treatments were not

460 significant (P= 0.14-0.20, Wilcoxon). However, the mean $\Delta^{14}C_{resp}$ of the girdled trees was

461 significantly higher than the control trees (represent current atmosphere) when

462 compared exclusively ($P= 0.04$, Wilcoxon). The mean age of the respired $\Delta^{14}C_{resp}$ in the

463 girdled trees was 1.2 ± 0.4 yr, compared to 0 ± 0.4 yr in the control roots.

464 The $\Delta^{14}C_{ws}$ values were higher than $\Delta^{14}C_{resp}$ for the same root samples, ranging between

465 0.8 and 90.7‰ (0 - 18.8 yr). The linear regression model equations for the $\Delta^{14}C_{ws}$ vs.

466 $\Delta^{14}C_{cell}$ relationship, its statistical information, and approaches 1 and 2 predictions are

467 presented in Table 2 and Figs. 5, 6. The variability in $\Delta^{14}C_{cell}$ explained the majority of

468 the variability in $\Delta^{14}C_{ws}$ ($r^2 > 0.8$ in most groups; Table 2; Fig. 6). The slope estimates

469 based on the linear fit equations were highly significant in most subgroups ($P < 0.01$),

470 except in the fine roots of the girdled trees due to one outlier with much higher $\Delta^{14}C_{ws}$

471 than $\Delta^{14}C_{cell}$ (Fig. 6). According to approach 2, F_{stored} equals the fitted line slope, hence

472 the overall F_{stored} ($\pm$ SE of the model) is 0.7 ± 0.05 and values in the subgroups range

473 between 0.55 ± 0.03 and 0.8 ± 0.1 (without the exceptional girdled fine roots). The

474 overall F_{active}, which equals $1 - F_{stored}$, is 0.3 with range of $0.2 - 0.45$ in the subgroups.

475 Significant intercept estimates were computed only for the coarse roots and coarse +

476 fine roots of the control trees ($P < 0.05$), while in the other subgroups the errors were

477 rather large (Table 2). The uncertainties in $\Delta^{14}C_{active}$ estimations (= Intercept / F_{active}) are

478 also large as a result of the propagation of the intercept errors. The overall $\Delta^{14}C_{active}$

479 calculated by approach 2 is 5.7 ± 5.9‰ with an estimated age of 0.7 ± 1.3 yr (Table 2).

480 Among the measured C pools the $\Delta^{14}C_{cell}$ values were the highest and ranged between

481 2.6 and 123.6‰ (0 - 25.8 years). Coarse roots had higher mean $\Delta^{14}C_{cell}$ (34.4 ± 5.9‰; n

482 = 23; 6.8 ± 1.3 yr) than fine roots (24.0 ± 2.7‰; n = 24; 4.6 ± 0.6 yr), but the relation

483 was not statistically significant (P= 0.40, Kruskal-Wallis).

Repeated incubations

485   Temporal changes in all measured parameters were observed between day 1 and day 7

486   of the repeated incubations (Fig. 7). Mean $\Delta^{14}C_{resp}$ ($\pm$ SE) increased more rapidly in the

487   fine roots (from 3.2 $\pm$ 0.8 to 16.1 $\pm$ 2.1‰) than in the coarse roots (from 4.4 $\pm$ 0.3 to 10.1

488   $\pm$ 0.9‰), corresponding to estimated ages of 2.9 $\pm$ 0.5 and 1.6 $\pm$ 0.2 yr (Fig 7a) at the

489   end of incubation, respectively. The CO_2 efflux rates declined over time, but remained

490   higher in fine roots compared to coarse roots (Fig 7b).

491   Following approach 1, any carbon with $\Delta^{14}C$ < 7‰ (the sum of $\Delta^{14}C_{atm}$ (2.3‰) and annual

492   decline (4.7‰)) was defined as younger than 1 yr. Using linear regression, we

493   estimated that the 7‰ value was exceeded after 2.3 and 4.3 days (= $t_{depletion}$) in fine and

494   coarse roots, respectively, indicating depletion of the young C pool (Fig. 7a). Integrating

495   the total CO_2 efflux over time (fitted curves in Figure 7b) between time = 0 and

496   time = $t_{depletion}$ gives an estimate of the active pool: 5.3 and 5.1 mg C g^{-1} for fine and

497   coarse roots, respectively. The total amount of C respired over the 7 days of incubation

498   was calculated as 13.4 and 7.1 mg C g^{-1} for fine and coarse roots, respectively, which is

499   equivalent to 66 and 35% of the total mean NSC-C in fine and coarse roots,

500   respectively.

501   The $\delta^{13}C$ of CO_2 respired by coarse-roots ($\delta^{13}C_{resp}$) did not change between the first (-

502   27.40 $\pm$ 0.16‰) and second incubation (-27.36 $\pm$ 0.06‰), while RQ decreased from

503   0.90 $\pm$ 0.15 to 0.65 $\pm$ 0.06 (Fig. 7c,d). Over the same time period the $\delta^{13}C_{resp}$ for fine

504   roots decreased by 0.83‰ (-27.53 $\pm$ 0.09 to -28.35 $\pm$ 0.12) and RQ declined to an even

505   greater degree than in the coarse roots, from 1.08 $\pm$ 0.08 to 0.55 $\pm$ 0.06.

δ^{13}C results

507   The δ^{13}C of the water soluble fraction extracted from roots varied significantly with

508   treatment (P < 0.001; two-way ANOVA) and root size (P < 0.01), with no interaction

509   effect (P = 0.833) (Fig. 8a, Table S2). The post-hoc tests (Tukey's HSD) showed δ^{13}C$_{ws}$

510   in the pre-girdling roots was significantly lower than the girdling and control roots that

511   didn't differ significantly. Water soluble C extracted from fine roots was 0.74‰ more

512   enriched than the coarse root extracts. The treatment had significant effect also for

513   δ^{13}C$_{resp}$ (P < 0.001, Kruskal-Wallis), and the post-hoc test (Wilcoxon) indicates significant

514   difference between all three campaigns: the pre-girdling roots had the lowest mean

515   value, with girdled trees on average 1.15‰ higher, and the control trees 2.64‰ higher

516   than the pre-girdling (Fig. 8b, Table S2). The coarse root δ^{13}C$_{resp}$ was 0.80‰ more

517   enriched than the fine roots (P = 0.052, Kruskal-Wallis). The mean Δ_R value (δ^{13}C$_{resp}$ -

518   δ^{13}C$_{ws}$) for fine roots was higher in 1.53‰ than for coarse roots (P < 0.01; one-way

519   ANOVA; Fig. 8c). The treatment had marginal effect (P = 0.053; Kruskal-Wallis), where

520   the Δ_R in the control roots was significantly lower (wilcoxon test) than in the pre-girdling

521   and girdling (Fig. 8c, Table S2).

522   ## Discussion

523   ### Size and age estimates for active and stored C pools using two approaches

524   Our results provide unique estimates for the size and age of the functional sub-pools of

525   NSC soluble in water. The computed slopes of the linear relation between Δ^{14}C$_{ws}$ and

526   Δ^{14}C$_{cell}$ suggest that the 'stored' pool, i.e. C stored over multiple years, makes up the

527   majority (averaging 70 ± 5%, with a range of 55-80%) of the extracted, water-soluble C

528   (Fig. 6). Sugars make up only a fraction of the water-soluble C used for the $\Delta^{14}C_{ws}$

529   measurement. Assuming sugars contribute in the same proportions as water-soluble C,

530   their total average of 17.1 ± 1.2 mg C g^{-1}, is represented by active pool (30%) of 5.15 ±

531   0.5 mg C g^{-1} and stored pool (70%) of 12.0 ± 0.5 mg C g^{-1} (Table 1; approach 2).

532   Separately, our repeated incubations provided estimates of the pool of C younger than

533   1 yr (approach 1). The C amounts were estimated to be 5.1 mg C g^{-1} for coarse roots

534   and 5.3 mg C g^{-1} for fine roots. The similarity between the different approaches in

535   estimating the 'active' pool size seems to indicate that the <1 yr criterion provides a way

536   to estimate the age and size of the active pool in intact roots. In agreement, the

537   estimates for the active pool age assessed by approaches 1 & 2 are both younger than

538   1 yr: 0.5 ± 0.2 and 0.7 ± 1.3 yr, respectively (Table 2). Richardson et al. (2013) provide

539   some references for our sub-pool estimations. They compared outputs of models for

540   whole tree C allocation and NSC dynamics with measured NSC concentrations and ^{14}C

541   ages in tree stems. When the active ('fast') NSC pool age was constrained to < 1yr its

542   percentage of the total NSC was 23 - 56% for the best fits, compared with our estimates

543   of 20 – 45% for roots (Table 2).

544   Despite the agreement in the overall age estimated by the two approaches, for

545   individual subgroups the two methods disagree (Fig. 5, Table 2). There are apparently

546   two reasons. The first reflects large uncertainties in the linear model's prediction of the

547   intercept value that approach 2 is based on, which leads to extreme and unrealistic

548    $\Delta^{14}C_{active}$ values, e.g. equivalent to -5.4 yr for coarse roots during pre-girdling (Table 2).

549   Still, considering the uncertainties, the two approaches to estimate $\Delta^{14}C_{active}$ mostly

550 agree (Fig, 5). Also, the tendency to use older C to fuel respiration (i.e. transferring

551 older C to the active pool) in the girdled-trees roots that was predicted and observed in

552 approach 1 ($\Delta^{14}C_{resp}$), was also observed in approach 2 (Fig. 5).

553 The second source for the disagreement between the methods is potentially true

554 difference, as apparent by the results for the control roots, where the estimate from

555 approach 2 for $\Delta^{14}C_{active}$ is significantly higher than approach 1 considering the

556 uncertainties (Fig. 5, Table 2). It suggests the active pool might be not perfectly mixed,

557 and can be further subdivided into two pools: the most active pool that supports

558 respiration (and is usually the youngest), and an intermediate pool with $\Delta^{14}C_{ws}$ that is not

559 explained by variability in $\Delta^{14}C_{cell}$.

560 The stored fraction should be also considered in driving Δ_R values

561 Water deficit in the soil was expected to increase from midsummer to the end of the

562 growing season because of the 2018 hot drought conditions (Fig. 1). In control (un-

563 girdled) trees, we observed enrichment in both $\delta^{13}C_{ws}$ and $\delta^{13}C_{resp}$ measured in Sept/Oct

564 compared to the pre-girdling roots measured in June-July. These $\delta^{13}C_{resp}$ increases are

565 in line with expected water shortage effects on leaf-level fractionation (Fig. 8) (Farquhar

566 & Sharkey, 1982; Madhavan, Treichel, & Oleary, 1991; Pate & Arthur, 1998; Scartazza,

567 Moscatello, Matteucci, Battistelli, & Brugnoli, 2015). Smaller enrichment in $\delta^{13}C_{ws}$ can be

568 explained by stored pool dilution. A simple mass balance calculation using the relative

569 enrichments in $\delta^{13}C_{ws}$ (+1.12‰) and $\delta^{13}C_{resp}$ (+2.64‰) between control and pre-girdling

570 provides an estimate for F_{active}: 1.12/2.64= 0.42, nearly equaling the 0.43 predicted by

571 approach 2 for the control trees (Table 2). This suggests that ^{13}C-enriched sugars

572 produced in control-tree leaves are mixed into the active pool in the roots where they

573 support respiration, while the stored water-soluble C integrates more depleted $\delta^{13}C$

574 values inherited from previous years. Thus the apparent decrease in Δ_R in the control

575 roots does not reflect a metabolic shift (e.g. reduced PEPC activity) in the roots. In

576 contrast, the enrichment between pre-girdled and girdled roots is larger for $\delta^{13}C_{ws}$

577 (+1.52‰) than for $\delta^{13}C_{resp}$ (+1.15‰), perhaps reflecting differences in transported

578 substrates.

579 The carbon balance in girdled-tree roots was maintained by translocation of stored NSC

580 The ability of roots to access older C for respiration when new C supply is cut off was

581 demonstrated in the repeated incubations and in the girdling experiments. However, the

582 supply of older C was not the same in both cases. Over the 7 days of the repeated

583 incubations, the coarse and fine roots respired 7.1 and 13.4 mg C g^{-1}, respectively,

584 roughly 35-70% of the 20.3 mg C g^{-1} of NSCs contained in the incubated roots (overall

585 sugars and starch mean, Table S2). The estimated ages of the respired C increased

586 from <1 yr to 1.6 and 2.9 yr, for coarse and fine roots, respectively. This is consistent

587 with roots respiring an initial active pool fed by freshly fixed C and switching to older

588 substrates as this pool is exhausted (Herrera-Ramirez et al., 2020). In the case of

589 excised roots, the source of the older C substrate was clearly in the roots themselves.

590 During the months that elapsed after girdling, root respiration rates did not decline as

591 they did for excised roots (Fig. 3b). During this period, assuming constant respiration

592 rates, an estimated 100 and 150 mg C g^{-1} were respired in the coarse and fine roots,

593 respectively. This was roughly 5-7 times higher than the C stored in the NSC pools,

594 which also did not decline following girdling (Fig. 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 may originate from storage in below-girdling parts of the tree, and from neighboring healthy trees. *Populus* trees are known to have root connections with shared root systems. *Populus* root systems can remain functional for at least 20 yr 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 yr stems and have disproportionally 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, Lieffers, & Landhausser, 2006; Gaspard & DesRochers, 2020; Mudge, Janick, Scofield, & Goldschmidt, 2009). Carbon transfer from neighboring trees can occur also via mycelial networks (Rog, Rosenstock, Korner, & Klein, 2020). Indeed, the girdled trees in our study have survived for two 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).

However they arrived, our isotopic measurements indicate that the C translocated into our girdled-tree roots are not fresh photo assimilates. Based on the $\Delta^{14}C_{resp}$ results the mean age of the respired C from girdled roots was older by ~1.2 yr than C respired from the control roots (Fig. 5). This is younger than the respired C at the end of the repeated incubations (1.6 – 2.9 yr). Difference in the $\delta^{13}C_{resp}$ between the girdling and control roots further indicates the roots are not sharing the same respiratory C source (Fig. 8b).

618 The increase in $\delta^{13}C_{resp}$ between pre-girdling and girdling can be explained by hydrolysis

619 of starch, which tends to be more enriched than sugars (Brugnoli, Hubick, von

620 Caemmerer, Wong, & Farquhar, 1988; Damesin & Lelarge, 2003; Gleixner, Danier,

621 Werner, & Schmidt, 1993; Maunoury-Danger et al., 2010).

622 Hints for high PEPC activity in the fine roots

623 The Δ_R mean values by root class and treatment ranged between (-0.35‰) – (-4.38‰)

624 (Fig. 8, Table S2), a slightly wider range than the (-0.7‰) – (-3.1‰) values measured in

625 mature Eucalyptus roots (Gessler et al., 2007). While some of this could be due to

626 differences in temperature effects, these likely do not explain the range in Δ_R values. Δ_R

627 was higher for fine (-1.1‰) than coarse (-2.67‰) roots, a difference reflecting lower

628 $\delta^{13}C_{resp}$ (-0.80‰) and higher $\delta^{13}C_{ws}$ (+0.74‰) in the fine roots (Fig. 8). This pattern is in

629 agreement with the expected net isotopic effect of refixation of internal CO_2 by PEPC: a

630 ^{13}C depletion of the respired CO_2 and a ^{13}C enrichment of the products (Werner &

631 Gessler, 2011).

632 Additional support for CO_2 fixation with high PEPC activity in roots comes from the

633 decline in the respiration quotient (RQ) from ~1 to ~0.6, during our repeated

634 incubations. PEPC CO_2 re-fixation would reduce CO_2 efflux and thereby RQ (Hilman et

635 al., 2019). The effect of PEPC on respiration grows as the fraction of its activity from

636 total respiration increases (Badeck et al., 2005), as might occur with repeated

637 incubations. Our results mirror those of Bathellier et al. (2009) who also observed a

638 decrease in RQ from 1.1 to 0.8-0.9 while $\delta^{13}C_{resp}$ remained stable in roots of French

639 bean during 6 days of carbohydrate starvation by darkening. Alternative explanations for

640 the change in RQ exist, including a shift from carbohydrates to lipids as the main

641 respiration substrate (Fig. 7d). However, we observed no simultaneous decline in

642 $\delta^{13}C_{resp}$ that would be expected to accompany such a substrate shift, given the depleted

643 $\delta^{13}C$ value of lipids (Fischer et al., 2015; Tcherkez et al., 2003). Other factors that could

644 cause a decline in RQ include a relative increase of O_2 uptake (e.g. through production

645 of reactive oxygen species associated with cell death during the experiment; (Chae &

646 Lee, 2001). While not conclusive, our results suggest that the potential role of PEPC in

647 roots deserves further exploration.

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Supporting information

892   Additional Supporting Information may be found online in the Supporting Information

893   section:

894   **Table S1** $\Delta^{14}C_{atm}$ measured at four ICOS sites in Europe during 2018 growing season.

895   **Table S2** A summary of the CO_2 efflux, NSC, and $\delta^{13}C$ results (mean ± SE) by

896   treatment and root class.

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Tables and Figures captions

Table 1 Methods for C pool size and age estimation. Approach 1 is based on the $\Delta^{14}C$ signature of root-respired CO_2 ($\Delta^{14}C_{resp}$), 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 ($\Delta^{14}C_{ws}$) and α-cellulose C ($\Delta^{14}C_{cell}$) extracted from roots. Those C extractions represent soluble sugars and structural C, respectively.

Table 2 A summary of approaches 1 & 2 results. The linear model equations are for the relationship between the $\Delta^{14}C$ signatures of the water-soluble C ($\Delta^{14}C_{ws}$) and α-cellulose C ($\Delta^{14}C_{cell}$) 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_{stored}) and active (F_{active}) C pools from the total water-soluble C, and the $\Delta^{14}C$ signature of the active pool ($\Delta^{14}C_{active}$).

Figure 1 Mean monthly rainfall (mm, bars) and air temperature (^{o}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.

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929　Figure 2 Conceptual model indicate the carbon (C) dynamics in a root, represented by

930　the box. Carbon transported into the root is the most accessible to metabolism and

931　respiration, while a portion is allocated to the stored pool. The $\Delta^{14}C$ signature of respired

932　CO_2 is assumed to be equal to the transported C signature. When photosynthesis is not

933　suppressed the transported C has $\Delta^{14}C$ signature of current atmospheric CO_2. Respired

934　CO_2 older than 1 yr might originate from stored pool C, or from transported C

935　translocated from root-external old storage. The $\Delta^{14}C$ of the total sugars, approximated

936　by $\Delta^{14}C_{ws}$, is the weighted age of the young active pool and the older stored pool.

937　Figure 3 Means ± SE of coarse (> 2 mm) and fine (2 ≤ mm) roots collected before

938　girdling (Pre-girdling), ~3 months after girdling (Girdling) and ~3 months after girdling

939　but in un-girdled trees (Control). (a) NSC concentrations separated by molecules.

940　Starch was not measured in pre-girdling roots. n = 11, 12, 6, 6, 6, 6, respectively; (b)

941　CO_2 efflux rates measured in two-day incubations in room temperature (empty bars)

942　and corrected to field temperature (full bars). n = 10, 11, 6, 6, 6, 6, respectively. Below

943　the legends the respective significant statistical tests.

944　Figure 4 The estimated atmospheric $\Delta^{14}C$ signature ($\Delta^{14}C_{atm}$) in the study site during the

945　last two decades. The atmospheric record in gray is the mean $\Delta^{14}C_{atm}$ of the northern

946　hemisphere zone 1 after Hua et al. (2016). Tree rings in orange is the α-cellulose $\Delta^{14}C$

947　signature measured for the late wood in annual rings from years 2010-2018. The blue

948　point is the $\Delta^{14}C$ signature of respired CO_2 from leaves harvested in July 2019, which

949　assumed to represent recent photo-assimilates thus current $\Delta^{14}C_{atm}$. The linear equation

950　indicates the mean annual decline in $\Delta^{14}C_{atm}$ is 4.7‰.

951   Figure 5 The $\Delta^{14}C$ signatures and mean age estimations of the active C pool according

952   to two approaches; approach 1 assumes $\Delta^{14}C_{active}$ equals to the $\Delta^{14}C$ signature of

953   respired CO_2 ($\Delta^{14}C_{resp}$); approach 2 is based on the intercept and slope estimates of the

954   fitted linear line between the $\Delta^{14}C$ signatures of the extracted water-soluble C ($\Delta^{14}C_{ws}$)

955   and α-cellulose C ($\Delta^{14}C_{cell}$). According to this approach the fraction of the active C pool

956   F_{active} = 1 - slope. Roots collected before girdling (Pre-girdling), ~3 months after girdling

957   (Girdling) and ~3 months after girdling but in un-girdled trees (Control). One set of roots

958   was used for respiration incubations (n = 12) and second set was used for the C

959   extractions (n = 23, 12, 11, respectively). Error bars of approach 1 are the standard

960   errors of the $\Delta^{14}C_{resp}$ results, and of approach 2 are the cumulative standard errors of the

961   slope and intercept estimates.

962   Figure 6 (a) A scatter plot of the $\Delta^{14}C$ signatures of the water-soluble C ($\Delta^{14}C_{ws}$) and α-

963   cellulose C ($\Delta^{14}C_{cell}$) extracted from roots, with the equation of the linear model for all

964   results pooled together. The shapes indicate the root class (coarse roots, > 2 mm, fine

965   roots, ≤ 2 mm), and colors indicate the treatment: before girdling (Pre-girdling), ~3

966   months after girdling (Girdling), and ~3 months after girdling in un-girdled trees

967   (Control). Error bars are the analitycal uncertainty; (b) the stored fraction (F_{stored}) from

968   the total water-soluble C as estimated from the slope when the linear model is applied

969   to the different subgroups (n = 46, 11, 12, 6, 6, 5, 6, respectively). For example, the

970   slope in the equation in panel (a) is 0.7 therefore F_{stored} for the subgroup 'All' is 0.7.

971   Labels within the bars are the r^2 of the linear regression. Error bars are the standard

972   error of the slope estimate.

973 Figure 7 Results (mean ± SE) for coarse roots (> 2 mm) and fine roots (≤ 2 mm) from

974 repeated respiration incubations during 7 days. (a) the $\Delta^{14}C$ signature of the respired

975 CO_2 ($\Delta^{14}C_{resp}$). Presented are equations of linear regression models; (b) CO_2 efflux rates.

976 Presented are equations that best fitted the results to the equation y = a × b^{-x}; (c) the

977 $\delta^{13}C$ signature of respired CO_2 ($\delta^{13}C_{resp}$); (d) RQ (ratio CO_2 efflux/O_2 uptake). Results in

978 panels (a) and (c) were measured in two-day flask incubations (n = 4, 3 for coarse and

979 fine roots, respectively), results in panels (b) and (d) were measured in short-term

980 incubations (~20 min, according the consecutive incubations order, n = 3, 4, 3 for

981 coarse roots, and n = 3, 4, 4 for fine roots).

982 Figure 8 Means ± SE of coarse (> 2 mm) and fine (≤ 2 mm) roots collected before

983 girdling (Pre-girdling), ~3 months after girdling (Girdling) and ~3 months after girdling in

984 un-girdled trees (Control). (a) the $\delta^{13}C$ signature of the water-soluble C ($\delta^{13}C_{ws}$).

985 According the order from left to right n = 10, 11, 6, 6, 5, 6; (b) the $\delta^{13}C$ signature of the

986 respired CO_2 ($\delta^{13}C_{resp}$). According the order from left to right n = 9, 10, 6, 6, 6, 6; (c) the

987 apparent fractionation factor ($\Delta_R = \delta^{13}C_{resp} - \delta^{13}C_{ws}$). According the order from left to right

988 n = 7, 9, 6, 6, 5, 6. Letters indicate significant differences between groups.

989