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Rapid report

Winter's bite: beech trees survive complete defoliation due to spring late-frost damage by mobilizing old C reserves

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Summary

• Late frost can destroy the photosynthetic apparatus of trees. We hypothesized that this can alter the normal cyclic dynamics of C-reserves in the wood.

• We measured soluble sugar concentrations and radiocarbon signatures (Δ^{14}C) of soluble nonstructural carbon (NSC) in woody tissues sampled from a Mediterranean beech forest that was completely defoliated by an exceptional late frost in 2016. We used the bomb radiocarbon approach to estimate the time elapsed since fixation of mobilized soluble sugars.

• During the leafless period after the frost event, soluble sugar concentrations declined sharply while Δ^{14}C of NSC increased. This can be explained by the lack of fresh assimilate supply and a mobilization of C from reserve pools. Soluble NSC became increasingly older during the leafless period, with a maximum average age of 5 yr from samples collected 27 d before canopy recovery. Following leaf re-growth, soluble sugar concentrations increased and Δ^{14}C of soluble NSC decreased, indicating the allocation of new assimilates to the stem soluble sugars pool.

• These data highlight that beech trees rapidly mobilize reserve C to survive strong source–sink imbalances, for example due to late frost, and show that NSC is a key trait for tree resilience under global change.

Introduction

The Mediterranean region is extremely vulnerable to climate change (Schröter *et al.*, 2005). One of the biggest changes attributed to global warming is the modification of leaf phenology (Rigby & Porporato, 2008; Gordo & Sanz, 2010). Warmer spring temperatures have led to earlier leaf unfolding in beech (Menzel *et al.*, 2006), increasing the risk of late spring frost damage to young leaves, especially at higher elevation (Vitasse *et al.*, 2018). Frost events with temperatures below −4°C can result in the death of the newly developing shoots and leaves of beech. Such events reduce the photosynthetic area and therefore the supply of newly assimilated C. Depending on the percentage of canopy lost this could result in C imbalance where new C supply no longer meets C demand,

especially since the tree needs additional C to replace lost leaves. Trees are thus potentially forced into using reserve C pools (Dittmar *et al.*, 2006).

Nonstructural carbon (NSC) stored in woody tissues (stem, roots, branches) is mainly constituted by soluble sugars (e.g. glucose, fructose and sucrose) and nonsoluble components (e.g. starch and lipids) (Carbone *et al.*, 2013). These compounds represent the primary C reserves in trees, contributing to plant survival during adverse conditions (Barbaroux *et al.*, 2003; Scartazza *et al.*, 2013; Gavrichkova *et al.*, 2019) and the production of new leaves after artificial defoliation (Cerasoli *et al.*, 2004). Their accumulation and mobilization depends on several internal and external factors including the availability of recent photosynthates and allocation to competing sinks (Moscatello *et al.*, 2017). The

amount of NSC available for recovery is sufficient to rebuild the whole canopy at least once (Würth *et al.*, 2005), but potentially up to four times (Hoch *et al.*, 2003).

Trees can mobilize NSC of different ages, depending on their physiological needs. Vargas *et al.* (2009) showed that C fixed on average up to 10 yr previously was used to produce new fine roots after defoliation caused by hurricane Wilma and suggested that these stored reserves play a critical role in the resilience of natural ecosystems. The age of NSC can be estimated on annual to decadal timescales by using the bomb-radiocarbon approach (Gaudinski *et al.*, 2001). Aboveground tests of thermonuclear weapons during the early 1960s released radiocarbon (^{14}C) into the atmosphere, nearly doubling its atmospheric concentration. Since 1964, when atmospheric nuclear weapon testing was banned, the amount of ^{14}C in atmospheric CO_2 has been declining due to net uptake of excess 'bomb' ^{14}C into oceanic and terrestrial C pools and the emission of ^{14}C-free CO_2 from the combustion of fossil fuels (Levin & Kromer, 2004). As Δ^{14}C data are corrected for mass-dependent isotope fractionation, newly assimilated plant C has the same signature (Δ^{14}C) as atmospheric CO_2 at the time of fixation (Levin & Hesshaimer, 2000).

A severe late frost event occurred in the night between 25 and 26 April 2016 and affected a large forest area in central and southern Italy. Satellite observations over Abruzzi, Basilicata and Calabria regions revealed that this event had a particularly strong impact on beech forests (Bascietto *et al.*, 2018; Greco *et al.*, 2018; Nolè *et al.*, 2018).

The main objective of this work was to characterize the physiological response of beech in terms of C reserve dynamics after a complete defoliation caused by the late frost. We focused on the soluble NSC, considering that this fraction can respond quickly to an altered source sink condition, and used the bomb radiocarbon approach to estimate the C age of mobilized C during the year affected by the late frost.

Materials and Methods

Study site

The study was carried out between April 2016 and May 2017 at the Selva Piana stand (41°50′58″N, 13°35′17″E, 1560 m elevation), an experimental area of the LTER (Long Term Ecological Research) Network established in 1991 in a pure mature beech (*Fagus sylvatica* L.) forest (*c.* 110-yr-old) in the Central Apennine in the municipality of Collelongo (Abruzzi region, central Italy). The Selva Piana stand is part of a 3000 ha community forest included in the wider forest area of the external belt of the Abruzzi National Park.

During the period 1950–2013 the mean annual temperature was 7.4°C, and the mean annual precipitation was 1024 mm (Rezaie *et al.*, 2018), of which *c.* 10% falls in summer (Guidolotti *et al.*, 2013). On the night between 25 and 26 April 2016 the temperature reached −6°C at the canopy level, destroying the whole stand canopy (Fig. 1).

Fig. 1 In the upper panel, photograph shows the situation at 19 May 2016 of the Selva Piana forest (Collelongo, Abruzzi region). In the lower panel, images of beech (*Fagus sylvatica*) tree canopies from April to August 2016.

Phenological data

Leaf area index (LAI) was monitored using the MODIS LAI (MCD15A3H.006, https://lpdaacsvc.cr.usgs.gov/appears) with a temporal resolution of 8 d and 500 m pixel size centred on the experimental site coordinates. Using the maximum rates of change in the curvature of the logistic models fitted to the LAI time trend we identified critical dates (see Fig. 2a) that represent transitions from one approximately linear stage to another: (1) green-up, photosynthetic activity onset; (2) senescence, sharp decrease of photosynthetic activity and green leaf area; (3) winter dormancy (Zhang *et al.*, 2003).

Tree selection, cellulose of tree ring, xylem sample

Five representative trees with diameters at breast height ranging from 49 to 53 cm were selected according to their similarity with site tree ring chronology (Gleichläufigkeit (GLK) ranging from 70 to 85; for more information on tree selection see Rezaie *et al.*, 2018).

The selected trees were located in proximity to each other (all in a ring with *c.* 50 m diameter) at the same elevation (1560 m above sea level (asl)) in a gently sloping area of the forest.

To ensure that the site was suitable for the bomb-radiocarbon approach, we reconstructed the local atmospheric $\Delta^{14}C$ by analysing $\Delta^{14}C$ in cellulose extracted from tree rings for the years 2005, 2007, 2009, 2011, and 2013. Cellulose was extracted according to Boettger *et al.* (2007).

For analysis, we extracted 1.5 cm long micro-cores with a diameter of 2 mm from the stem at 1.1–1.5 m above the ground using the Trephor tool (Rossi *et al.*, 2005) on 12 dates from April 2016 to May 2017. This sampling depth is considered as the most representative for characterization of soluble NSC age (Richardson *et al.*, 2013). Collected samples were immediately put on ice in a cooler for transport and stored at −20°C until processing. Frozen wood samples were manually milled in a mortar with liquid nitrogen before biochemical analyses (Scartazza *et al.*, 2013; Moscatello *et al.*, 2017).

Radiocarbon analysis

For ^{14}C analyses, soluble NSC was extracted by heating 15–50 mg of milled wood in 5 ml of water at 65 °C for 10 min for three times in pre-baked (550 °C for several hours) glass vials. Ethanol or methanol, as suggested in other methods, are not suitable for isolating NSC for ^{14}C analysis because they introduce exogenous C that affects the ^{14}C content of the NSC pool (Carbone *et al.*, 2013). The extract material was composed mainly by soluble sugars, but likely contained several additional C compounds as reported by Trumbore *et al.* (2015).

For radiocarbon measurements of tree ring cellulose and soluble NSC, *c.* 0.5 mg C were transferred into the tin cups, air-dried in a fume-hood, processed according to Steinhof *et al.* (2017) and then measured by accelerator mass spectrometry (MICADAS, Ionplus, Dietikon, Switzerland) at the Max-Planck Institute for Biogeochemistry in Jena (Germany). Radiocarbon data are expressed as $\Delta^{14}C$, which is the per mil deviation from the $^{14}C : ^{12}C$ ratio of oxalic acid standard in 1950. A measurement precision and accuracy of 3 to 4‰ was estimated based on repeated analysis of laboratory standards. The sample $^{14}C : ^{12}C$ ratio has been corrected to a $\delta^{13}C$ value of −25‰ to account for any mass-dependent fractionation effects (Stuiver & Polach, 1977). Due to this correction, $\Delta^{14}C$ is identical for atmospheric CO_2 and the photosynthetic products fixed from it, allowing us to compare samples $\Delta^{14}C$ values directly to the atmospheric $\Delta^{14}CO_2$ record.

Soluble sugar concentrations

Soluble sugars are the most abundant compounds among NSC. Mainly in this form C is directly involved in plant primary metabolism after mobilization. For measuring soluble sugar concentrations, we extracted 10 mg of milled wood with 1.5 ml of 80% ethanol at 80 °C for 45 min. After cooling and centrifugation at 13 000 *g* for 5 min, the supernatant was analysed by high-performance anion exchange chromatography, with pulsed amperometric detection (HPAEC-PAD, Thermo Scientific™Dionex™ICS-5000, Sunnyvale, CA, USA). For details see Proietti *et al.* (2017).

Statistical analysis

Linear regression was used to assess the decline in $\Delta^{14}C$ of tree ring cellulose over the years before 2016, as well as to search for trends in soluble sugar concentrations and $\Delta^{14}C$ of soluble NSC. We tested data normality and constant variance using Shapiro–Wilk test and the Spearman rank correlation between the absolute values of the residuals and the observed value of the dependent variable, respectively. Analysis and graphics were performed using R 3.5.0 (R Development Core Team, 2018).

Results

Leaf phenology

A rapid increase in LAI on day of the year (DOY) 95 in 2016 and DOY 120 in 2017 (Fig. 2a) indicated the green-up of the canopy. In 2016, a second green-up occurred on DOY 178 (28 June), after the canopy was destroyed by the late frost of 25 April. Thus, the stand experienced a late spring leafless period of 53 d. In 2017, senescence started on DOY 252 (8 September) and the start of dormancy was assigned to DOY 287 (13 October). The maximum LAI observed was lower in 2016 (4.79 m² m⁻²) than in 2017 (5.37 m² m⁻²).

Radiocarbon data

The $\Delta^{14}C$ of cellulose that we used to reconstruct the local atmospheric $\Delta^{14}C$ decreased from *c.* 60‰ in 2005 to *c.* 28‰ in 2013, corresponding to an annual decline of *c.* −3.9‰ yr⁻¹ ($r^2 = 0.985$). Comparison of tree ring cellulose $\Delta^{14}C$ with unpolluted atmospheric $\Delta^{14}CO_2$ conditions measured at the Schauinsland observatory (Hammer & Levin, 2017) indicated no
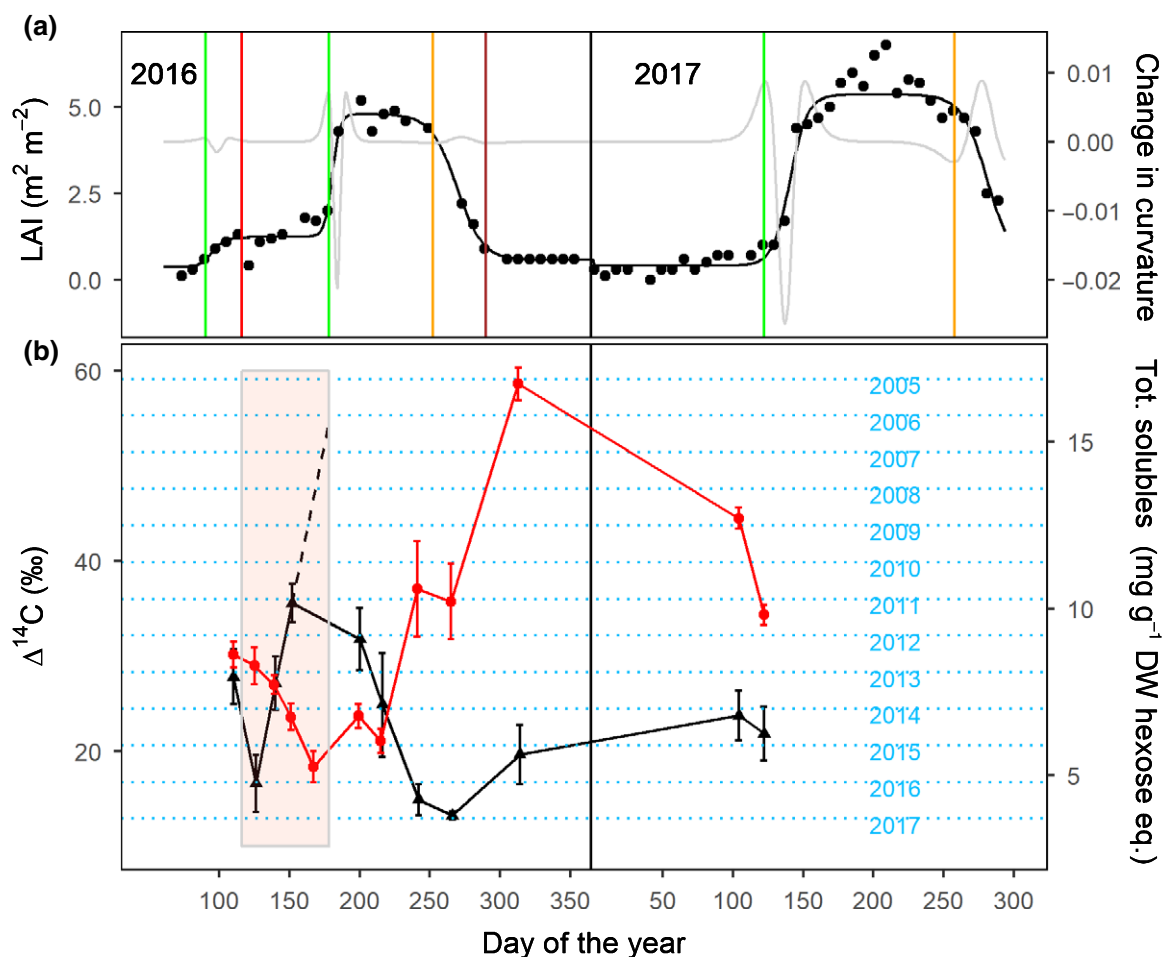
Fig. 2 (a) Leaf area index (LAI) of the experimental area derived from Moderate Resolution Imaging Spectroradiometer (Modis) with 8-d of temporal resolution and 500 m pixel size in 2016 and 2017. Dots are raw Modis LAI values. Solid black line is the modelled pattern of LAI, using two logistic for the increasing and decreasing phases. Solid grey line is the rate of change in curvature. Red vertical line represents the late frost occurred during the night between 25 and 26 April 2016. Green vertical line is the green-up; orange vertical line is the onset of senescence; brown vertical line represents the dormancy. (b) Mean ($\pm$ SE) of Δ^{14}C of soluble nonstructural carbon (NSC, black) and the concentration of soluble sugars (red) extracted from wood samples of beech trees (*Fagus sylvatica*). The light red rectangular is the leaf less period after the late frost. The horizontal dotted cyan lines represent the local atmospheric $\Delta^{14}CO_2$ for the given year and allow an estimation for the time elapsed since fixation (e.g. a sample from 2016 with the same Δ^{14}C as the atmosphere in 2011 contains C on average fixed 5 yr previously). Black dashed line represents the relationship between day of the year and Δ^{14}C of soluble NSC during leaf less period. The bars represent the standard errors of the mean.

differences (*T*-test, $t = 0.192$, *P*-value $= 0.853$) (Fig. 3). This suggests that the study site is unaffected by pollution from local fossil fuel combustion sources that can dilute $\Delta^{14}CO_2$ and complicate the application of bomb-radiocarbon approach.

We observed large changes in the Δ^{14}C of soluble NSC (Fig. 2b). Immediately following the canopy green-up in April 2016, the Δ^{14}C of soluble NSC was *c.* 28 ± 3‰. This value is close to what was observed for tree rings or atmospheric $\Delta^{14}CO_2$ in 2013. Thus, the soluble NSC was fixed on average 3 yr before sampling, that is we assign it an 'age' of 3 yr.

Just after the frost event, the Δ^{14}C of soluble NSC equalled that of the current year's atmospheric $\Delta^{14}CO_2$ with a value of 16 ± 3‰. However, Δ^{14}C of soluble NSC extracted subsequently increased with time during the leafless period. The maximum observed value was 36 ± 2‰, corresponding to atmospheric $\Delta^{14}CO_2$ in 2011, that is 5 yr old. After the re-growth of the canopy starting from 28 June, the Δ^{14}C of NSC steadily declined until it approached the

value of the current year's atmosphere *c.* 3 months later. During the dormant season and before leaf-out 2017, Δ^{14}C of NSC was elevated again compared to the atmospheric $\Delta^{14}CO_2$.

Soluble sugar concentrations

Soluble sugar concentrations ranged from 5.24 ± 0.46 to 16.74 ± 0.46 mg g^{-1} DW (Fig. 2b). A linear decline was observed after the spring late frost ($y = -0.0591x + 15.507$, $R^2 = 0.928$, *P*-value < 0.01) and a linear increase was measured starting from the second green-up until winter maximum ($y = 0.0905x - 12.256$, $R^2 = 0.913$, *P*-value $= 0.01$).

Discussion

This study demonstrates for the first time that beech trees mobilize several-year-old C reserves for maintaining metabolic activity and

Fig. 3 Reconstruction of local atmospheric $\Delta^{14}CO_2$ from average (± SE) $\Delta^{14}C$ of tree-ring specific cellulose of two beech trees (*Fagus sylvatica*) (black circles). Red circles show $\Delta^{14}C$ modelled by the regression (black solid line) for the period 2014–2017. Blue lines are 95% confidence interval. Blue triangles represent the mean atmospheric $\Delta^{14}CO_2$ data for the growing season (15 May–15 September) measured at Schauinsland observatory, representing unpolluted conditions. The bars represent the SE of the mean.
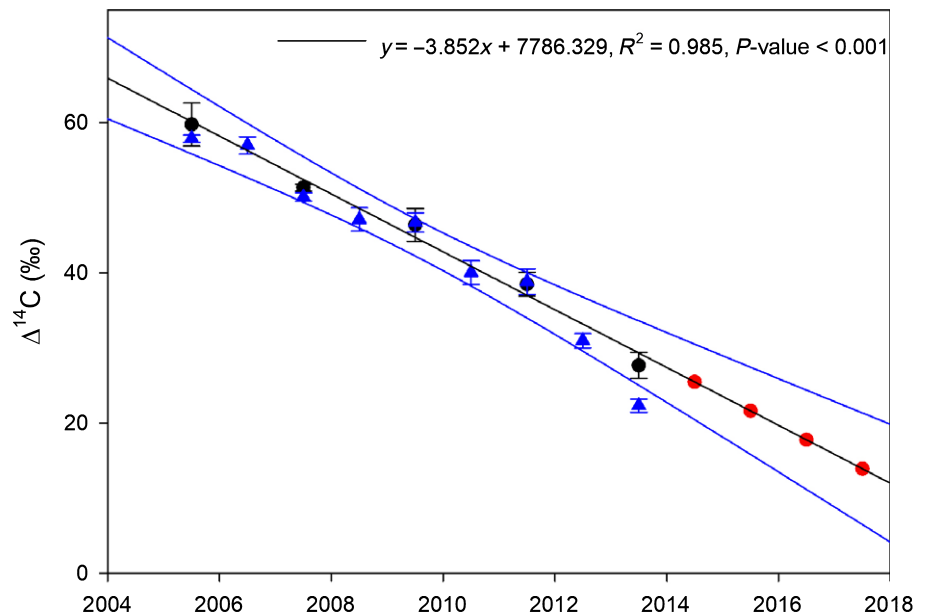
for re-growth after having lost the whole canopy during a late frost in spring.

Soluble NSC in close proximity to the phloem had $\Delta^{14}C$ values equal to or greater than those of local atmospheric $\Delta^{14}CO_2$ throughout the whole measurement period. This clearly demonstrates that soluble NSC mix newly assimilated with older sources (Carbone *et al.*, 2013; Richardson *et al.*, 2013; Trumbore *et al.*, 2015). The increase in $\Delta^{14}C$ of NSC and decline in soluble sugar concentrations observed following the destruction of the canopy suggests that in the absence of new assimilate supply the trees mobilized NSC reserves that got increasingly older with time. Such a 'reverse chronological mobilization' following a disturbance event has been described previously by Muhr *et al.* (2018) in reaction to complete stem girdling in tropical *Scleronema* trees.

Interestingly, 10 d after the loss of all leaves the soluble NSC was dominated by recently assimilated C as demonstrated by its $\Delta^{14}C$ values close to those of the atmospheric background. The effect of the late frost on the source of soluble NSC became detectable as a clear increase in $\Delta^{14}C$ values by the second sampling date, 17 d after the frost event. By the time of the third sampling in the leafless period, soluble NSC was already 5 yr old on average, and soluble sugar concentrations had reached a minimum. As the leafless period continued for another 27 d, the mean age of mobilized reserves likely increased even further, though we did not measure it. Based on the simplified assumption that the ageing trend observed during the three post-frost sampling dates continued at the same rate until canopy regrowth, we estimate that at the end of the leafless period the trees might have mobilized reserves fixed on average 9 yr ago (linear extrapolation, shown by the dashed back line in Fig. 2b). Mobilization of reserves that old and even older in mature trees has been reported before in several other studies and shows that trees build up reserve pools over several seasons and can keep them accessible for years. Carbone *et al.* (2013) reported mobilization of NSC as old as 17 yr to support re-sprouting from the stump of felled trees. NSC as old as 10 yr was mobilized to produce new roots in a tropical forest after hurricane damage (Vargas *et al.*, 2009). In

girdled *Scleronema* trees in a tropical rainforest, the maximum average age of mobilized NSC was 14 yr (Muhr *et al.*, 2018).

Canopy re-growth occurred 53 d after the late frost event, and was followed by a decrease of $\Delta^{14}C$ and the increase of soluble sugar concentrations that can be attributed to the delivery of new assimilates via the phloem. Interestingly, it took almost 63 d before the $\Delta^{14}C$ recovered to the same level as the atmospheric background, suggesting that older reserves still contribute to the soluble sugar pool weeks after the canopy has recovered. This could either be storage C that had been mobilized during the stress and now is still available, or it could be ongoing mobilization even after recovery. Regular mixing of reserve C with new assimilates even during periods that are not characterized by measureable stress has been suggested previously (Carbone *et al.*, 2013; Muhr *et al.*, 2013). While our data clearly show the contribution of C reserve, we have no way of identifying the origin of these NSC which might either be mobilized locally in the stem or in other organs (e.g. branches and roots).

We would like to point out that the exceptional reserve mobilization following the late-frost damage seemed to have no effects beyond the growing season of 2016. The $\Delta^{14}C$ of soluble NSC and soluble sugar concentrations measured during the following dormant season were not different from the values assessed in the dormant season before the frost event. As an additional test, in the year following the frost event (April, June and August 2017), we sampled stem respired $\Delta^{14}CO_2$ from trees affected by the frost vs trees at lower elevations and hence not affected by the event (data not shown) and found no differences between the two groups, with $\Delta^{14}C$ of CO_2 emitted from the stem always being the same as the atmospheric background. These data exclude a long-term effect of the frost event on C age of the emitted CO_2. Also, overall the seasonal dynamics of soluble sugar concentrations showed the pattern typical for beech (Barbaroux & Bréda, 2002; Scartazza *et al.*, 2013), with a decrease at the beginning of the growing season, a peak at the beginning of dormancy and a reduction during the winter. We would like to

especially point out the high concentration at the beginning of the dormant season; despite the exceptional C shortage caused by almost two leafless months at the beginning of the growing period, the trees were still capable of building up the soluble sugars pool typically observed at the start of the dormant season. Moreover, at the start of the 2017 growing season, this C pool was at the same level as 2016. This confirms that the pattern and size of NSC accumulation in beech trees is a conservative trait with relevant homeostasis even in the presence of adverse events like late frost or summer drought (Scartazza *et al.*, 2013).

We conclude that the trees were able to fully recover from the temporary shortage in assimilate supply, though we do not know the effect on long-term NSC stores that may be deeper in the stem or in other organs like roots and branches.

In addition to the NSC mobilization as a reaction to canopy loss after frost, we also observed increased $\Delta^{14}C$ during the dormant season, when beech also has no leaves. In both sampling years, the soluble NSC was on average 3 yr old around the time of leaf-out in early spring, clearly showing that beech mobilizes NSC older than just from the very last growing season during winter. The mobilization of old NSC thus is not restricted to disturbance events but occurs on a regular basis at least during certain phenophases, suggesting that reserve pools and more recent assimilates can regularly exchange and might be also well mixed as suggested by Richardson *et al.* (2013). Similar findings have been reported for sugar maple trees in Canada which mobilize on average 4–5 yr old NSC at the end of winter during sap season (Muhr *et al.*, 2015).

Summarizing, our study illustrates that mature beech trees have access to old reserve pools that can rapidly be mobilized to survive exceptional periods of assimilate shortage like for example late frost leaf damage or other extreme weather events. This finding also highlights the importance of the NSC for tree species fitness in an environment rapidly modified by climate change.

Author contributions

ED'A, NR, OG, GM, AS and JM contributed to the design of the research. Fieldwork was carried out by ED'A, NR and OG. Soluble sugars content analysis were performed by SM, AB, SP and AS. The

^{14}C analysis was performed by IK, NR, JM and ST. Data analysis was done by ED'A, with data interpretation by all authors. The manuscript was written by ED'A with contributions by all co-authors.

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