# The fate of recently fixed carbon after drought release: towards unravelling C storage regulation in *Tilia platyphyllos* and *Pinus sylvestris*

***Running Head: The fate of recently fixed C after drought release***

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**ABSTRACT**

Carbon reserves are important for maintaining tree function during and after stress. Increasing tree mortality driven by drought globally has renewed the interest in how plants regulate allocation of recently fixed C to reserve formation. Three year-old seedlings of two species (*Tilia platyphyllos, Pinus sylvestris)* were exposed to two intensities of experimental drought during ~ 10 weeks and $^{13}C$ pulse labelling was subsequently applied with rewetting. Tracking the $^{13}C$-label across different organs and C compounds (soluble sugars, starch, myo-inositol, lipids and cellulose), together with the monitoring of gas exchange and C mass balances over time, allowed for the identification of variations in C allocation priorities and tree C balances that are associated to drought effects and subsequent drought release. The results demonstrate that soluble sugars accumulated in *P. sylvestris* under drought conditions independently of growth trends; thus NSC formation cannot be simply considered a passive overflow process in this species. Once drought ceased, C allocation to storage was still prioritized at the expense of growth, which suggested the presence of 'drought memory effects', possibly to ensure future growth and survival. On the contrary, NSC and growth dynamics in *T. platyphyllos* were consistent with a passive (overflow) view of NSC formation.

**KEYWORDS**

allocation; carbon isotope; carbon storage; drought; growth; pulse labelling; recovery

## INTRODUCTION

Tree and forest mortality driven by drought and heat have become a global concern in the last few decades (Allen et al. 2010; Choat et al. 2012). Climate models project global increases in drought frequency, intensity and duration (IPCC 2014; Allen et al. 2015). In addition, the interaction between drought stress and damage caused by pathogens and pests may intensify forest vulnerability (Oliva et al. 2014; Anderegg et al. 2015). Although the mechanistic basis underlying drought-induced tree death is still not well identified, there is agreement in that it involves the impairment of the vascular transport system and/or the carbon (C) economy (McDowell et al. 2008; Sala et al. 2010; McDowell et al. 2011, 2013). Not surprisingly, given the role of C storage for tree survival under stressful conditions (O'Brien et al. 2014), there is a growing interest in understanding how C storage is affected by drought and how plants regulate allocation of recent C assimilates to storage pools (Sala et al. 2012; Willey & Helliker 2012; Dietze et al. 2014).

Trees fix atmospheric $CO_2$ via photosynthesis (source) and distribute assimilates as substrate for growth, maintenance, storage, defence, export and reproduction (sinks; Chapin et al. 1990; Körner 2003). C storage is a major plant function that has been defined as the build-up of carbon in the plant that can be mobilized in the future to support biosynthesis when C supply and demand are not synchronous (Chapin et al. 1990). Mature trees may therefore store large amounts of mobile C pools, mostly non-structural carbohydrates (NSC) and neutral lipids (triacylglycerols) (see Hoch et al. 2003; Würth et al. 2005). Three storage processes have been recognized (Chapin et al. 1990): (1) accumulation when C supply simply exceeds demands (passive overflow); (2) reserve formation when the synthesis of storage compounds is up-regulated so that it competes for resources with growth and defence (active storage); and (3) recycling of compounds primarily involved in growth or defence to support

future growth, which is normally considered unimportant for carbon. Although this distinction inherently involved passive overflow and actively regulated processes, the use of 'passive' and 'active' storage has become more common only recently (Dietze et al. 2014). The current discussion about the role of C storage under drought stress has renewed the interest in disentangling whether C storage is either a 'passive' or 'active' process or both (Sala et al. 2012; Wiley & Helliker 2012; Hartmann & Trumbore 2016; Martínez-Vilalta et al. 2016).

Stomatal closure during drought to prevent desiccation and hydraulic failure also reduces photosynthetic C uptake. It has been hypothesized that trees under drought stress are eventually forced into C storage dependency to meet continuous metabolic demands for maintenance, osmotic adjustments, tissue repair, and defence against pathogens (McDowell et al. 2008, 2011; Sala et al. 2012). However, numerous observational and experimental studies have analysed NSC dynamics in response to drought without any conclusive pattern emerging to date, as NSC concentrations may decrease (e.g. Galiano et al. 2011; Mitchell et al. 2013; Sevanto et al. 2014; Aguadé et al. 2015), increase or not change at all (Sala & Hoch 2009; Anderegg et al. 2012; Gruber et al. 2012; Hartmann et al. 2013a). Several non-exclusive explanations have been proposed to explain these mixed results: (1) cell division and expansion underlying growth are more sensitive to drought than photosynthesis (Muller et al. 2011; Dosio et al. 2011), and thus NSC pools may increase under drought, at least in early stages (McDowell 2011; Hagedorn et al. 2016); as a result, the degree of asynchrony between growth and photosynthesis decline determine the 'carbon safety margin' during drought, which seems to be species-specific (Mitchell et al. 2014); (2) NSC responses may vary across organs within a plant (Galvez et al. 2013; Hartmann et al. 2013b) reflecting the mobile nature of NSC and the need to consider the whole plant in this type of studies; (3) negative effects of drought on C mobilization and phloem transport may lead to C starvation

at the cellular level irrespective of stored reserves (Sala et al. 2010); (4) NSC are likely never fully depleted because soluble sugars are required to remain above a certain threshold to sustain immediate plant functions (e.g., osmoregulation, transport, signalling; Sala et al. 2010; Hartmann & Trumbore 2016; Martínez-Vilalta et al. 2016); (5) NSC may be actively accumulated at the expense of short-term growth to optimize growth and survival in the long term (Sala et al. 2012; Wiley & Helliker 2012).

Observational and experimental studies thus far have mostly focused on plant physiological responses during the drought period (see Mencuccini 2014), whereas the tree's ability to recover physiological functions once drought is released remains much less investigated (Hagedorn et al. 2016). Here, we applied $^{13}C$ pulse labelling to determine the fate of newly produced C assimilates directly after drought release. Seedlings of two species (*Tilia platyphyllos*, *Pinus sylvestris*) with contrasted leaf habit and wood anatomy were subjected to two intensities of experimental drought during ~ 10 weeks, and a subsequent $^{13}C$ pulse labelling was simultaneously applied with rewetting. Tracking the fate of $^{13}C$-label across different plant organs (leaves, stem, coarse roots) and C compounds (soluble sugars, starch, myo-inositol, lipids and cellulose), together with the monitoring of light-saturated photosynthesis ($A_{sat}$), stomatal conductance ($g_s$), organ biomass and NSC concentrations, facilitated the identification of variations in C allocation priorities and tree C balances during recovery from drought. In addition, gas exchange, organ biomass, NSC concentrations and natural $^{13}C$ abundances in C compounds were also monitored during drought in order to relate the recovery effects to the drought-induced changes in the tree C balance. Specifically, our objectives were to determine: (1) whether NSC concentrations increase under drought conditions; (2) whether this increase is caused by active NSC formation (i.e. 'active' storage); and (3) to what extent C allocation strategies prioritize C storage at the expense of growth in seedlings recovering from drought, giving raise to 'drought memory effects'.

## MATERIAL AND METHODS

### Plant material and experimental design

*Tilia platyphyllos* (Scop.) is a deciduous broad-leaved tree with diffuse-porous wood anatomy, native to central and southern Europe. *T. platyphyllos* was observed to strongly reduce transpiration fluxes in response to the intense natural drought experienced in Central Europe in 2003 (see Scherrer et al. 2011), but recovered particularly well once drought ceased (Leuzinger et al. 2005). *Pinus sylvestris* (L.) is an evergreen coniferous tree native to Eurasia that grows under a wide range of environmental conditions and has great ability in adjusting its hydraulic architecture to water availability (Martínez-Vilalta et al. 2009; Poyatos et al. 2013; Salmon et al. 2015). Drought-driven mortality has been documented in *P. sylvestris* at the driest edge of its distribution in dry alpine valleys (Bigler et al. 2006; Rigling et al. 2013) and in the Mediterranean basin (Martínez-Vilalta & Piñol 2002; Vilà-Cabrera et al. 2013), and has been associated with depletion of NSC reserves (Galiano et al. 2011; Aguadé et al. 2015).

The study was carried out during summer 2014 in the greenhouse of the Swiss Federal Institute WSL (47º 21′ 37″ N, 08º 27′21″ E; 500 m a.s.l.) where temperature and relative air humidity varied along with the outside conditions and ranged from 9 to 37 ºC (mean 21 ºC) and from 22 to 74 % (mean 62 %), respectively. Additional artificial illumination was applied (Master Green Power CG T 400W Mogul 1SL/12, Philips Lighting Holding B.V., Eindhoven, The Netherlands) to simulate the average summer photoperiod (~ 15 h light) at Swiss latitudes. Photosynthetic active radiation (PAR) at canopy level was never < 200 µmol $m^{-2}$ $s^{-1}$. A total of 108 three year-old seedlings of each of the two species were obtained from a commercial tree nursery (Kressibucher, Berg, Switzerland). At the beginning of May 2014, 216 pots were numbered and one seedling was planted into each 3.5-l pot filled with 1.8 kg

soil substrate (mixture of 65 % clay mineral components and 35 % hummus; ökohum GmbH, Herrenhof, Switzerland) before leaves developed in *T. platyphyllos*. At planting time, seedling height and stem diameter at the base were 29.5±0.62 cm and 0.7±0.01 cm for *T. platyphyllos* and 27.5±0.30 cm and 0.50±0.006 cm for *P. sylvestris*. The bulk density of the mineral soil substrate was 0.51±0.0001 g $cm^{-3}$. During the acclimation period in the greenhouse, all seedlings were watered every second day to field capacity. All pots were fertilized with a standard controlled-release fertilizer every second month throughout the entire experiment (N:P:K – 18:6:12).

Equal numbers of pots from each of the two species (N = 36) were randomly allocated to one of three treatments: control/well-watered, moderate drought and severe drought. Soil volumetric water content (VWC) in control treatment was maintained at field capacity (see Figure 1). Soil VWC was monitored gravimetrically by weighting ten random pots per treatment every third day. Soil water potential ($\psi_s$) was derived from soil VWC according to Priesack & Durner (2006). Drought treatments were applied when seedlings showed vigorous condition and leaves were fully developed, starting 14 July 2014 in *T. platyphyllos* and 28 July 2014 in *P. sylvestris*. From this point onwards, no water was added until the moderate drought treatment reached the soil VWC threshold of 15 % ($\psi_s$ ~ -0.12 MPa) and the severe drought treatment reached the soil VWC threshold of 8% ($\psi_s$ ~ -1.3 MPa). In *T. platyphyllos*, moderate and severe soil VWC thresholds were reached after 5 and 24 days of irrigation cessation, respectively. In *P. sylvestris*, moderate and severe soil VWC thresholds were reached after 7 and 26 days, respectively. After approximately two months of water shortage, pots from both drought treatments were rewetted every second day to field capacity for a period of 20 days.

**$^{13}CO_2$ pulse-labelling**

$^{13}C$ pulse labelling allows the investigation of C allocation dynamics, i.e. how fast and where the labelled C assimilates are allocated among different sink tissues and activities (Epron et al. 2012; Hartmann & Trumbore 2016) and has been successfully applied to assess the distribution of new assimilates under drought conditions (Ruehr et al. 2009; Blessing et al.2015, Hommel et al. 2016). $^{13}CO_2$ labelling was applied here two days after seedlings were rewetted, on 18 September 2014 in *T. platyphyllos* and 9 October 2014 in *P. sylvestris*. 18 pots per treatment were randomly selected and placed inside an airtight transparent plastic chamber (2.20 m x 1 m x 0.90 m; volume ca. 2000 l). The $^{12}CO_2$ concentrations within the chamber were continuously monitored with the GFS-3000 portable IRGA system (Heinz Walz GmbH, Effeltrich, Germany). For the labelling application, 5 g 99 % $^{13}C$ sodium bicarbonate (Cambridge Isotope Laboratories Inc, Tewksbury, MA, USA) was mixed with 5 g standard $^{12}C$ sodium bicarbonate and hydrochloric acid in an airtight sealed beaker outside the chamber to generate the 50 % labelled $^{13}CO_2$ gas. The $^{13}C$ labelled gas was pumped into the chamber as soon as the $CO_2$ concentration inside reached approx. 300 ppm due to photosynthetic $CO_2$ uptake. Labelling started for both species at 09:00 h. After 30 minutes the $CO_2$ concentration inside the chamber reached approx. 1700 ppm (i.e. ~ 700 ppm $^{13}CO_2$ and ~ 1000 ppm $^{12}CO_2$), which was maintained during the following 3.5 h. The use of fans inside the labelling chamber ensured a good mixing of the air. The mean air temperature inside the chamber during the labelling increased by no more than 4 ºC relative to the ambient air temperature in the greenhouse, and was 23.4 ºC for *T. platyphyllos* and 20.6 ºC for *P. sylvestris*. After the labelling, the transparent plastic chamber was removed and the greenhouse atmosphere was flushed with ambient air ensuring rapid removal of the remaining $^{13}CO_2$.

**Sample collection and physiological measurements**

Harvest time points were distributed unequally throughout the course of the experiment: 2-4 week intervals during the drought phase; and 1 hour, 6 h, 1 day/24 h, 2 d/48 h, 8 d/ 192 h and 20 d/480 h after the labelling and during the rewetting phase. Note that there was a time lag of 2 weeks between the harvest of the two species, starting 28 July 2014 in *T. platyphyllos* and 11 August 2014 in *P. sylvestris* (see Figure 1). At each harvest time, 3 individuals per species and treatment (control, moderate drought, severe drought) were entirely harvested and separated into leaves/needles, stem (10 cm from the root collar), coarse roots (> 2 mm) and fine roots (≤ 2 mm). Fresh weight (FW) was determined in all samples to compute tissue water content (WC; g $g^{-1}$) of each organ and at the whole-plant level (difference between FW and dry weight (DW) divided by DW). All samples were subsequently microwaved for 90 s at 600 W to stop enzymatic activity, oven-dried for 72 h at 65 ºC and ground to fine powder with a ball mill (MM400, Retsch GmbH, Haan, Germany). Leaf gas exchange measurements were conducted on mature, fully developed leaves immediately before destructive sample collection. Light-saturated photosynthesis ($A_{sat}$) and stomatal conductance ($g_s$) were measured between 12:00 and 16:00 h using a GFS-3000 portable IRGA system (Heinz Walz GmbH, Effeltrich, Germany) at a photosynthetic photon flux of 2000 µmol $m^{-2}$ $s^{-1}$, a $CO_2$ concentration of 400 ppm, and a cuvette temperature and relative humidity of 24 ºC and 60 %, respectively. Pre-dawn ($\Psi_{PD}$) and mid-day ($\Psi_{MD}$) leaf water potentials were measured at three time points during the drought phase of the experiment using a Scholander-type pressure chamber (PMS instruments, Corvalis, OR, USA).

Carbon isotope composition of bulk material and specific carbon compounds and NSC concentrations were assessed at different harvest time points: $\delta^{13}C$ in bulk plant material was analysed after 2 months of drought prior to the $^{13}C$ pulse labelling (baseline) and at all harvest

time points after labelling in three organs (leaves, stem, coarse roots) in all treatments and for both species. Compound-specific $\delta^{13}C$ signatures in cellulose, non-structural carbohydrates (NSC; including glucose, fructose and sucrose as soluble sugars, plus starch), myo-inositol (sugar alcohol) and lipids were measured after 2 months of drought prior to labelling (baseline) and at 6 h, 24 h and 192 h after labelling in three organs (leaves, stem, coarse roots) in control and severe drought treatments and for both species. $\delta^{13}C$ signatures in cellulose were only measured in lignified organs (stem, coarse roots) as we assumed that C allocation to leaf cellulose was quantitatively less important once leaves are fully developed. NSC concentrations were quantified at all harvest time points during the drought phase of the experiment and at 20 d after labelling in three organs (leaves, stem, coarse roots) in all treatments and for both species.

**Extraction of carbon compounds for isotope analyses**

Water-soluble carbon compounds (soluble sugars i.e. glucose, fructose and sucrose; and myo-inositol) were extracted from approx. 100 mg of homogenised dried material using 1.5 ml of Milli-Q water (18.2 MΩ cm at 25 ºC). The samples were heated at 85 ºC for 30 min, centrifuged at 10000 g for 2 min and 800 µl of the supernatant was transferred into 2-ml reaction vials. Neutral carbon compounds were then purified from ionic compounds using anion and cation exchange cartridges (OnGuard II H 1 cc, OnGuard II A 1 cc, Dionex Corporation, Sunnyvale, CA, USA; Rinne et al. 2012). Lipids and starch were extracted from the pellet that remained from the previous water-soluble fraction extraction, following the method described by Wanek et al. (2001) as modified by Göttlicher et al. (2006). The pellet was re-suspended by adding a monophasic mixture of methanol, chloroform and water (MCW; 12:3:5, v/v/v) at 70 ºC for 30 min. After three cycles of centrifugations at 10000 g for 2 min and further chloroform and water additions, the water and the lipophilic phases

separated. Lipids were then extracted by transferring the lower lipophilic phase into glass vials. Before starch extraction, the remaining pellet was washed three more times with water to remove all soluble compounds. Starch was then gelatinized at 100 ºC for 15 min and broken down to glucose at 85 ºC for 120 min using α-Amylase from *Bacillus licheniformis* (Sigma Aldrich A4551 1 g). Hydrolysed starch was finally transferred into pre-washed centrifugal devices (Vivaspin 500, Sartorius, Göttingen, Germany) and centrifuged at 12000 g for 50 min to remove enzymes. After the centrifugation, 140 µl of the filtrated starch samples were transferred into pre-weighted tin capsules, which were stored in an oven at 60 ºC overnight. When the samples were dried, the tin capsules were re-weighted again to obtain a difference in weight before and after which is actually an approximate sample weight before isotopic measurements. In the case of lipids, 30 µl of sample was transferred into the tin capsules for drying before isotopic measurements, and the same procedure was applied to obtain the weight of each sample.

For cellulose extraction, approx. 15 mg of homogenised dried material was weighted and packed within heat-sealed Teflon bags (Fiber Filter Bags F57, Ankom Technology, Macedon, USA). In order to remove hemicelluloses from the samples, Teflon bags were soaked in a 5 % NaOH solution within Pyrex Erlenmeyer flasks at 60 °C for 2 h and washed three times with boiling distilled water. Afterwards, the samples were soaked in a 7 % $NaClO_2$ solution at 60 ºC for 30 h to remove resin and lignin and washed again three more times with boiling distilled water. All extracted solutions were stored at minus 20 ºC for further analyses.

**Isotope analyses and calculations**

$\delta^{13}C$ in the bulk plant material, cellulose, starch and lipids was determined by combustion method using an elemental analyser (EA: EA1110 CHN, Carlo Erba, Milan, Italy) linked in the HE downstream mode via a CONFLO II opensplit interface to an isotope ratio mass

spectrometer (IRMS: Delta S, Finnigan MAT, Bremen, Germany) from 0.4-0.6 mg of sample material. Laboratory plant standards and international standards with known $\delta^{13}C$ values were used for calibration of the measurements resulting in a precision of 0.1 ‰. Compound-specific isotope analyses (CSIA) of soluble sugars and myo-inositol were conducted using a high-performance liquid chromatography (HPLC) coupled to the IRMS (delta V Advantage; Thermo, Bremen, Germany) with a LC Isolink interface (Thermo, Bremen, Germany). The mobile phase was 1 mM NaOH. For chromatographic separation a CarboPac PA20 anion-exchange column (3 × 150 mm, Dionex) at a temperature of 20°C was used (Rinne et al. 2012). Myo-inositol and pinitol co-elute due to their similar biochemistry as sugar alcohols and this combined peak is in the following referred to as myo-inositol. The $\delta^{13}C$-standard deviation for repeated analysis of standard mixtures was on an average 0.3 ‰ for myo-inositol, 0.3 ‰ for glucose, 0.4 ‰ for fructose and 0.4 ‰ for sucrose.

The isotopic $^{13}C/^{12}C$ ratio in all samples was expressed in δ notation (‰) relative to the international Vienna Pee Dee Belemnite standard (VPDB). Thereafter $\delta^{13}C$ in bulk plant material was converted to atom% as follows (Ruehr et al. 2009):

$$\text{atom\%} = \frac{100 \cdot 0.0111802 \cdot \left(\frac{\delta}{1000}+1\right)}{1+0.0111802 \cdot \left(\frac{\delta}{1000}+1\right)} \qquad \text{Eqn 1}$$

where 0.0111802 is the standard value for the isotopic $^{13}C/^{12}C$ ratio of VPDB. To calculate excess $^{13}C$ (mg $m^{-2}$) for each organ (i.e. the amount of $^{13}C$ added to the respective organ due to $^{13}C$ labelling), atom% was normalized per organ DW and its natural isotope baseline prior labelling (Ruehr et al. 2009):

$$\text{excess } ^{13}C = \frac{\text{atom\%}_s - \text{atom\%}_b}{100} \cdot B \cdot \frac{C\%}{100} \qquad \text{Eqn 2}$$

where atom%$_s$ is the atom% of the organ sample; atom%$_b$ is the atom% of the natural unlabelled background averaged per species, treatment and organ (n = 3); B (mg m$^{-2}$) is the biomass DW averaged per species, treatment and organ (n = 3) and referred to the pot ground area (0.02 m$^2$); and C% is the percentage of C in the organic sample.

Isotopic concentrations in specific C compounds (cellulose, NSC, myo-inositol and lipids) from the samples collected after pulse-labelling were expressed as $\Delta\delta^{13}C$ (‰) and calculated as follows:

$$\Delta\delta^{13}C_s = \delta^{13}C_s - \delta^{13}C_b \quad \text{Eqn 3}$$

where $\delta^{13}C_s$ is the $\delta^{13}C$ of the organ sample and $\delta^{13}C_b$ is the $\delta^{13}C$ of the natural unlabelled baseline averaged per species, treatment and organ. Excess $^{13}C$ as described above for bulk plant material was not computed for specific C compounds because C concentrations would be needed and were only analysed in this study for NSC and lipids (see Blessing et al. 2015).

**Non-structural carbohydrates concentrations**

Non-structural carbohydrates (NSC) were defined as soluble sugars (glucose, fructose and sucrose) plus starch. A recent study on the comparability of NSC measurements across laboratories concluded that water extraction and quantification using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) are the methods with the least variable results (Quentin et al. 2015); and this was the methodology applied here. Soluble sugars were extracted from 6 mg of homogenised dried material using 1 ml of Milli-Q water (18.2 MΩ cm at 25 ºC). The samples were centrifuged at 14000 g for 10 min and the supernatant was diluted 1:50 (v/v) with distilled water within glass vials for sugar quantification. Glucose, fructose and sucrose were separated and quantified by HPAEC-PAD (DX-300, Dionex, USA) on an analytical Carbo-Pac PA20 Column from

ThermoFisher using a NaOH elution gradient. Starch was extracted from another 6 mg fraction of homogenised dried material and gelatinized at 100 ºC for 60 min using 1 ml of Milli-Q water (18.2 MΩ cm). Starch was then incubated with an amyloglucosidase from *Aspergillus niger* (Sigma Aldrich 10115 5 g) at 50 ºC overnight, to break down starch and sucrose into glucose and glucose and fructose, respectively. Total soluble sugars resulting from the amyloglucosidase digestion were also separated and quantified by HPAEC-PAD. Starch was calculated as the total soluble sugars minus the soluble sugars resulting from the water extraction. All NSC values are expressed as mg $g^{-1}$ of dry mass. A plant standard with known NSC concentrations was measured every 20 samples of the analysis sequence and used as an internal standard (mean NSC 64.03±1.18 mg $g^{-1}$ across all NSC analyses).

**Statistics**

Statistical tests were computed independently for each combination of species, organ and harvest time point. Species responses cannot be directly compared because watering regimes and pulse-labelling set-ups were not simultaneous in *T. platyphyllos* and *P. sylvestris* and climatic conditions in the greenhouse changed over time. Response variables (soil VWC, plant WC, $A_{sat}$, $g_s$, leaf $\psi_{PD}$ and $\psi_{MD}$, $\delta^{13}C$, $\Delta\delta^{13}C$, biomass and NSC) were thus tested for mean differences between treatments (control, moderate drought, severe drought) by using generalised least squares (GLS) models for each combination of species, organ and harvest time point. Function 'weights' was applied to the models in order to account for heterogeneity of variances across treatments. The residuals of all models were normally distributed.

The decrease of $^{13}C$ excess in leaves was described by the following exponential decay function:

$$N(t) = N_0 e^{-\lambda t} \quad \text{Eqn 4}$$

where $t$ is the time in hours after labelling; $\lambda$ is the decay constant; $N_0$ is the initial quantity of $^{13}C$ excess at time = 0 ($^{13}C$ peak); and $N(t)$ is the quantity of $^{13}C$ excess after time $t$. Mean residence time (MRT = 1 /$\lambda$) and half life time (HLT = ln (2) / $\lambda$) were then computed for each species and drought treatment. All statistical analyses were performed with the R statistical software v3.2.5. (R Development Core Team, 2016) with the nlme package for GLS models. Significant differences were considered when $P < 0.05$.

## RESULTS

### Water status and gas exchange

In general, whole-plant water content during drought followed the same pattern as soil volumetric water content for both species (Figure 1). After two weeks of treatment, whole-plant WC in seedlings under both drought intensities significantly differed from whole-plant WC in control seedlings for both species; and these differences were maintained or increased throughout the course of the drought. In *T. platyphyllos*, WC decreases in fine roots were clear under both drought intensities during the entire drought period and only after one month of severe drought in coarse roots (Figure S1). In *P. sylvestris*, WC decreases in fine roots also occurred under both drought intensities during the entire drought period, and only after one month of moderate and severe drought in stem and coarse roots (Figure S1). In both species, midday leaf water potentials ($\psi_{MD}$) were consistently lower under the moderate and, particularly, the severe drought treatments than for the control plants (Table 1).

Soil volumetric water content was completely recovered to field capacity after ten days of rewetting in *T. platyphyllos* (26 Sept 2014), but was still not recovered in *P. sylvestris* after 20 days (Figure 1). Whole-plant WC largely mirrored soil VWC and, consequently, the water status of *P. sylvestris* did not fully recover for any of the drought intensities by the end of the rewetting phase. In *T. platyphyllos*, coarse and fine roots equalled the water status of control individuals by the end of the experiment (Figure S1). In *P. sylvestris*, however, WC in fine roots did not reach the values of the controls for any of the drought treatments at the end of the experiment, and probably determined WC dynamics at the whole-plant level.

Stomatal conductance ($g_s$) responded differently to moderate drought in the two species (Figure 1). In *T. platyphyllos*, $g_s$ under moderate drought only differed from control after two months of water shortage. By contrast, $g_s$ under moderate drought in *P. sylvestris* and under severe drought for both species differed from the control after two weeks of water shortage. Thus, $g_s$ patterns in *P. sylvestris* during the drought phase mainly mirrored WC in plants and soil. $g_s$ recovered after 2-3 days of rewetting in those seedlings from both species that experienced moderate drought conditions during the drought phase of the experiment (Figure 1). Nonetheless, *T. platyphyllos* seedlings that experienced severe drought only recovered $g_s$ after approximately ten days of rewetting (26 Sept 2014). In *P. sylvestris*, seedlings that experienced severe drought recovered $g_s$ after three days of rewetting (10 Oct 2014), despite a small significant relapse occurring one day later. Light-saturated photosynthesis ($A_{sat}$) exhibited the same dynamics as $g_s$ for both species and drought intensities (Figure 1).

**NSC and biomass changes**

Overall, NSC concentrations were 4-fold higher in *T. platyphyllos* (79.5±2.6, 180.4±5.6 and 308.4±11.4 mg $g^{-1}$ for leaves, stem and roots, respectively; Figure 2A) than in *P. sylvestris* (55.5±3.6, 42.5±2.2 and 40.7±1.9 mg $g^{-1}$ for needles, stem and roots, respectively; Figure

2B). Nonetheless, soluble sugars were proportionally more abundant in *P. sylvestris* (Figure 2).

In *T. platyphyllos*, soluble sugars in lignified organs tended to increase under severe drought over time but only significantly after one month of drought in stems (the increase was only marginally significant, $0.05 < P < 0.1$, for coarse roots after one month of drought, and for stem and coarse roots by the end of the drought; Figure 2A). Similarly, starch increased in coarse roots under both drought intensities by the end of the drought. By contrast, starch concentrations in stems were significantly lower under both drought intensities at the beginning of the drought, and equalled control values by the end of the drought period. Overall decreases in biomass under both drought intensities (relative to the control plants) were observed in *T. platyphyllos* at the end of the drought period, despite being significant only for coarse and fine roots (and marginally for stems; Figure S2).

In *P. sylvestris*, an overall increase in soluble sugar concentrations under both drought intensities was observed in all organs during the entire drought period (Figure 2B). A similar pattern was observed for the absolute content of soluble sugars, which increased specially in needles and stems under both drought intensities over time (Figure S2; only results for NSC are shown). Starch concentrations were significantly higher under both drought intensities only at the end of the drought period in needles and coarse roots (Figure 2B). As for *T. platyphyllos*, overall decreases in biomass under both drought intensities were observed in *P. sylvestris* at the end of the drought period. These differences were significant for fine roots and marginally significant for stem and coarse roots; Figure S2).

In *T. platyphyllos* leaves, starch concentrations after 20 days of rewetting were significantly higher in the seedlings that experienced severe drought during the drought phase of the experiment (Figure 2A). In coarse roots, starch remained high in previously drought treated

seedlings compared to the controls. After 20 days of rewetting, *T. platyphyllos* seedlings equalled biomass in coarse and fine roots across all watering regimes, despite a general (non-significant) tendency towards smaller organs in previously drought-stressed seedlings was still observed (Figure S2).

In *P. sylvestris*, soluble sugar concentrations in coarse roots were still significantly higher after 20 days of rewetting in those seedlings that had experienced severe drought compared to the control ones (Figure 2B). After 20 days of rewetting, *P. sylvestris* seedlings equalled biomass in fine roots across all watering regimes, but biomass reductions in coarse roots of drought-stressed seedlings became statistically significant compared to the controls (Figure S2).

**Natural $^{13}C$ abundance**

Overall, bulk $\delta^{13}C$ signatures in plant organs exposed to both drought intensities were significantly higher than in control organs for the two species (Table 2). *P. sylvestris* under severe drought exhibited significant $^{13}C$ enrichment in soluble sugars and starch in all organs. Similarly, *T. platyphyllos* under severe drought showed significant higher $\delta^{13}C$ signatures in all organs for soluble sugars, but only in leaves for starch. In both species, myo-inositol was significantly $^{13}C$ enriched under severe drought only in roots. Although increases in $\delta^{13}C$ in cellulose and lipids were common under severe drought in all organs and for both species, the differences were not significant relative to the control.

**$^{13}C$ pulse-labelling**

Mean residence time (MRT) and half-life time (HLT) for bulk $\delta^{13}C$ in leaves of both species were similar between seedlings that experienced control and moderate drought conditions (Figure S3 and Table 3). Seedlings under severe drought, however, exhibited longer MRT

and HLT. In *T. platyphyllos* stems, the $^{13}C$ label peaked simultaneously in bulk organic matter for control and moderate drought 1 h after labelling, while no obvious peak was observed for the severe drought treatment (Figure S3). In coarse roots, a first $^{13}C$ label peak occurred after 24 h in all watering regimes. In *P. sylvestris* stems, the $^{13}C$ label peaked earlier in the moderate drought treatment (1 h after labelling), and subsequently in the severe drought (6 h after labelling) and control (6-24 h after labelling) treatments (Figure S3). In coarse roots, the $^{13}C$ label showed the highest value earlier in the control and moderate drought treatments (6-24 h after labelling), and only 192 h after labelling in the severe drought treatment. In all cases, the amount of $^{13}C$ label allocated to the coarse roots was larger in those seedlings that previously experienced well-watered (control) conditions relative to moderate and severe drought ($P < 0.05$ for moderate and severe drought in *T. platyphyllos*; $P = 0.16$ for moderate drought and $P < 0.05$ for severe drought in *P. sylvestris*).

Compound specific carbon isotope analyses showed that $^{13}C$-enrichment due to labelling was most strongly expressed in soluble sugars and starch compared to much lower values in myo-inositol, lipids, and cellulose for both species. Nonetheless, dissimilar patterns of C allocation were revealed across compounds and watering regimes (control vs. severe drought; Figure 3). In *T. platyphyllos* leaves, $\Delta\delta^{13}C$ in soluble sugars, starch and lipids were highest 6 h after labelling and afterwards diminished over time (Figure 3A). The $^{13}C$ label in leaf soluble sugars and starch remained significantly longer in the severe drought than in the control treatment. $\Delta\delta^{13}C$ in stem soluble sugars (24 h after labelling) and starch (192 h after labelling) was also higher in the severe drought treatment relative to the controls. Larger amounts of $^{13}C$ label contained in soluble sugars, myo-inositol and lipids were allocated to coarse roots in the control treatment compared to severe drought. By contrast, $^{13}C$ label in starch of coarse roots increased similarly over time for both watering regimes. Interestingly,

192 h after labelling, the $^{13}C$ label peaked in cellulose of coarse roots only in control seedlings.

In *P. sylvestris*, the $^{13}C$ label in soluble sugars of needles was also highest 6 h after labelling, and greater amounts of $^{13}C$ label appeared in stems and coarse roots of control seedlings in comparison to the severe drought-stressed ones (Figure 3B). In contrast, the $^{13}C$ label in myo-inositol was highest 192 h after labelling in needles and coarse roots of severe drought-stressed seedlings, whilst $\Delta\delta^{13}C$ remained rather constant over time in the controls. $^{13}C$ label in starch was also highest 192 h after labelling in stem and coarse roots of severe drought-stressed seedlings. Similar patterns were observed for lipids in all organs and for both watering regimes, with larger amounts of $^{13}C$ label in the severe drought treatment 192 h after labelling. The $^{13}C$ label was also highest in cellulose of stems and coarse roots 192 h after labelling for both watering regimes, but in this case larger amounts were found in the control treatment.

**DISCUSSION**

The dynamics, role and regulation of C storage remain a debated topic in the context of drought-induced tree death (Sala et al. 2012; Wiley & Helliker 2012; Hartmann & Trumbore 2016; Martínez-Vilalta et al. 2016). Tree's ability to recover the C reserves after drought has been much less investigated (but see Galiano et al. 2011; Zang et al. 2014; Jakob 2016). By combining the monitoring of NSC and biomass measurements at the organ and whole-plant level with $^{13}C$ pulse labelling and gas-exchange measurements, this experiment facilitated the identification of variations in C allocation priorities and tree C balances that are associated to drought effects and subsequent drought release.

**C allocation to storage during drought**

Our results indicate that NSC concentrations increased in *P. sylvestris* under drought conditions and this increase is caused by active NSC formation. For this species, we observed two different phases of NSC formation during drought (Figure 2 and Figure S2): (1) early in the drought period (after two weeks), $A_{sat}$ was 50 % lower in seedlings exposed to both drought intensities relative to the control plants; even though overall growth was similar under all watering regimes and total NSC (both concentrations and absolute amounts) accumulated in all organs under both drought intensities; (2) late in the drought period (after two months), total NSC (both concentrations and absolute amounts) still accumulated, especially under severe drought conditions, although $A_{sat}$ decreased to ~ zero under severe drought and growth also declined under both drought intensities relative to the controls. Interestingly, soluble sugars were always the NSC fraction that increased the most with increasing drought. These NSC patterns suggest that, at least in the short-term, soluble sugars may be actively accumulated as an osmotic response to maintain vascular integrity as water availability decreases (Woodruff & Meinzer 2011; Sala et al. 2012). These results contradict the prediction that isohydric species such as *P. sylvestris* (Irvine et al. 1998) rapidly deplete their C reserves under drought conditions (McDowell et al. 2008) and suggest that NSC formation for this species cannot be simply considered a passive overflow process (Hartmann et al. 2015; see also Salmon et al. 2015). Nonetheless, under prolonged drought, the relatively isohydric strategy of *P. sylvestris*, together with reduced canopy leaf area, may eventually force trees to draw from stored carbohydrates to meet the continued metabolic demands (McDowell et al. 2008, 2011; Galiano et al. 2011; Poyatos et al. 2013).

Increases in absolute contents of NSC were not commonly observed in *T. platyphyllos* under drought conditions, and NSC concentration dynamics were thus likely more related to mere

changes in biomass and $A_{sat}$ over time (Figure 2 and Figure S2): (1) early in the drought period (after two weeks), $A_{sat}$ was 50 % lower in seedlings exposed to severe drought relative to the controls, and total NSC concentrations tended to diminish under drought whilst growth remained comparable to that of control seedlings; (2) late in the drought period (after two months), $A_{sat}$ decreased to ~ zero under severe drought and growth also tended to decline under both drought intensities. Consequently, total NSC concentrations under drought remained similar (stem) or even increased (coarse roots) relative to those in control seedlings. This pattern could be explained by the fact that, contrary to *P. sylvestris*, *T. platyphyllos* contained large NSC amounts (mostly starch; Hoch et al. 2003) in all organs and additional NSC formation may not be important in this species to maintain physiological functioning under drought conditions (at least up to the drought intensities studied here; see Morris et al. 2016; Martínez-Vilalta et al. 2016).

Natural $^{13}C$ abundances indicated that overall NSC became isotopically heavier in drought-stressed seedlings after two months of drought (Table 1). $^{13}C$ enrichment in soluble sugars under drought conditions can be explained by two main processes: (1) decreases in the intercellular $CO_2$ partial pressure induced by stomatal closure that leads to decreasing $^{13}CO_2$ discrimination by Rubisco (Farquhar et al. 1982); and/or (2) increases in the amount of $^{13}C$-enriched soluble sugars originating from degradation of the generally $^{13}C$-enriched starch (see Tcherkez et al. 2003). Considering the starch dynamics observed in this study, the $^{13}C$ enrichment in soluble sugars of *P. sylvestris* was more likely due to the former process, which would indicate once again that new $^{13}C$ enriched assimilates were still allocated to C storage and thus further enriching starch in $^{13}C$ under drought conditions. By contrast, NSC dynamics in *T. platyphyllos* indicated some degree of starch breakdown under drought conditions, which means that in this species $^{13}C$ enrichment in soluble sugars could occur by either of the two processes described above.

Myo-inositol is an organic osmolyte that serves as a precursor to a number of metabolites related to membrane biogenesis, cell signalling and biosynthesis of other organic osmolytes directly involved in osmotic adjustment (e.g. pinitol; Loewus & Murthy, 1999). It should be noted, however, that myo-inositol here was not distinguished from pinitol due to their similar biochemistry (see Material and Methods). Myo-inositol was also $^{13}C$-enriched in the coarse roots of seedlings from both species exposed to severe drought (Table 2). Considering the low growth rates of those coarse roots by the end of the drought period, its $^{13}C$ enrichment might indicate yet again that osmoprotectant synthesis pathways may be activated under harsh conditions, in this case to protect root cells against drought stress (Nguyen & Lamant 1988; Rathinasabapathi 2000).

**C allocation to storage during drought release**

Stomatal conductance and $A_{sat}$ recovered rapidly to control values after rewetting, despite *T. platyphyllos* lagged by a few days relative to *P. sylvestris*. Nevertheless, MRT and HLT of $\delta^{13}C$ in leaves after pulse labelling indicated prolonged retention of recent assimilates aboveground in seedlings previously exposed to drought conditions for both species, suggestive of phloem transport impairment. Other studies have also documented reduced phloem transport velocities under drought conditions (Ruehr et al. 2009; Barthel et al. 2011) and subsequent rewetting (Zang et al. 2014). Similarly, and especially in *T. platyphyllos*, less C was translocated belowground to the coarse roots of previously drought-stressed seedlings, which could have led to NSC accumulation in the leaves of *T. platyphyllos* by the end of the experiment.

Interestingly, $^{13}C$-label tracking in specific C compounds revealed opposite C allocation patterns between control and previously drought-stressed seedlings for both species. In *T. platyphyllos*, previously drought-stressed seedlings incorporated most $^{13}C$-label into storage

(starch) in stem and coarse roots eight days after labelling. Simultaneously, control seedlings incorporated C into both storage (starch) and growth (cellulose) in coarse roots. Six and 24 hours after labelling, simultaneous incorporation of $^{13}$C-label into myo-inositol and cellulose of control coarse roots might indicate that biosynthetic pathways are being activated for growth production (cf. see above). In *P. sylvestris*, seedlings recovering from drought incorporated $^{13}$C-label into storage (starch and lipids), osmoprotection (myo-inositol) and, to a lower extent, growth (cellulose) in the stem and coarse roots eight days after labelling. Simultaneously, control seedlings mainly incorporated C into growth (cellulose) and, to a lower extent, storage (lipids) in the stem and coarse roots.

Storage formation in seedlings recovering from drought may be the result of two processes: (1) plant 'memory effects' after drought may still prioritize C storage at the expense of growth to optimize growth and survival in the long term (i.e. 'active' storage; Sala et al. 2012; Wiley & Helliker 2012); (2) growth may be constrained by other limitations than insufficient C availability and surplus C may overflow to storage (McDowell 2011; Palacio et al. 2014). The dynamics of C mass balance during drought did not support active storage for *T. platyphyllos*, and thus the latter process appears more plausible in this species. On the contrary, the fact that growth was not constrained in *P. sylvestris* and NSC still accumulated suggest that 'drought memory effects' may still favour C storage over growth during recovery to ensure future growth and survival (Sala et al. 2012). These results support that C storage may be an important sink under drought conditions and subsequent recovery in *P. sylvestris* and highlight that other C pools (lipids, sugar alcohols) should be considered as potential competing C sinks (Fischer & Höll 1992; Hoch et al. 2003).

$^{13}$C pulse labelling, together with the monitoring of gas exchange and C mass balances over time, is a powerful tool to understand how plants regulate C partition to storage and growth

among other sink activities (Hartmann & Trumbore 2016). At least in the short-term, soluble sugars may be actively accumulated in *P. sylvestris* as an osmotic response to drought effects, and NSC formation cannot be simply considered a passive overflow process for this species (Sala et al. 2012). Once drought ceased, C allocation to storage was still prioritized at the expense of growth in *P. sylvestris*, which suggested that 'drought memory effects' may increase future growth and survival. Our study, however, did not allow the distinction whether C storage was directly upregulated or indirectly stimulated through downregulation of growth ('active' versus 'quasi-active' storage, respectively; Wiley & Helliker 2012). Research using molecular and genetic tools may shed more light on these questions to completely understand C storage regulation in woody plants and its role in stress responses at different time scales (Dietze et al. 2014).

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**CONFLICT OF INTEREST**

The authors have no conflict of interest to declare.

**REFERENCES**

**Aguadé D., Poyatos R., Gómez M., Oliva J. & Martínez-Vilalta J. (2015) The role of defoliation and rot pathogen infection in driving the mode of drought-related physiological decline in Scots pine (*Pinus sylvestris* L.). *Tree Physiology* 35, 229–242.**

**Allen C.D., Breshears D.D. & McDowell N.G. (2015) On underestimation of global vulnerability to tree mortality and forest die-off from hotter drought in the Anthropocene. *Ecosphere* 6, art129.**

**Allen C.D., Macalady A.K., Chenchouni H., Bachelet D., McDowell N., Vennetier M., ..., Cobb N. (2010) A global overview of drought and heat-induced tree mortality reveals emerging climate change risks for forests. *Forest Ecology and Management* 259, 660–684.**

**Anderegg W.R.L., Berry J.A., Smith D.D., Sperry J.S., Anderegg L.D.L. & Field C.B. (2012) The roles of hydraulic and carbon stress in a widespread climate-induced forest die-off. *Proceedings of the National Academy of Sciences of the United States of America* 109, 233–237.**

**Anderegg W.R.L., Hicke J.A., Fisher R.A., Allen C.D., Aukema J., Bentz B., …, Zeppel M. (2015) Tree mortality from drought, insects, and their interactions in a changing climate. *New Phytologist* 208, 674-683.**

**Barthel M., Hammerle A., Sturm P., Baur T., Gentsch L. & Knohl A. (2011) The diel imprint of leaf metabolism on the $\delta^{13}C$ signal of soil respiration under control and drought conditions. *New Phytologist* 192, 925–938.**

**Bigler C., Bräker O.U., Bugmann H., Dobbertin M. & Rigling A. (2006) Drought as an inciting mortality factor in Scots pine stands of the Valais, Switzerland.** ***Ecosystems*** **9, 330–343.**

**Blessing C.H., Werner R.A., Siegwolf R. & Buchmann N. (2015) Allocation dynamics of recently fixed carbon in beech saplings in response to increased temperatures and drought.** ***Tree Physiology*** **35, 585–598.**

**Chapin F.S., Schulze E. & Mooney H.A. (1990) The ecology and economics of storage in plants.** ***Annual Review of Ecology and Systematics*** **21, 423–447.**

**Choat B., Jansen S., Brodribb T.J., Cochard H., Delzon S., Bhaskar R., ..., Zanne A.E. (2012) Global convergence in the vulnerability of forests to drought.** ***Nature*** **491, 752–755.**

**Dietze M.C., Sala A., Carbone M.S., Czimczik C.I., Mantooth J.A., Richardson A.D. & Vargas R. (2014) Nonstructural carbon in woody plants.** ***Annual Review of Plant Biology*** **65, 667–687.**

**Dosio G.A.A., Tardieu F. & Turc O. (2011) Floret initiation, tissue expansion and carbon availability at the meristem of the sunflower capitulum as affected by water or light deficits.** ***New Phytologist*** **189, 94–105.**

**Epron D., Bahn M., Derrien D., Lattanzi F.A., Pumpanen J., Gessler A., ..., Buchmann N. (2012) Pulse-labelling trees to study carbon allocation dynamics: a review of methods, current knowledge and future prospects.** ***Tree Physiology*** **32, 776–798.**

**Farquhar G.D., O'Leary M.H. & Berry JA. (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves.** ***Australian Journal of Plant Physiology*** **9, 121–137.**

**Fischer C. & Höll W. (1992) Food reserves of Scots pine (*Pinus sylvestris* L.). II. Seasonal changes and radial distribution of carbohydrate and fat reserves in pine wood.** ***Trees*** **6, 147–155.**

**Galiano L., Martinez-Vilalta J. & Lloret F. (2011) Carbon reserves and canopy defoliation determine the recovery of Scots pine 4 yr after a drought episode.** ***New Phytologist*** **190, 750–759.**

**Galvez D.A., Landhaeusser S.M. & Tyree M.T. (2013) Low root reserve accumulation during drought may lead to winter mortality in poplar seedlings.** ***New Phytologist*** **198, 139–148.**

**Göttlicher S., Knohl A., Wanek W., Buchmann N. & Richter A. (2006) Short-term changes in carbon isotope composition of soluble carbohydrates and starch: from canopy leaves to the root system.** ***Rapid Communications in Mass Spectrometry*** **20, 653–660.**

**Gruber A., Pirkebner D., Florian C. & Oberhuber W. (2012) No evidence for depletion of carbohydrate pools in Scots pine (*Pinus sylvestris* L.) under drought stress.** ***Plant Biology*** **14, 142–148.**

**Hagedorn F., Joseph J., Peter M., Luster J., Pritsch K., Geppert U., ..., Arend M. (2016) Recovery of trees from drought depends on belowground sink control.** ***Nature Plants*** **2, art 16111.**

**Hartmann H., McDowell N.G. & Trumbore S. (2015) Allocation to carbon storage pools in Norway spruce saplings under drought and low $CO_2$. *Tree Physiology* 35, 243–252.**

**Hartmann H. & Trumbore S. (2016) Understanding the roles of nonstructural carbohydrates in forest trees – from what we can measure to what we want to know. *New Phytologist* 211, 386–403.**

**Hartmann H., Ziegler W., Kolle O. & Trumbore S. (2013a) Thirst beats hunger – declining hydration during drought prevents carbon starvation in Norway spruce saplings. *New Phytologist* 200, 340–349.**

**Hartmann H., Ziegler W. & Trumbore S. (2013b) Lethal drought leads to reduction in nonstructural carbohydrates in Norway spruce tree roots but not in the canopy. *Functional Ecology* 27, 413–427.**

**Hoch G., Richter A. & Körner C. (2003) Non-structural carbon compounds in temperate forest trees. *Plant, Cell and Environment* 26, 1067–1081.**

**Hommel R., Siegwolf R., Zavadlav S., Arend M., Schaub M., Galiano L., ..., Gessler A. (2016) Impact of interspecific competition and drought on the allocation of new assimilates in trees. *Plant Biology* 18, 785–796.**

**IPCC (2014) Climate Change 2014: Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. (eds O. Edenhofer, R. Pichs-Madruga, Y. Sokona, E. Farahani, S. Kadner, K. Seyboth, ..., J.C. Minx), Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.**

**Irvine J., Perks M.P., Magnani F. & Grace J. (1998) The response of *Pinus sylvestris* to drought: stomatal control of transpiration and hydraulic conductance. *Tree Physiology* 18, 393-402.**

**Jakob S. (2016) Water relations during drought and the recovery from drought in two *Eucalyptus* species with contrasting water-use strategies. PhD thesis, The University of Western Australia, Crawley, WA, Australia.**

**Körner C. (2003) Carbon limitation in trees. *Journal of Ecology* 91, 4–17.**

**Leuzinger S., Zotz G., Asshoff R. & Körner C. (2005) Responses of deciduous forest trees to severe drought in Central Europe. *Tree Physiology* 25, 641–650.**

**Loewus F.A. & Murthy P.P.N. (1999) Myo-inositol metabolism in plants. *Plant Science* 150, 1–19.**

**Martínez-Vilalta J., Cochard H., Mencuccini M., Sterck F., Herrero A., Korhonen J.F.J., …, Zweifel R. (2009) Hydraulic adjustment of Scots pine across Europe. *New Phytologist* 184, 353–364.**

**Martínez-Vilalta J. & Piñol J. (2002) Drought-induced mortality and hydraulic architecture in pine populations of the NE Iberian Peninsula. *Forest Ecology and Management* 161, 247–56.**

**Martínez-Vilalta J., Sala A., Asensio D., Galiano L., Hoch G., Palacio S., Piper F.I. & Lloret F. (2016) Dynamics of non-structural carbohydrates in terrestrial plants: a global synthesis. *Ecological Monographs* 86, 495–516.**

**McDowell NG. (2011) Mechanisms linking drought, hydraulics, carbon metabolism, and vegetation mortality. *Plant Physiology* 155, 1051–1059.**

**McDowell N.G., Beerling D.J., Breshears D.D., Fisher R.A., Raffa K.F. & Stitt M. (2011) The interdependence of mechanisms underlying climate-driven vegetation mortality.** ***Trends in Ecology and Evolution*** **26, 523–532.**

**McDowell N.G., Fisher R.A., Xu C., Domec J.C., Hölttä T., Mackay D.S., ..., Pockman W.T. (2013) Evaluating theories of drought-induced vegetation mortality using a multimodel-experiment framework.** ***New Phytologist*** **200, 304–321.**

**McDowell N.G., Pockman W.T., Allen C.D., Breshears D.D., Cobb N., Kolb T., ..., Yepez E.A. (2008) Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought?** ***New Phytologist*** **178, 719–739.**

**Mencuccini M. (2014) Temporal scales for the coordination of tree carbon and water economies during droughts.** ***Tree Physiology*** **34, 439–442.**

**Mitchell P.J., O'Grady A.P., Tissue D.T., White D.A., Ottenschlaeger M.L. & Pinkard E.A. (2013) Drought response strategies define the relative contributions of hydraulic dysfunction and carbohydrate depletion during tree mortality.** ***New Phytologist*** **197, 862–872.**

**Mitchell P.J., O'Grady A.P., Tissue D.T., Worledge D. & Pinkard E.A. (2014) Co-ordination of growth, gas exchange, and hydraulics define the carbon safety margin in tree species with contrasting drought strategies.** ***Tree Physiology*** **34, 443–458.**

**Morris H., Plavcová L., Cvecko P., Fichtler E., Gillingham M.A.F., Martínez-Cabrera H.I., ..., Jansen S. (2016) A global analysis of parenchyma tissue fractions in secondary xylem of seed plants.** ***New Phytologist*** **209, 1553–1565.**

**Muller B., Pantin F., Génard M., Turc O., Freixes S., Piques M. & Gibon Y. (2011) Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs.** ***Journal of Experimental Botany*** **62, 1715–1729.**

**Nguyen A. & Lamant A. (1988) Pinitol and myo-inositol accumulation in water-stressed seedlings of maritime pine.** ***Phytochemistry*** **27, 3423–3427.**

**O'Brien M.J., Leuzinger S., Philipson C.D., Tay J. & Hector A. (2014) Drought survival of tropical tree seedlings enhanced by non-structural carbohydrate levels.** ***Nature Climate Change*** **4, 710-714.**

**Oliva J., Stenlid J. & Martínez-Vilalta J. (2014) The effect of fungal pathogens on the water and carbon economy of trees: Implications for drought-induced mortality.** ***New Phytologist*** **203, 1028–1035.**

**Palacio S., Hoch G., Sala A., Körner C. & Millard P. (2014) Does carbon storage limit tree growth?** ***New Phytologist*** **201, 1096–1100.**

**Poyatos R., Aguadé D., Galiano L., Mencuccini M. & Martínez-Vilalta J. (2013) Drought-induced defoliation and long periods of near-zero gas exchange play a key role in accentuating metabolic decline of Scots pine.** ***New Phytologist*** **200, 388–401.**

**Priesack E. & Durner W. (2006) Closed-form expression for the multi-modal unsaturated conductivity function.** ***Vadose Zone Journal*** **5, 121–124.**

**Quentin A.G., Pinkard E.A., Ryan M.G., Tissue D.T., Baggett L.S., Adams H.D., ..., Woodruff D.R. (2015) Nonstructural carbohydrates in woody plants compared among laboratories.** ***Tree Physiology*** **35, 1146–1165.**

**Rathinasabapathi B. (2000) Metabolic engineering for stress tolerance: installing osmoprotectant synthesis pathways.** ***Annals of Botany*** **86, 709–716.**

**Rigling A., Bigler C., Eilmann B., Feldmeyer-Christe E., Gimmi U., Ginzler C., ..., Dobbertin M. (2013) Driving factors of a vegetation shift from Scots pine to pubescent oak in dry Alpine forests.** ***Global Change Biology*** **19, 229–240.**

**Rinne K.T., Saurer M., Streit K. & Siegwolf R.T.W. (2012) Evaluation of a liquid chromatography method for compound-specific $\delta^{13}C$ analysis of plant carbohydrates in alkaline media.** ***Rapid Communications in Mass Spectrometry*** **26, 2173–2185.**

**Ruehr N. K., Offermann C. A., Gessler A., Winkler J. B., Ferrio J. P., Buchmann N. & Barnard R. L. (2009) Drought effects on allocation of recent carbon: from beech leaves to soil CO2 efflux.** ***New Phytologist*** **184, 950–961.**

**Sala A. & Hoch G. (2009) Height-related growth declines in ponderosa pine are not due to carbon limitation.** ***Plant, Cell and Environment*** **32, 22–30.**

**Sala A., Piper F. & Hoch G. (2010) Physiological mechanisms of drought-induced tree mortality are far from being resolved.** ***New Phytologist*** **186, 274–281.**

**Sala A., Woodruff D.R. & Meinzer FC. (2012) Carbon dynamics in trees: feast or famine?** ***Tree Physiology*** **32, 764–775.**

**Salmon Y., Torres-Ruiz J.M., Poyatos R., Martínez-Vilalta J., Meir P., Cochard H. & Mencuccini M. (2015) Balancing the risks of hydraulic failure and carbon starvation: a twig scale analysis in declining Scots pine.** ***Plant, Cell and Environment*** **38, 2575–2588.**

**Scherrer D., Karl-Friedrich Bader M. & Christian Körner C. (2011) Drought-sensitivity ranking of deciduous tree species based on thermal imaging of forest canopies.** ***Agricultural and Forest Meteorology*** **151, 1632–1640.**

**Sevanto S., McDowell N.G., Dickman L.T., Pangle R. & Pockman W.T. (2014) How do trees die? A test of the hydraulic failure and carbon starvation hypotheses.** ***Plant Cell and Environment*** **37, 153–161.**

**Tcherkez G., Nogués S., Bleton J., Cornic G., Badeck F. & Ghashghaie J. (2003) Metabolic origin of carbon isotope composition of leaf dark-respired $CO_2$ in French bean.** ***Plant physiology*** **131, 237–244.**

**Vilà-Cabrera A., Martínez-Vilalta J., Galiano L. & Retana J. (2013) Patterns of forest decline and regeneration across Scots Pine populations.** ***Ecosystems*** **16, 323–335.**

**Wanek W., Heintel S. & Richter A. (2001) Preparation of starch and other carbon fractions from higher plant leaves for stable carbon isotope analysis.** ***Rapid Communications in Mass Spectrometry*** **15, 1136–1140.**

**Wiley E. & Helliker B. (2012) A re-evaluation of carbon storage in trees lends greater support for carbon limitation to growth.** ***New Phytologist*** **195, 285–289.**

**Woodruff D.R. & Meinzer F.C. (2011) Water stress, shoot growth and storage of non-structural carbohydrates along a tree height gradient in a tall conifer.** ***Plant, Cell and Environment*** **34, 1920–1930.**

**Würth M.K.R., Peláez-Riedl S., Wright S.J. & Körner C. (2005) Non-structural carbohydrate pools in a tropical forest.** ***Oecologia*** **143, 11–24.**

**Zang U., Goisser M., Grams T.E., Häberle K-H., Matyssek R., Matzner E. & Borken W. (2014) Fate of recently fixed carbon in European beech (*Fagus sylvatica*) saplings during drought and subsequent recovery. *Tree Physiology* 34, 29–38.**

**Table 1.** Means and standard errors (n=3) of $\Psi_{PD}$ and $\Psi_{MD}$ (MPa) values for both species and all watering regimes during the drought phase of the experiment. Different letters indicate significant differences between watering regimes for $\Psi_{PD}$ and $\Psi_{MD}$ at each time point ($P < 0.05$).

| | Control | | Moderate D | | Severe D | |
|---|---|---|---|---|---|---|
| | $\Psi_{PD}$ | $\Psi_{MD}$ | $\Psi_{PD}$ | $\Psi_{MD}$ | $\Psi_{PD}$ | $\Psi_{MD}$ |
| *Tilia platyphyllos* | | | | | | |
| 28 July 2014 | -0.20±0.00 a | -1.53±0.06 a | -0.23±0.03 b | -1.63±0.09 a | -0.33±0.03 c | -1.70±0.06 a |
| 11 August 2014 | -0.26±0.03 a | -1.16±0.09 a | -0.36±0.03 a | -1.40±0.15 a | -0.66±0.09 b | -1.80±0.06 b |
| 14 September 2014 | -0.33±0.03 a | -1.10±0.06 a | -0.43±0.09 a | -1.23±0.09 a | -1.06±0.16 b | -2.53±0.09 b |
| *Pinus sylvestris* | | | | | | |
| 11 August 2014 | -0.60±0.06 a | -1.10±0.06 a | -0.76±0.06 a | -1.53±0.12 b | -0.70±0.11 a | -1.60±0.10 b |
| 28 August 2014 | -0.36±0.06 a | -0.86±0.03 a | -0.56±0.06 a | -1.26±0.03 b | -0.96±0.09 b | -1.56±0.09 c |
| 6 October 2014 | -0.36±0.03 a | -1.10±0.06 a | -0.83±0.09 b | -1.43±0.12 b | -0.76±0.12 b | -1.63±0.06 b |

**Table 2.** Means and standard errors (n=3) of $\delta^{13}C$ values for bulk material and carbon compounds (cellulose, soluble sugars, myo-inositol, starch and lipids) extracted from leaves/needles, stem and coarse roots of both species at the end of the drought phase (10 weeks) and before labelling (14 September 2014 for *Tilia platyphyllos*, and 6 October 2014 for *Pinus sylvestris*). Carbon compounds were only extracted in control and severe drought treatments. Cellulose was only extracted from lignified organs (stem and coarse roots). Different letters indicate significant differences among watering regimes for each organ and carbon compound ($P < 0.05$).

| Organ | Watering regime | $\delta^{13}C$ (‰) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Bulk | Cellulose | Soluble sugars | Myo-inositol | Starch | Lipids |
| *Tilia platyphyllos* | | | | | | | |
| Leaves | Control | -26.85±0.09 a | — | -25.81±0.88 a | -28.50±0.79 a | -25.99±0.33 a | -27.99±0.46 a |
| | Moderate D | -26.28±0.14 b | — | — | — | — | — |
| | Severe D | -25.43±0.46 b | — | -19.73±0.41 b | -27.33±0.70 a | -23.22±0.31 b | -25.81±1.32 a |
| Stem | Control | -27.26±0.20 a | -26.00±0.20 a | -25.48±0.54 a | -30.94±0.34 a | -24.57±0.37 a | -27.63±0.17 a |
| | Moderate D | -26.00±0.21 b | — | — | — | — | — |
| | Severe D | -25.92±0.78 ab | -24.67±1.23 a | -19.80±0.47 b | -29.61±0.84 a | -23.62±0.86 a | -27.38±0.15 a |
| Coarse roots | Control | -26.51±0.14 a | -25.88±0.14 a | -23.71±0.78 a | -29.55±0.58 a | -24.54±0.11 a | -26.46±0.41 a |
| | Moderate D | -25.27±0.40 b | — | — | — | — | — |
| | Severe D | -25.09±0.85 ab | -23.93±1.88 a | -18.40±0.19 b | -26.08±0.78 b | -22.68±0.71 a | -24.81±3.00 a |
| *Pinus sylvestris* | | | | | | | |
| Needles | Control | -30.30±0.46 a | — | -31.66±0.06 a | -30.16±0.76 a | -29.33±0.55 a | -27.50±0.48 a |
| | Moderate D | -29.12±0.27 b | — | — | — | — | — |
| | Severe D | -29.16±0.20 b | — | -22.60±1.08 b | -29.46±0.12 a | -24.48±0.19 b | -26.87±0.03 a |
| Stem | Control | -30.16±0.50 a | -28.38±0.49 a | -30.19±0.16 a | -30.93±0.49 a | -29.24±0.57 a | -25.71±1.58 a |
| | Moderate D | -28.91±0.31 b | — | — | — | — | — |
| | Severe D | -29.21±0.19 ab | -28.07±0.11 a | -23.12±0.55 b | -31.20±0.24 a | -25.70±0.46 b | -24.44±1.55 a |
| Coarse roots | Control | -29.30±0.46 a | -27.78±0.57 a | -29.86±0.34 a | -31.68±0.12 a | -27.44±0.36 a | -26.00±0.16 a |
| | Moderate D | -27.88±0.30 b | — | — | — | — | — |
| | Severe D | -28.04±0.44 ab | -27.25±0.19 a | -22.45±0.47 b | -30.83±0.15 b | -26.00±0.35 b | -25.64±0.65 a |

**Table 3.** Mean residence time (MRT) and half life time (HLT) in hours derived from the decay constant (λ) for recent assimilates ($^{13}C$ excess) in leaves/needles of both species. The coefficient of determination ($R^2$) expresses the goodness of the fit for each watering regime, and P-values indicate that fitted coefficients ($N_0$, λ) were always significantly different from zero.

| Organ | Watering regime | λ | Std error λ | MRT | HLT | $R^2$ | P-value $N_0$ | P-value λ |
|---|---|---|---|---|---|---|---|---|
| *Tilia platyphyllos* | | | | | | | | |
| $^{13}C$ excess bulk leaves | Control | 0.0471 | 0.0047 | 21.20 | 14.69 | 0.95 | <0.001 | <0.001 |
| | Moderate D | 0.0454 | 0.0063 | 22.01 | 15.26 | 0.91 | <0.001 | <0.001 |
| | Severe D | 0.0389 | 0.0052 | 25.68 | 17.80 | 0.91 | <0.001 | <0.001 |
| *Pinus sylvestris* | | | | | | | | |
| $^{13}C$ excess bulk needles | Control | 0.0439 | 0.0048 | 22.76 | 15.77 | 0.94 | <0.001 | <0.001 |
| | Moderate D | 0.0407 | 0.0052 | 24.52 | 16.99 | 0.92 | <0.001 | <0.001 |
| | Severe D | 0.0259 | 0.0040 | 38.58 | 26.74 | 0.89 | <0.001 | <0.001 |

**FIGURE LEGENDS**

Figure 1

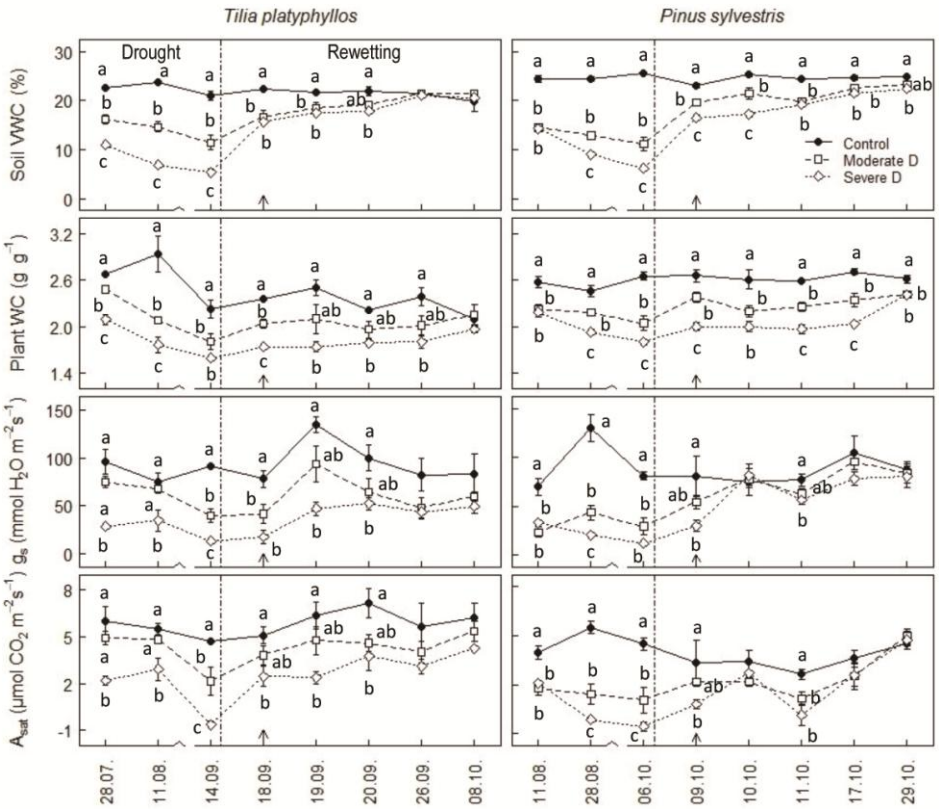


**Figure 1.** Temporal dynamics of soil volumetric water content (Soil VWC), whole-plant water content (Plant WC), stomatal conductance for $H_2O$ ($g_s$) and light-saturated leaf photosynthesis ($A_{sat}$) for control (closed circles), moderate drought (open squares) and severe drought (open diamonds) treatments in both species. The dotted vertical lines denote the starting point of rewetting to field capacity after 10 weeks of moderate or severe drought (16 September 2014 in *T. platyphyllos*, and 7 October 2014 in *P. sylvestris*). Upward arrows on X-axes indicate the labelling date. Dates after labelling on X-axes correspond to the harvest time points: 1 day/24 h, 2 d/48 h, 8 d/ 192 h and 20 d/480 h. Error bars represent standard errors (n=3). Different letters indicate significant differences among watering regimes at each harvest time point ($P < 0.05$).

Figure 2A

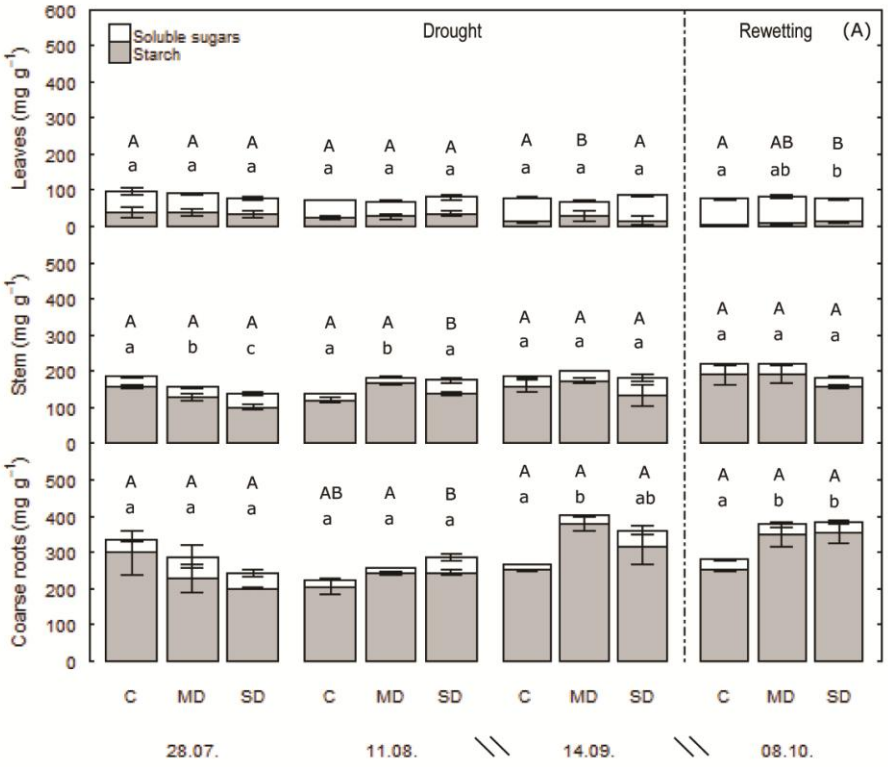

Figure 2B

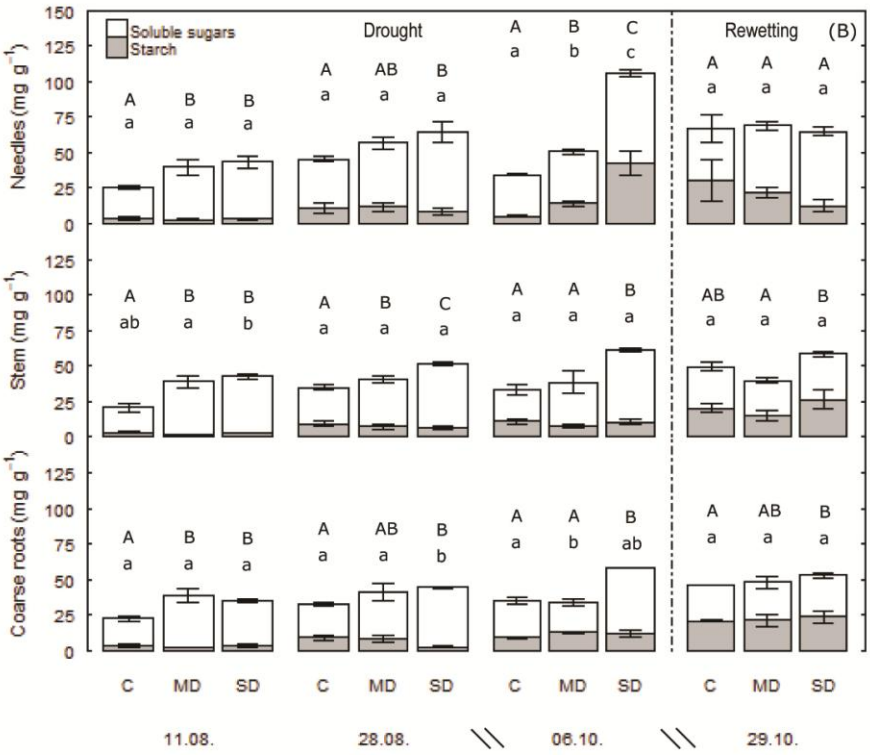


**Figure 2.** Concentrations of soluble sugars and starch in leaves/needles, stem and coarse roots of *Tilia platyphyllos* (A) and *Pinus sylvestris* (B) seedlings for control, moderate drought and severe drought treatments (C, MD and SD in X-axes, respectively). The dotted vertical line separates the drought (10 weeks) and the rewetting (20 days) phases of the experiment. Error bars represent standard errors (n=3). Different capital and lower case letters indicate significant differences among watering regimes at each harvest time point for soluble sugars and starch, respectively ($P < 0.05$).

Figure 3A

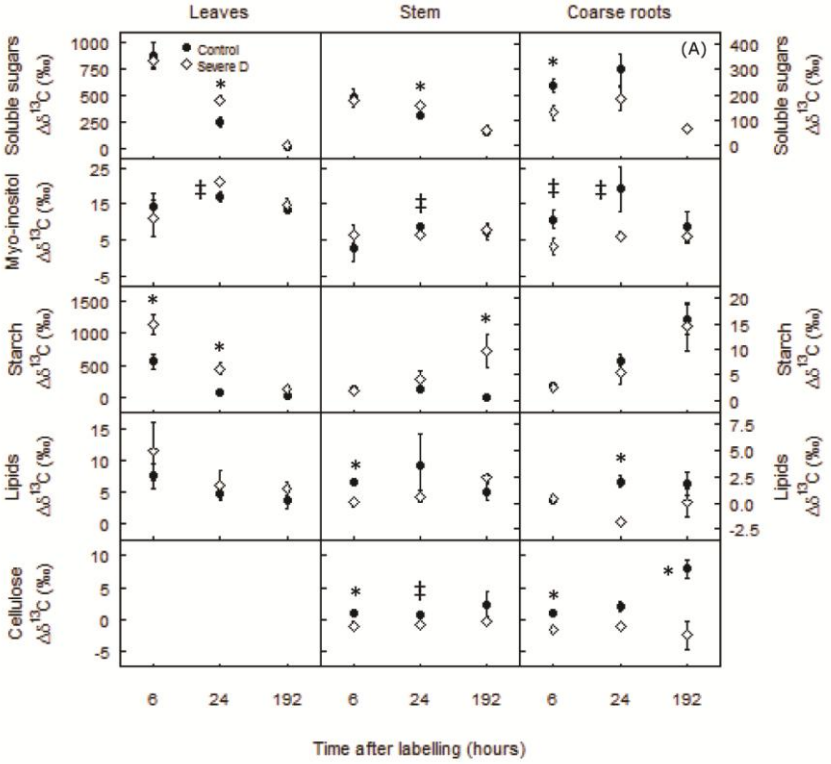


Figure 3B

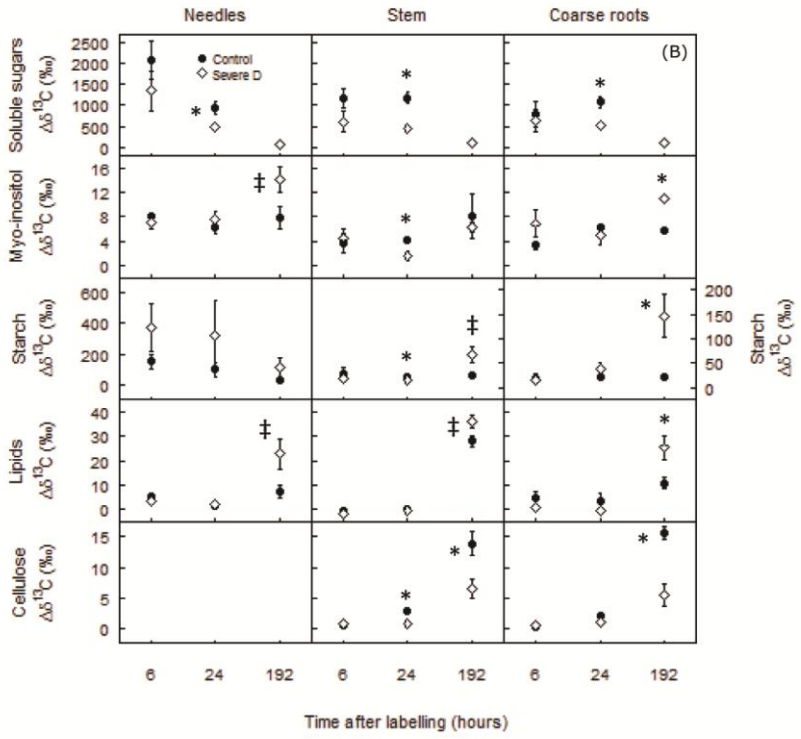

**Figure 3.** Temporal dynamics of $\Delta\delta^{13}C$ ($\delta^{13}C_{sample}$ - $\delta^{13}C_{background}$) for soluble sugars, myo-inositol, starch, lipids and cellulose extracted from leaves/needles, stem and coarse roots of *Tilia platyphyllos* (A) and *Pinus sylvestris* (B) seedlings 6 hours, 24 h/1 day and 192 h/8 d after the labelling (i.e. rewetting phase) in control (closed circles) and severe drought (open diamonds) treatments. $\delta^{13}C$ of cellulose was only measured in lignified organs (stem and coarse roots). Error bars represent standard errors (n=3). Left/right position of tick marks on y-axis inside each panel indicates relevant left/right y-axis scale. Significant differences between watering regimes at each harvest time point are given (*$P < 0.05$; ‡$0.05 < P < 0.1$).