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Research paper

Allocation to carbon storage pools in Norway spruce saplings under drought and low CO_2

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Non-structural carbohydrates (NSCs) are critical to maintain plant metabolism under stressful environmental conditions, but we do not fully understand how NSC allocation and utilization from storage varies with stress. While it has become established that storage allocation is unlikely to be a mere overflow process, very little empirical evidence has been produced to support this view, at least not for trees. Here we present the results of an intensively monitored experimental manipulation of whole-tree carbon (C) balance (young *Picea abies* (L.) H Karst.) using reduced atmospheric [CO_2] and drought to reduce C sources. We measured specific C storage pools (glucose, fructose, sucrose, starch) over 21 weeks and converted concentration measurement into fluxes into and out of the storage pool. Continuous labeling (^{13}C) allowed us to track C allocation to biomass and non-structural C pools. Net C fluxes into the storage pool occurred mainly when the C balance was positive. Storage pools increased during periods of positive C gain and were reduced under negative C gain. ^{13}C data showed that C was allocated to storage pools independent of the net flux and even under severe C limitation. Allocation to below-ground tissues was strongest in control trees followed by trees experiencing drought followed by those grown under low [CO_2]. Our data suggest that NSC storage has, under the conditions of our experimental manipulation (e.g., strong progressive drought, no above-ground growth), a high allocation priority and cannot be considered an overflow process. While these results also suggest active storage allocation, definitive proof of active plant control of storage in woody plants requires studies involving molecular tools.

Keywords: carbohydrates, carbon starvation, carbon storage control, stress physiology, tree mortality.

Introduction

Trees are among the largest, longest-lived organisms on earth (Petit and Hampe 2006). Their long life span requires that trees survive stressful environmental conditions and attack by pathogens and insects, often multiple times within their lifespan (Gutschick and BassiriRad 2003). To minimize loss of biomass and to maintain life-sustaining functions, trees must store resources (Chapin et al. 1990). Despite a long history of research on how plants survive stressful conditions, fundamental processes underlying how exactly trees regulate carbon storage remain highly debated (Sala et al. 2010).

A tree's mass provides considerable capacity for storage of resources such as water, nutrients and carbon that can be drawn upon as needed to survive periods of low resource acquisition (Bloom et al. 1985, Hoch et al. 2003). Non-structural carbohydrates (NSCs) may accumulate in live tissues of stems, branches and roots during periods when carbon (C) gain exceeds use (Körner 2003) and the amount of C stored in carbohydrates may equal more than half of the requirement for annual stem growth or for several canopy refoliations (Hoch et al. 2003). Non-structural carbohydrates can be retained in mature tree tissues for decades (Carbone et al. 2013, Richardson et al. 2013) and carbon dioxide respired in tree stems may be several years to more than a decade old (Muhr et al. 2013), indicating the use of stored NSCs for respiration. It is well established that NSCs are critical to supply energy to fuel growth, respiration and other functions (see review

in McDowell et al. 2011), and their depletion results in cessation of metabolism and mortality (e.g., Marshall and Waring 1985, Marshall 1986). However, in comparison to other resources such as nutrients and water, and carbon fluxes such as photosynthesis, growth and respiration, there is relatively less understanding about storage and utilization of NSC in woody plants (Sala et al. 2010, Stitt and Zeeman 2012, Dietze et al. 2014).

Research on the mechanisms of recent drought-induced forest mortality has raised several questions about the function and regulation of carbon storage. Accounts of increased NSC content during drought suggest that drought-induced declines in sink activity (i.e., growth) can outweigh declines in carbon uptake if photosynthesis continues during drought (Muller et al. 2011). There is no common pattern in how carbohydrate levels vary during lethal drought; in numerous cases declines occurred (Galiano et al. 2011, Galvez et al. 2013, Poyatos et al. 2013), but increases in NSC concentration have also been observed prior to death (Galvez et al. 2011, Anderegg et al. 2012). Manipulations of tree C balance via defoliation substantially reduced NSC concentration in pine (Li et al. 2002) but not in poplar (Anderegg and Callaway 2012), while in oak allocation to starch was increased by defoliation and at the expense of growth (Wiley et al. 2013). The lack of a clear pattern has led to questions regarding the usefulness of NSC concentration measurements alone, i.e., without information about the fluxes, as indicators for the carbon balance (Ryan 2011). Such an approach requires going beyond measures of NSC concentration for determining C balance at the level of the whole-plant or organ/C reservoir in order to elucidate the role of C storage and its regulatory mechanisms in trees (Dietze et al. 2014). Very few studies have manipulated and assessed the whole-tree C balance so far (but see Hartmann et al. 2013*a*, Zhao et al. 2013) and we are not aware of any study that directly relates measured net C balance to NSC storage fluxes (but see Klein and Hoch 2015 for a top-down scaling approach).

Here, we present a highly controlled experimental approach in which we monitored the C balance of tree saplings with manipulations that limited carbon availability by imposing drought or lowering the atmospheric CO_2 content. We monitored the net plant C balance, as well as the amount of C in storage pools, and used a continuous $\delta^{13}C$ label to trace C flow into growth and NSC following the start of the treatment. We integrated NSC concentration measurements over whole trees and converted these into fluxes over the experimental period. Our objective was to test whether allocation to storage reserves occurred only when the net plant C balance is positive (i.e., allocation to storage is an 'overflow' process).

Materials and methods

Experimental design

The study was carried out in July through November 2012 in the greenhouse of the Max-Planck Institute for Biogeochemistry in Jena, Germany. Temperature in the greenhouse was maintained at ~22 °C during the day and ~13 °C at night, natural sunlight was augmented with greenhouse lighting (400 W Gro-Lux®, Osram Sylvania Ltd, Danvers, MA, USA) yielding an average of ~5 mol PAR m^{-2} day^{-1} (measured). Under normal daylight conditions, light flux density levels were beyond light saturation (~400 µmol PAR m^{-2} day^{-1}, measured on similar seedlings).

We placed 12 individually ventilated glass chambers (45 cm wide × 75 cm long × 80 cm high) in one of the greenhouse bays. Daily average temperatures in the glass chambers deviated from greenhouse temperatures by several degrees (usually between 21 and 24 °C during days, and 12 and –16 °C during nights) with a seasonal average of 22.5 °C (day) and 13.5 °C (night). In each glass chamber, we placed four small Norway spruce saplings (*Picea abies* (L.) H Karst., ~75 cm high) in separate 2.5 l pots. Because changes in biomass are difficult or even impossible to measure at the required resolution for the purpose of our study (i.e., partitioning C balance), we started the experiment only after longitudinal growth had ceased and lignification of newly grown branch biomass was completed. During this period all chambers were flushed with the same air and all trees were given equal amounts (200 ml) of water once per week.

Trees were grown in a 2 : 1 vermiculate-sand mixture (i.e., with no native soil organic matter) and were supplied with nutrients (Manna® Wuxal Super 8-8-6 with microelements and a slow-release conifer fertilizer Substral® Osmocote 11-8-17; Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany and Scotts Celaflor GmbH, Mainz, Germany). Chambers were subdivided into above- and below-ground chambers (flush rate 25 and 5 l min^{-1}, respectively) allowing separate measurements of root respiration and above-ground net gas exchange (see Hartmann et al. 2013*a* for more information). Flow rates were set to achieve a measurable draw-down of CO_2 in the chamber while avoiding any limitation to carboxylation and/or ^{13}C-enrichment of the chamber air by photosynthesis.

Treatments

Treatments were initiated on 1 July 2012. From this point onwards, one-third of the chambers were supplied with air of reduced [CO_2] by first removing CO_2 using a molecular sieve (Schnyder 1992, Gamnitzer et al. 2009). Depending on the specified treatment, CO_2 from a pressurized tank was then added to the CO_2-free air at concentrations of either 350 or 40 ppm, depending on treatment. The high concentration was meant to simulate current ambient [CO_2], i.e., concentrations >350 ppm, but these levels could not be achieved due to the limited capacity of our mass flow controller. The lower concentration (40 ppm) was established in a pre-experiment as a treatment causing a permanent negative daily carbon balance. The concentration was continuously assessed and re-adjusted with a computer-controlled mass flow controller (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). In the pre-treatment

period, trees were grown in ambient air with a $\delta^{13}C$ of approximately −9‰ (on VPD scale), during the experiment the CO_2 supplied to the CO_2-free air had a $\delta^{13}C$ of approximately −42‰.

Normally irrigated trees were given 200 ml of water once a week, and trees in the drought treatment were given only 50 ml at the same time. We therefore had three treatments: (i) 200 ml water per week and 350 ppm [CO_2] (Ambient-C), (ii) 200 ml water per week and 40 ppm [CO_2] (Low-C) and (iii) 50 ml water per week and 350 ppm [CO_2] (Drought). There were three glass chambers per treatment. Trees in the drought treatment died after ~14 weeks, while trees in the Low-CO_2 treatment survived until Week 21, at which time Ambient-C trees were still alive and healthy-looking. Tree death was determined by complete foliage browning, near-zero respiration and cambial necrosis in branches and stems. Trees in the drought treatment showed very low relative tissue water content at the end of the experiment (Table S1 available as Supplementary Data at *Tree Physiology* Online). We concluded the experiment after trees in the Low-C treatment had died.

Measurements of carbon fluxes

Weekly net carbon gain We measured above-ground net carbon exchange and root respiration as the difference between [CO_2] of air entering and air leaving the chambers. To do so, the in- and outlet air stream of each chamber was sampled for 2.5 min each with a Picarro® 2131-*i* (above-ground) and 2101-*i* (below-ground) before switching to the next chamber. The rotation between chambers was achieved with a logger-controlled valve switching unit (Campbell Scientific® CR 1000 micrologger, Campbell Scientific Inc., Logan, UT, USA, see Figure S1 available as Supplementary Data at *Tree Physiology* Online), completing a whole cycle within 1 h.

Each 5-min measurement cycle was converted to hourly carbon flux (C) at time j using the following equation:

$$C_j(\text{g h}^{-1}) = \Delta[CO_2]_j(\mu\text{mol mol}^{-1}) \times \frac{\text{VFR (l min}^{-1}) \times 60\text{ (min)}}{22.4\text{ (l mol}^{-1})} \times 10^{-6} \times \text{MW (g mol}^{-1}) \quad (1)$$

where $\Delta[CO_2]_j$ is the difference in [CO_2] between inlet and outlet air stream at time j for a given chamber, VFR the normalized volumetric flow rate of air going through the above- and below-ground chamber (25 and 5 l min^{-1}, respectively) and MW the molecular weight of carbon per mole of CO_2.

We computed the whole-chamber (above- and below-ground) net carbon gain at week i (mg C per week, NCG_i) as the differences in carbon assimilation and respiration. To do so, we summed hourly carbon fluxes (above- and below-ground) on a daily basis and over week i (Day 1–7).

$$NCG_i = \sum_{d=1}^{7}\sum_{j=0}^{23} {}_A C_{ij} - \sum_{j=0}^{23} {}_B C_{ij} \quad (2)$$

where the subscript A and B in C_{ij} denotes above- and below-ground, respectively.

NSC measurements Carbon storage was estimated by assessing tissue-specific concentrations of NSC and by upscaling to total NSC content with biomass measurements. We measured soluble sugars (SS), glucose (Glu), fructose (Fru) and sucrose (Suc) as the main mobile compounds and starch (Star) as the main non-mobile NSC compound in needles, branches and roots. Because repeatedly opening the below-ground chambers may have severely disturbed the tree root systems we only collected root samples when trees were placed in the chambers and at the end of the experiment. Branch and needles were sampled on average every 2.5 weeks using a sharp branch cutter. Samples were frozen immediately by immersion in liquid nitrogen and then placed in a −80 °C freezer for longer storage.

For NSC extraction, frozen samples were vacuum freeze-dried for 72 h and milled with a ball mill (Retsch® MM200, Haan, Germany) to a fine, consistent powder. To extract Glu, Fru and Suc we added 50 mg of ground sample to 1-ml distilled water. The mixture was vortexed, incubated for 10 min at 65 °C in a thermomixer (1050 rpm) and then centrifuged for 15 min at 2300*g*. The supernatant was removed with a pipette, stored on ice and the procedure was repeated twice. The supernatants were pooled and stored frozen at −20 °C for later measurement. For starch analysis, 50 mg of ground sample was added to 0.35-ml distilled water, vortexed for 1 min and treated for 10 min in a thermomixer at 65 °C (1050 rpm). For starch hydrolysis we then added 0.5 ml of 33% perchloric acid and let it incubate in an orbital shaker for 20 min. After centrifuging at 14,300*g* for 6 min, the supernatant was removed with a pipette and the procedure repeated on the remaining pellet. The supernatants from the two extractions were pooled and stored frozen at −20 °C for later measurement.

Sugar and starch extracts were diluted (1 : 20 and 1 : 55, respectively) before measurement with high-pressure liquid chromatography pulsed amperometric detection (HPLC–PAD) on a Dionex® ICS 3000 ion chromatography system equipped with an autosampler (Raessler et al. 2010). Starch concentrations were then computed as the differences in Glu concentration in the hydrolyzed extract minus the Glu and half of the Suc concentration in the water-SS extract multiplied by a conversion factor of 0.9 (Sullivan 1935).

Biomass measurement and whole-tree carbon storage flux estimation At the end of the experiment, we harvested and dried all the trees and measured dry biomass of each tissue type (needles, branches and stems, roots). Biomass samples taken for NSC measurements were also dried and weighed and these were added to the final biomass estimates.

Tissue-specific NSC (NCC_i) was estimated by multiplying tissue-specific NSC concentration at period i with tissue mass at period i (accounting for biomass reduction from sampling) and

by multiplying the tissue-specific NSC content by the mass proportion of carbon in NSC (0.4 for SS, 0.42 for Star). We obtained whole-tree NCC by summing over all tissues. Because of the small stature of the studied trees, stems and branches were quite similar in size and we treated them as one category.

Fluxes to and from storage (S_i) were estimated with the following equation:

$$S_i = NCC_i - NCC_{i+1} \quad (3)$$

where NCC_i is the non-structural carbon content at period i. Hence, storage fluxes were the change in NCC from one period to the following. Please note that this definition of storage also includes transitory pools (i.e., SS) that are translocated prior to utilization. However, such pools can be depleted during stressful periods (Hartmann et al. 2013*a*) and hence may provide similar functionality as immobile compounds like starch. Potential changes in biomass (e.g., fine root turnover, allocation to secondary metabolites, lignin deposition) were not assessed in our study. Because above-ground longitudinal growth had ceased before the start of the experiment and because secondary growth did not occur (Hartmann et al. 2013*a*), potential changes in biomass can be considered minor. However, as we were unable to account for changes in root biomass during the experiment period, we estimated allocation to a 'residual pool' (B_i, comprising mainly root biomass increases) by assuming mass balance (sensu, McDowell 2011):

$$B_i = NCG_i - S_i \quad (4)$$

Error propagation Error of measurements were propagated throughout the experimental period for computed (e.g., storage flux) or cumulated variables using standard error propagation rules.

Allocation to storage and biomass using $\delta^{13}C$ as indicator

During the experiment trees were assimilating CO_2 with a very different isotopic composition (−42‰) compared with the ambient air of the pre-experimental period (−9‰). Knowing the two different sources, one can compute the fractions of each source in plant carbon pools (i.e., mobile sugars and starch, biomass) using a mixing model (Dawson et al. 2002). This approach requires either assuming constant fractionation factors across treatments (not realistic in our experiment) or measuring isotopic discrimination during the experiment (difficult to measure in situ and online). We therefore use the $\Delta\delta$ notation as an indicator for such proportions, where

$$\Delta\delta^{13}C_i = \delta^{13}C_i - \delta^{13}C_{start} \quad (5)$$

where $\Delta\delta^{13}C_i$ is the difference in $\delta^{13}C$ of a particular pool (e.g., NSC, biomass) at period i minus $\delta^{13}C$ at the beginning of the experiment ($\delta^{13}C_{start}$). Since the CO_2 source used during the experiment had a much lower $\delta^{13}C$ than the ambient air trees were growing in prior to the experiment, negative values indicated the strength of incorporation of 'newly assimilated' (i.e., during the experiment) carbon into the pool. Decreases in fractionation during drought may cause an upward shift in $\delta^{13}C$ of leaf metabolites (approximately +6‰, Duranceau et al. 1999, Ghashghaie et al. 2001) but such a shift would not offset the approximately −33‰ shift in the source signal. We measured $\delta^{13}C$ of the SS pool as an indicator for allocation to NSC and of the remaining pellet (which includes also starch and lipids but mainly, i.e., >95%, structural biomass, data not shown) as an indicator for allocation to structural biomass.

Results

Net carbon assimilation

Trees growing at 350 ppm [CO_2] assimilated more carbon than they respired throughout most of the entire experimental period (Figure 1). The seasonal decline in assimilation may be attributed to decreasing daytime length from July until November that was not compensated by the artificial greenhouse lighting. Droughted trees showed a sharp decline in net carbon gain during the first 4 weeks and a net carbon loss from Week 5 onwards. Trees in the Low-C treatment showed no positive net carbon gain at all during the experiment (Figure 1).

Carbohydrate pool measurements

Generally, concentrations of starch were substantially lower than of SS in all tissues and treatments. NSC pools (both starch and SS) increased in needles of Ambient-C trees during the experiment. This trend was similar in Drought trees except for a decline prior to their death (Figure 2). Branch NSC showed a similar

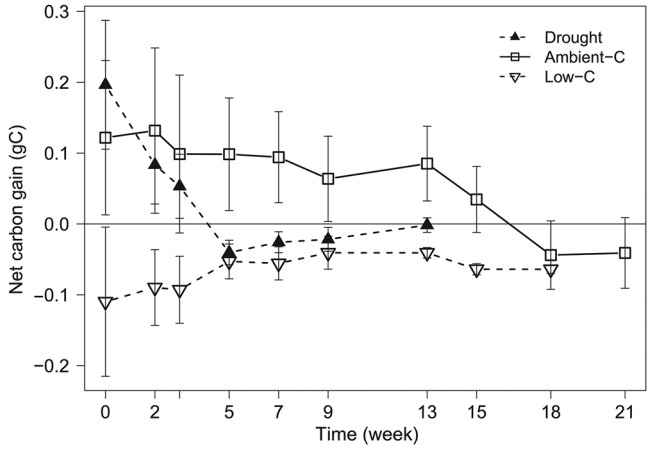

Figure 1. Net weekly carbon gain (g C, ±1 SE) during the experimental period. Droughted trees (filled triangles) died during Week 14, trees in the Low-C (inverted triangles) treatment during Week 20 while trees in Ambient-C (squares) survived the experiment. Data are shown only for weeks for which NSC measurements were taken.

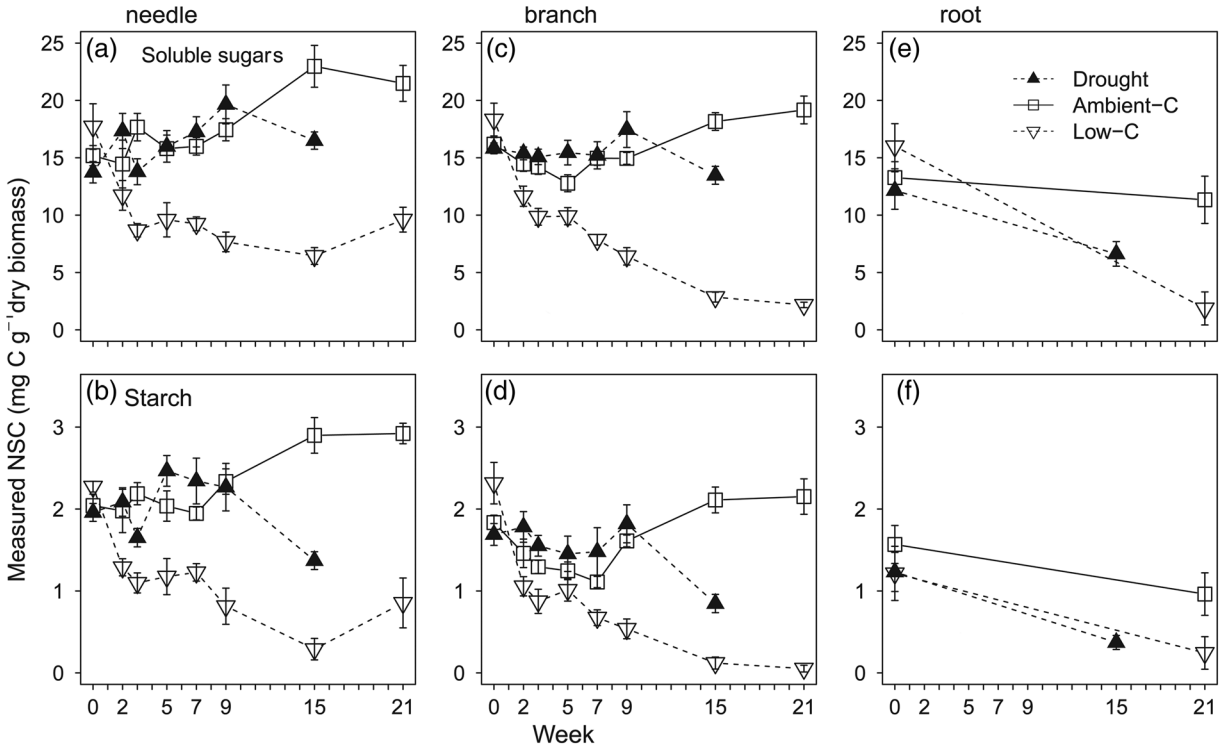

Figure 2. Weekly concentrations (mg C g^{-1} dry biomass, ±1 SE) of SS (a, c and e) and starch (b, d and f) in needles (a and b), branches (c and d) and roots (e and f) (Drought: filled triangles, Ambient-C: squares, Low-C: inverted triangles). Note that the last measurements in Drought and Low-C trees were at final harvest after their death (Weeks 12 and 20, respectively).

Table 1. Averages (g) and standard deviation (SD) of tissue and total dry biomass of sample trees as well as B_{cum} (estimated residual biomass cumulated over experimental period) and the percentage proportion of B_{cum} over total biomass.

	Needle		Branch		Root		Total		B_{cum}		B_{cum}/total
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	(%)
Ambient-C	31.21	2.35	46.41	5.83	30.17	1.80	107.79	6.54	0.38	0.49	0.35
Low-C	32.37	4.28	46.27	6.67	21.03	2.91	99.67	8.44	1.00	0.27	1.00
Drought	37.34	5.50	51.89	5.48	30.33	7.92	119.56	11.09	0.52	0.42	0.43

seasonal trend to needle NSC but both SS and starch declined almost to zero in Low-C trees. Root NSC declined in all treatments, but most severely in Low-C trees (Figure 2).

Biomass and residual of mass balance

There were no significant differences in tissue or in total biomass of sample trees across treatments. The within-treatment variability was greatest in needle, root and total biomass of Drought and Low-C trees (Table 1).

As noted previously, while we assume there is no biomass change above-ground, we cannot assess below-ground change; hence we have estimated the residual of the mass balance as the difference between measured plant C mass balance (from gas exchange) and the changes in C reserves. Due to multiple error propagation these estimates are highly uncertain and show very small positive values, ranging from 0.38 g (±0.49 g, Ambient-C) to 1.00 g of carbon (±0.27 g, Low-C). Weighted on biomass, this residual growth makes up between 0.35% (Ambient-C) and 1.00% (Low-C) of the total biomass (Table 1).

Changes in carbon storage

Overall changes in whole-tree SS and starch were small compared with variability between trees within a given treatment. For Drought and Ambient-C treatments, storage pools remained essentially constant during the first few weeks of treatment, and the only period with substantial storage/remobilization occurred during Week 9. Trees in the Low-C treatment were losing carbon from SS and starch throughout the experiment (Figure 3).

Allocation to storage and biomass

Trees at Ambient-C allocated more of the C fixed after the start of the experiment to storage compounds and to biomass than trees in the other treatments (Figure 4). Relative allocation (per unit of biomass) of newly assimilated carbon was greater in roots

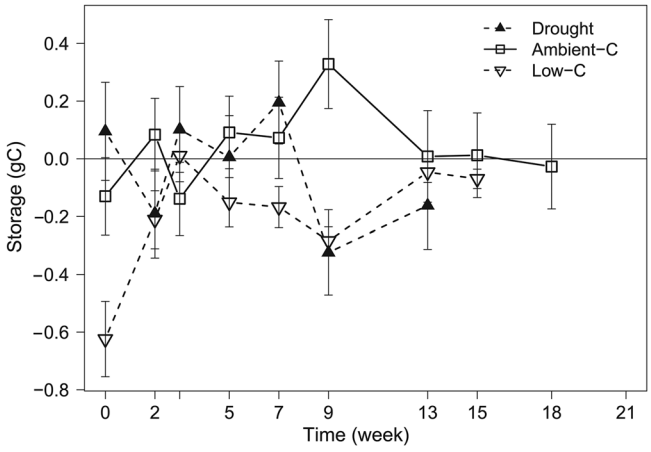

Figure 3. Net weekly storage fluxes (g C, ±1 SE) during the experimental period (Drought: filled triangles, Ambient-C: squares, Low-C: inverted triangles). Positive values indicate allocation to storage, negative values storage mobilization. Data are shown only for weeks before final harvest.

than in above-ground tissues in all treatments. Positive $\Delta\delta^{13}C$ values in branch NSC of Drought and Low-C trees indicate mobilization of stored, heavier compounds like starch. There was no substantial carbon allocation to above-ground storage or biomass in Drought and Low-C trees, while $\Delta\delta^{13}C$ of root NSC and biomass in Drought trees show indication for allocation of newly assimilated carbon. A small fraction of newly assimilated carbon was incorporated into root biomass in Low-C trees (Figure 4).

During the experimental period, the strong declines in storage pools in the Low-C treatment were greater than net C losses measured from gas exchange and hence the residual pool in this treatment was greatest across treatments (Figure 5).

Discussion

Plant allocation to storage remains a debated issue (Sala et al. 2012) and is plausibly controlled by active regulation within

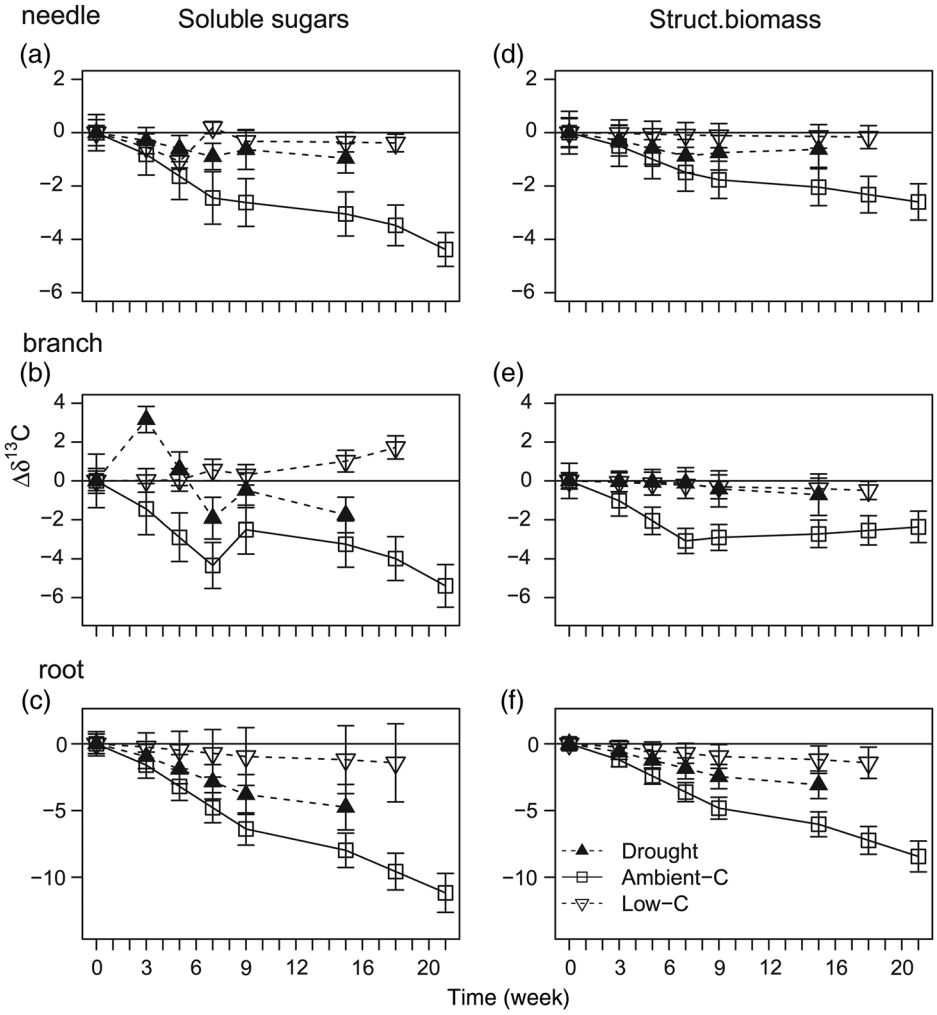

Figure 4. Weekly $\Delta\delta^{13}C$ (‰, ±1 SE) of SS (a, b and c) and in the remaining pellet following the extraction (structural biomass + starch, d, e and f) in needles (a and d), branches (b and e) and roots (c and f) (Drought: filled triangles, Ambient-C: squares, Low-C: inverted triangles). Negative values indicate incorporation of carbon assimilated during the experimental period in a given pool, positive values can indicate allocation of previously stored compounds.

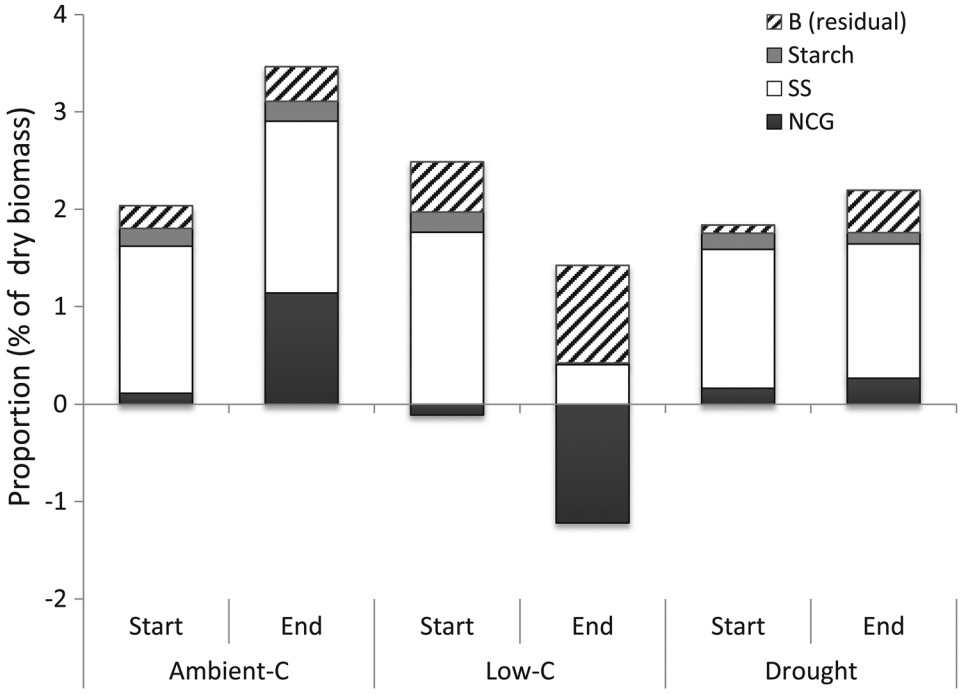

Figure 5. Summary figure showing components of the carbon balance (as % of dry biomass) between beginning (first week of experiment, Start) and end (End) of the experiment. Net C gain (NCG, Eq. (2)) and residual (*B*, Eq. (4)) were cumulated throughout the experiment period.

biophysical constraints (Dietze et al. 2014). By combining measures of whole-plant mass balance with measurements of carbon storage pools (SS and starch) at the whole-plant and organ level and use of a stable carbon isotope label to identify the fate of pre- versus post-treatment C, our experiment allows us to draw inferences on storage allocation of newly assimilated carbon. While we are aware that measurements of carbohydrates are highly uncertain (A.G. Quentin et al., submitted) likely making our pool estimates and computed fluxes inaccurate (i.e., show true values), we are confident that our methods are precise enough to yield reliable qualitative estimates of flux direction, i.e., storage allocation or mobilization.

Continuous losses in SS and starch pools in the Low-C treatment indicated a net depletion of storage pools. While the stable carbon isotope data corroborate storage depletion in above-ground tissues, root $\Delta\delta^{13}C$ data suggest that both the root SS pool and the root starch/biomass contained small proportions of newly assimilated carbon. Given the very low carbon availability and the consistently negative carbon balance in the Low-C treatment, these trees had little carbon to allocate to storage (but apparently did) and rather remobilized existing pools for survival. The observed allocation to storage under these conditions indicates a high storage allocation priority consistent with active storage regulation. Similar to our findings, manipulations of carbon availability via defoliation showed a relative increase in storage pools (compared with growth) in half-defoliated trees, suggesting that storage allocation was independent of carbon availability and potentially under active control (Wiley et al. 2013).

Droughted trees showed a substantial net increase in the storage pool at the whole-plant level after entering negative net carbon balance between Weeks 5 and 7, followed by rather strong storage remobilization (Figure 3). As in the Low-C treatment, $\Delta\delta^{13}C$ changes support a continuous incorporation of newly assimilated carbon into SS and starch/biomass, at least in below-ground tissues. This is particularly interesting because previous investigations on changes in carbohydrate concentrations in the same species showed that reduced hydration may prevent translocation of above-ground carbon storage to the root system and cause a decoupling of above- and below-ground tissues (Hartmann et al. 2013*a*). The isotopic data here suggest that newly assimilated carbon was transported into the root system even during later phases of the drought treatment and hence do not corroborate reduced translocation. Drought causes a strong increase in $\delta^{13}C$ of SS in above-ground tissues (Hartmann et al. 2013*b*) and such an increase may have partially offset the observed $\Delta\delta^{13}C$ signal in needle and branch storage pools of droughted trees. Positive $\Delta\delta^{13}C$ in SS of branches in the early phase of the experiment corroborate this idea and, if so, droughted trees may have also been allocating carbon to storage pools in above-ground tissues. Klein et al. (2014) showed that drought-stressed trees maintained storage pool size by dramatically decreasing growth rates and interpreted the decrease in growth as a regulatory mechanism to maintain a positive C balance (Klein et al. 2014). A similar mechanism may have acted in our trees, at least in below-ground tissues.

Trees in the Ambient-C treatment maintained a net positive C balance through most of the experimental period. Newly assimilated C was incorporated into SS and starch/biomass throughout the experiment, with most pronounced incorporation of new C into the root system, even though net fluxes into storage were not always positive. Declines of $\Delta\delta^{13}C$ during these periods could also occur if C from pre-treatment storage reserves were preferentially used for C sinks (like respiration) that removed 'old', i.e., heavier, C from the plant system.

Our mass balance approach relies on the assumption that all components are accurately assessed. While measurements of $[CO_2]$ with sophisticated technology and verified against calibration gases of known concentration can be considered accurate, carbohydrate concentrations cannot be accurately assessed due to a lack of standards (A.G. Quentin et al., submitted). This implies that the absolute concentrations presented here (Figure 2) are potentially wrong. However, a large international study of carbohydrate assessments across 31 laboratories (including our own) also revealed a high intra-laboratory precision (repeatability) (A.G. Quentin et al., submitted). This means that our differential measurements (between periods) and resulting flux estimations are valid and accurate and hence the partitioning of available carbon into different pools based on mass balance (i.e., residual pool) is also accurate. Allocation to this residual pool is greatest in the Low-C and Drought treatment (strongest change Pre vs Post in Figure 5) suggesting either below-ground biomass production or stress-induced synthesis of other compounds. Drought and shading studies have shown increases of several amino acids (Cyr et al. 1990, Vance and Zaerr 1990, Busing and Mailly 2004, Ditmarová et al. 2010) that may alleviate physiological stress during drought (Ashraf and Foolad 2007) or act as nitrogen storage compounds during C limitation (Miflin and Lea 1977, Llácer et al. 2008). Future research on storage processes should include assessments on such secondary metabolites but also other storage compounds like lipids as potential competing sinks in the C balance. In our study, lipid synthesis was unlikely to be the sink accounting for the residual pool (see Table S2 available as Supplementary Data at *Tree Physiology* Online) but we have no data allowing us to exclude allocation to amino acids.

While it could be argued that our $\Delta\delta^{13}C$ data merely indicate mixing of newly assimilated C into a transient carbohydrate pool (Keel et al. 2007) and not allocation to a storage pool, we agree that this may be the case for SS but not for starch/biomass, which must be synthesized. Starch synthesis can be triggered by high carbohydrate supply to reduce osmolyte accumulation (Koch 1996) and could be considered a protective mechanism rather than storage allocation under high carbohydrate availability. Given the very low SS concentrations in tissues of Low-C trees such a process would be very unlikely and the incorporation of newly assimilated carbon into root starch/biomass appears to be active allocation.

The difference between active and passive allocation to storage is likely anchored in the regulation of growth and storage: (i) passive allocation to storage may occur when plants up-regulate growth but carbon supply is sufficiently large to allow storage allocation (concurrent flux to growth and to storage), (ii) 'quasi-active' allocation to storage may be achieved via down-regulation of growth, which allows diversion of carbon to storage (flux to storage under reduced flux to growth) and (iii) active allocation would occur via direct up-regulation of allocation to storage independent of growth (Dietze et al. 2014). Trees in our study allocated newly assimilated C into storage pools even during periods when they experienced a negative net C balance. Such a behavior would be very unlikely if storage was a mere overflow process and we have good reason to refute passive storage as a sole acting mechanism. Our data do not allow us to distinguish whether the observed allocation to storage was directly up-regulated or via indirectly down-regulation of growth (Wiley and Helliker 2012), especially since we deliberately avoided the period of strong above-ground growth during the experiment. Further advances in this domain clearly depend on the application of genetic and biochemical tools in investigations on trees, similar to what has been carried out in studies on *Arabidopsis* (e.g., Smith and Stitt 2007, Stitt and Zeeman 2012). Such investigations have become feasible by recent advances in genome sequencing of common tree species like eucalypts, spruce or cottonwood (Hogberg et al. 2001, Tuskan et al. 2006, Myburg et al. 2011) and can identify whether direct or indirect up-regulation of storage occurs even under C limitation. To better define C limitation, a more complete assessment of the competing C pools is required, including structural biomass changes, secondary metabolites, alternative storage compounds like lipids and proteins, defense compounds, volatile organic substances and also C sinks like root exudates and biotic interactions (herbivory, symbiotic exchanges). Maybe most important, further research is required to develop more accurate assessments of carbohydrates, the most abundant storage compound family, in plant tissues (A.G. Quentin et al., submitted).

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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