Effects of soil moisture, needle age, and leaf morphology on C and O uptake, incorporation, and allocation: A dual labelling approach with $^{13}\text{CO}_2$ and H_2^{18}O in foliage of a coniferous forest

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Abstract

The carbon and oxygen isotopic composition of water and assimilates in plants reveals valuable information on plant response to climatic conditions. Yet, the C and O uptake, incorporation, and allocation processes determining isotopic compositions are not fully understood.

We carried out a dual-isotope labelling experiment at high humidity with ^{18}O -enriched water (H_2^{18}O) and ^{13}C -enriched CO_2 ($^{13}\text{CO}_2$) with attached Scots pine (*Pinus sylvestris*) branches and detached twigs of hemi-parasitic mistletoes (*Viscum album ssp. austriacum*) in a naturally dry coniferous forest, where also a long-term irrigation takes place. After 4 hours of label exposure, we sampled previous and recent year leaves, twig phloem, and twig xylem over 192 h for the analysis of isotope ratios in water and assimilates.

For both species, the uptake into leaf water and the incorporation of the ^{18}O -label into leaf assimilates was not influenced by soil moisture, while the ^{13}C -label incorporation into assimilates was significantly higher under irrigation compared to control dry conditions. Species-specific differences in leaf morphology or needle age did not affect ^{18}O -label uptake into leaf water, but the incorporation of both tracers into assimilates was two times lower in mistletoe than in pine. The ^{18}O -label allocation in water from pine needles to twig tissues was two times higher for phloem than for xylem under both soil moisture conditions. In contrast, the allocation of both tracers in pine assimilates were similar and not affected by soil moisture, twig tissue or needle age.

Soil moisture effects on ^{13}C -label but not on ^{18}O -label incorporation into assimilates can be explained by the stomatal responses at high humidity, non-stomatal pathways for water, and isotope exchange reactions. Our results suggest that non-photosynthetic ^{18}O -incorporation processes may have masked prevalent photosynthetic processes. Thus, isotopic variation in leaf water could also be imprinted on assimilates when photosynthetic assimilation rates are low.

Keywords: sugars, assimilates, non-structural carbohydrates (NSC), carbon isotopes, oxygen isotopes, drought

Introduction

Tracer experiments with stable carbon and oxygen isotopes have been widely applied in plant ecophysiological research to determine uptake, incorporation, and allocation processes of isotopic signals into water and assimilates (Cernusak et al. 2003; Ruehr et al. 2009; Epron et al. 2012; Lehmann et al. 2018). A better understanding of these processes under changing environmental conditions supports the interpretation and the modelling of the physio-biochemical and hydro-climatic information that is laid down in the natural isotopic signature of plant material (Gessler et al. 2014; Treydte et al. 2014; Saurer et al. 2016; Bögelein et al. 2019).

Recent studies have shown that ^{18}O -labelled water (H_2^{18}O) applied via fogging or leaf wetting (i.e. at high humidity conditions) can serve as a pulse-labelling technique (Lehmann et al. 2018; Lehmann et al. 2020). The ^{18}O -signature of gaseous or liquid water is transferred to leaf water within minutes, likely via bidirectional exchange of water molecules between atmosphere and leaves that occur independently of the plant water status (Goldsmith et al. 2017). The rate of bidirectional exchange and thus the time when steady-state conditions are reached differs between species and growth forms (Lehmann et al. 2020), but might also be modified by leaf traits such as the increasing suberization of needles with plant age (Roden et al. 2015; Dubbert et al. 2017). Moreover, the ^{18}O -labelled leaf lamina water has been observed to diffuse into the main vein and twigs under low transpiration at high humidity (Lehmann et al. 2018), however, the question remains whether water diffusion from leaves to twigs differs between phloem and xylem.

Given the rapid isotopic equilibration between oxygen in CO_2 and surrounding water (Gillon and Yakir 2000; Uchikawa and Zeebe 2012), the isotopic composition of leaf water is subsequently incorporated into sugars within a couple of hours, with the rate dependent on photosynthetic assimilation (Lehmann et al. 2018). However, the ^{18}O -label uptake into leaf water and the ^{18}O -label incorporation into leaf assimilates during high humidity conditions were not affected by water restrictions, although treatment differences in plant water status have been observed (Lehmann et al. 2018). The reasons for this remain not fully understood but may depend on gas-

exchange responses to the ^{18}O -labelling conditions at high humidity and leaf wetness. These conditions may affect the diffusion of H_2O and CO_2 through stomatal pathways to the sites of carbon assimilation and photosynthetic assimilation rates (Aparecido et al. 2017; Dawson and Goldsmith 2018; Berry and Goldsmith 2020). On the other hand, non-photosynthetic processes are known to result in an additional incorporation of isotopic signals of plant water into assimilates. The original leaf water (or more precisely the water signal at the site of synthesis) can be modified by other, mostly more ^{18}O -depleted plant water signals (e.g. source water) during biosynthesis of leaf or tree-ring cellulose (Farquhar et al. 1998; Roden et al. 2000; Sternberg 2009; Lehmann et al. 2017). For example, it is evident that water signals can also be incorporated into sugars and other compounds via hydrolysis reactions in metabolic pathways or via oxygen isotope exchange between water and aldehyde and ketone (i.e. carbonyl) groups (Schmidt et al. 2001; Werner 2003). Previous studies indirectly inferred about this non-photosynthetic processes by determining the isotopic signature of cellulose, however, studies investigating the actual oxygen isotopic composition of water-soluble plant assimilates are still rare (Barbour et al. 2000; Cernusak et al. 2003; Gessler et al. 2013; Lehmann et al. 2017).

For disentangling the processes leading to the ^{18}O -label incorporation and thus to the formation of the oxygen isotopic composition of plants, we propose a dual isotope labelling with ^{18}O -labelled water (H_2^{18}O) and ^{13}C -labelled CO_2 ($^{13}\text{CO}_2$). This is due to the fact that $^{13}\text{CO}_2$ -labelling is a measure for CO_2 diffusion through the stomata and photosynthetic incorporation of CO_2 into plant assimilates in response to environmental conditions, which is likely different from the H_2^{18}O -labelling response. The $^{13}\text{CO}_2$ -labelling approach is particularly helpful to understand plant physiological responses at high humidity, when classical gas-exchange measurements are extremely challenging. Labelling of foliar material with ^{13}C -enriched CO_2 is a commonly applied tracer technique (Epron et al. 2012; Salmon et al. 2019), but also ^{13}C -depleted CO_2 from fossil sources have been used to trace carbon uptake and allocation in plants (Mildner et al. 2014; Klein et al. 2016). The ^{13}C -labelled CO_2 is assimilated by photosynthesis and integrated first into triose phosphates, which are subsequently metabolized to sugars and starch. Both carbohydrate sources are used for the daily supply of carbon skeletons for all metabolic processes in the leaf and thus ^{13}C can be found in many different compounds already shortly after uptake (Galiano et al. 2017). To determine timing and extent of transfer processes from source to sink tissues in response to

environmental conditions such as drought stress (Salmon et al. 2019), the tracer can be followed over time by analyzing the isotopic composition of plant assimilates in different tree organs. For instance, $^{13}\text{CO}_2$ pulse-labelling studies with trees showed lower ^{13}C -label incorporation into leaf assimilates under water restriction (Ruehr et al. 2009).

To our knowledge, Studer et al. (2015) conducted the only dual simultaneous isotope labelling experiment with $^{13}\text{CO}_2$ and H_2^{18}O so far. The authors showed that ^{13}C in organic matter was more rapidly transferred from leaves towards different sink tissues than ^{18}O . A dual isotope labelling approach thus also enable a direct comparison of allocation processes from source to sink tissues (e.g. from leaves to twigs) between both tracers under different environmental conditions. This may provide a better understanding how well the carbon and oxygen isotopic composition of recent leaf assimilates are linked to translocation and metabolic processes in sink tissues under water restrictions, which is particularly important for the interpretation of isotopic signals in tree-rings (Gessler 2011; Pflug et al. 2015).

For our proposed dual isotope labelling approach, we chose a forest site in Switzerland where a long-term irrigation experiment has been conducted since 2003. The forest is dominated by 100-year old Scots pine trees (*Pinus sylvestris*) growing in one of the driest areas (657 mm in precipitation) of the European Alps. The pine forests in the area are regularly subjected to drought-induced mortality events (Bigler et al. 2006; Allen et al. 2010; Rigling et al. 2013), indicating that the conditions are close to the dry limits of distribution of Scots pine. The pine trees showed distinct responses to the irrigation treatment at the whole plant level from leaves towards roots, including increased stem growth and fine root biomass, and reduced defoliation (Dobbertin et al. 2010; Eilmann et al. 2011; Herzog et al. 2014). We therefore expect to observe significant differences in plant physiological responses at high humidity levels in the tree crown between both soil moisture conditions. In addition, a large part of the pine trees is severely infested by mistletoes (*Viscum album ssp. austriacum*), a hemi-parasite, increasing the drought stress of its host through consumption of water (Dobbertin and Rigling 2006; Dobbertin et al. 2010; Zweifel et al. 2012). The anatomical and morphological differences of mistletoe leaves compared to pine needles, as well as their different gas-exchange rates, with generally higher transpiration and lower CO_2 assimilation rates make them an ideal contrasting species to investigate isotope uptake and incorporation processes.

The general purpose of this study was to provide a better understanding of the drivers and processes (e.g. soil moisture, leaf traits, non-photosynthetic reactions) influencing the ^{13}C - and ^{18}O -dynamics (i.e. uptake, incorporation, and allocation) in plants. Therefore, we pulse-labelled pine branches and detached mistletoes from control dry and irrigation plots simultaneously with ^{13}C -enriched CO_2 and ^{18}O -enriched water over 4 hours and sampled leaf and twig tissues over 192 hours for analysis of isotope ratios in water and assimilates. We hypothesize that (1) the diffusion of CO_2 and H_2O proceeds with a lower rate in the non-irrigated control, in older needles and in the conifer (compared to the mistletoe); (2) non-photosynthetic processes contribute significantly to ^{18}O -label incorporation into sugars; and (3) the allocation rates of both tracers are higher in the irrigation treatment, in the twig phloem (compared to the xylem), and in younger needles (compared to the older ones).

Material & Methods

Study site

Our study was performed in a natural, mature Scots pine (*Pinus sylvestris*) forest in Pfynwald, Valais, Switzerland close to Leuk (46°19'27"N, 7°34'40"E, 610 m a.s.l.). The forest site is located within the driest part of Switzerland, with a mean annual precipitation of 657 mm and large interannual variation, and a mean annual temperature of 9.7°C (Dobbertin et al. 2010). A long-term irrigation experiment is being conducted since 2003, where four plots of 1000 m² within the forest are irrigated with water (+700 mm year⁻¹, resulting in 1300-1400 mm total precipitation per year) from a nearby water channel. Four other plots of the same size are used as a naturally dry control. This coniferous forest (> 10 km²) is dominated by 90–100 years old *P. sylvestris*, with a diameter at breast height (DBH) of ≥ 12 cm and a mean height of ~ 11 m with occasional *Quercus pubescens* (Schaub et al. 2016). The soil type is a Rendzic Leptosol with limestone as the parent material (Rigling et al. 2010). The Scots pine trees are severely invested with pine mistletoe (*Viscum album* ssp. *austriacum*) (Dobbertin et al. 2010), with variations in density and age (mostly more than 10 years-old). Environmental conditions at the site are continuously monitored (DecentLab data logger, DecentLab GmbH, Dübendorf, Switzerland), including temperature/humidity (Sensirion STS21, Stäfa, Switzerland), precipitation (Tipping Bucket Raingauge 52203, RM Young, Traverse City, USA), and soil water potential (MPS-2, Decagon Devices, Pullmann, USA).

Dual-isotope labelling experiment

The ^{13}C - and ^{18}O -labelling was performed from 29th August to 7th September in 2017 on scaffolds that are placed next to pine trees in control and irrigated plots, allowing sampling and measurements in the canopy. Figure 1 shows the environmental conditions for the experimental period, with minimum and maximum temperature and relative air humidity ranging between 11.2 ± 2.6 to 24.7 ± 5.3 °C and 39.6 ± 13.6 and $85.6 \pm 5.1\%$, respectively. Although a minor rain event occurred on the third day of the experiment, the soil moisture conditions were consistently different as indicated by soil water potential data.

Individual branches of the sun exposed crown (length = ca. 80 cm) of three different trees from each soil moisture condition (i.e. control naturally dry vs. irrigated conditions) were selected ($n = 3$). Each branch held several twigs with at least two needle generations. During the first three days of the experiment, one branch per treatment was pulse-labelled per day. Pulse-labelling was started at ca. 09:00 CET each day. Each branch was fully enclosed by a cylindrical labelling chamber (ca. 200 L) consisting of PVC tubes stabilized by metal wires and light-permeable plastic foil, allowing the basipetal part of the branch to protrude. One mistletoe branch from a neighboring tree of the same treatment was cut and the detached end sealed with parafilm to avoid uptake of any label via the xylem. Detached mistletoes were then fixed on a twig in the middle of the enclosed pine branch. Subsequently, ^{18}O -enriched water ($\delta^{18}\text{O} = 1650\text{‰}$) was sprayed with a handheld spraying device until all parts of the branch including all needles and twigs and mistletoe leaves were visibly wet. Subsequently, the chamber was fully sealed and 20 ml 99% $^{13}\text{CO}_2$ and 20 ml 99% $^{12}\text{CO}_2$ injected, resulting in an initial total CO_2 concentration of ca. 600 ppm ($^{13}\text{CO}_2 + ^{12}\text{CO}_2$). Constant labelling conditions were provided by re-wetting the branch with ^{18}O -labelled water through a resealable window in the plastic foil and by injections of 20 ml 99% $^{13}\text{CO}_2$ and 20 ml 99% $^{12}\text{CO}_2$ every hour. Mixing of air within the chamber was facilitated by a fan. On average, $^{12}\text{CO}_2$ concentration of 386 ± 85 ppm (MI70 handheld with GMP343 probe; Vaisala, Vantaa, Finland) and $\delta^{18}\text{O}$ of water vapour of 440.3 ± 36.9 ‰ (see methods below) were observed during the labelling period across all experiments (mean $\pm$ SD). Relative air humidity and temperature were $85.7 \pm 14.2\%$ and 28.0 ± 7.8 °C (mean $\pm$ SD), respectively (HP23-A handheld with HygroClip2 probe; Rotronic, Bassersdorf, Switzerland). Variations in both environmental conditions were mainly caused by decreasing ambient temperatures during the rain

event on the third day of the experiment (Fig. 1). After 4 h, the labelling was stopped by completely removing the chamber and foil from the branches.

Sampling of plant material

Leaf and twig samples from pine branches that had been enclosed in the chamber were taken over the course of 192 h: first in the morning (ca. 08:30 CET) before the labelling started, once during the labelling period (i.e. 2 h after labelling start), and several times after the labelling over the course of 8 days (i.e. 4, 7, 10, 24, 48, 72, 192 h after labelling start). At each point in time, a twig that was enclosed in the chamber was cut from the branch. Samples from the recent (i.e. 2017, “New needle”) and previous year needles (i.e. 2016, “Old needle”) were transferred separately to 12 ml gas-tight glass vials (“Exetainers”, Labco, Lampeter, UK). About 10 cm of twig bark including the phloem (“Twig phloem”) was peeled off the xylem (“Twig xylem”) and both tissues were also transferred separately to glass vials. In addition, mistletoe leaves were also cut and transferred to glass vials before the labelling, as well as 2 and 4 h after the labelling started. All samples were immediately placed into a -20°C freezer on the site to stop metabolic activity and kept frozen until further analyses. The last sampling event was conducted on the same day for all experiments and thus the time after start of labelling slightly varies for this time point (i.e. 192 ± 24 h).

Isotope analysis of water vapour and water

Water vapour ca. 30 cm above the pine tree canopy was constantly analyzed during the experimental period with a water vapour isotope laser (WVIA-45r-EP, Los Gatos Research, San Jose, CA, US). Air with an average of ca. 13000 ppm H₂O was pumped with a standard instrumental flow rate (ca. 480 ml min⁻¹) through a Teflon tubing into the analyzer and data was collected every ten seconds. Calibration was performed with dry air that was mixed with two standard waters of known isotope ratios ($\delta^{18}\text{O} = -5\text{‰}$ and 21 ‰). Besides that, $\delta^{18}\text{O}$ of water vapour in the chambers was determined at the end of each labelling experiment by manually injecting 20 ml of chamber air in syringes into an injection port (Swagelock t-connector) that was connected to the same tubing and laser system.

Water was extracted from the frozen leaf and twig samples by using a cryogenic vacuum distillation method (West et al. 2006). In brief, glass vials with samples were boiled for a minimum of 2 hours in a water bath at 80 °C and the evaporating water was constantly trapped in

liquid-nitrogen cooled U-tubes under vacuum (10^{-2} mbar). Subsequently, the U-tubes were dissembled from the vacuum line, closed with rubber stoppers, and the thawed water was transferred to new reaction vials for isotope analysis. $\delta^{18}\text{O}$ values of all liquid water samples were determined using a temperature conversion elemental analyzer that was connected via a ConFlo III to a DELTA^{Plus} XP isotope ratio mass spectrometer (TC/EA-IRMS, all supplied by Finnigan MAT, Bremen, Germany), with a typical precision of $\leq 0.2\text{‰}$ (SD).

Isotope analysis of water-soluble content

The dry plant material from the glass vials (after water extraction) was powdered by a steel-ball mill (MM400, Retsch, Haan, Germany). 60 mg of leaf and 100 mg of twig material were transferred to 2 ml reaction vials and mixed with 1.5 ml deionized hot water ($\sim 80^\circ\text{C}$), and the water-soluble compounds (WSC) extracted for 30 min at 85°C in a water bath (Lehmann et al. 2018). After centrifugation, the supernatant (containing the WSC fraction) was transferred to a new reaction vial. An aliquot was then transferred to silver capsules (ca. 0.5mg WSC), frozen, and freeze-dried. The WSC fraction in twig xylem likely originates from living ray parenchyma cells (von Arx et al. 2017). $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values of all WSC containing capsules were simultaneously determined using a TC/EA (Vario pyrocube, Elementar, Hanau, Germany) that was coupled to the same IRMS as described above (Weigt et al. 2015). Typical precision for $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ were 0.3‰ and 0.2‰ (both SD), respectively. All isotope values are normalized to the international scales V-SMOW ($\delta^{18}\text{O}$, ‰) and V-PDB ($\delta^{13}\text{C}$, ‰).

Calculations and statistics

All carbon and oxygen isotope ratios were corrected for pre-labelling isotope ratios to facilitate the extent of ^{13}C and ^{18}O -label incorporation in different tissues.

$$\Delta\delta\text{X} = \delta\text{X}_\text{L} - \delta\text{X}_\text{NA}$$

Eqn. 1

where X reflects ^{13}C or ^{18}O , δX_L is the isotope ratio after start of the labelling, and δX_NA the isotope ratio at natural isotope abundances (see Table S1).

The transfer of ^{13}C or ^{18}O -label from leaf to twig tissues in pine in water and assimilates was calculated as:

$$\text{LTT (\%)} = \Delta\delta X_{\text{Twig}} / \Delta\delta X_{\text{Leaf}} * 100$$

Eqn. 2

where LTT (%) is the leaf-to-twig transfer of both tracers in percent and X reflects ^{13}C or ^{18}O . For water, $\Delta\delta X_{\text{Leaf}}$ and $\Delta\delta X_{\text{Twig}}$ reflect the $\Delta\delta^{18}\text{O}$ values in leaves and twigs at 4 h, respectively. We assumed that the LTT of ^{18}O -label in water is best observed at the end of the pulse labelling, when transpiration is still low due to high humidity (Lehmann et al. 2018). For assimilates, $\Delta\delta X_{\text{Leaf}}$ and $\Delta\delta X_{\text{Twig}}$ are $\Delta\delta^{13}\text{C}$ or $\Delta\delta^{18}\text{O}$ values in leaves at 4 h and in twigs at 24 h, respectively. To facilitate the isotopic signal comparison between leaf and twig tissues, we assumed a common delay of 24 h for the pulse-labelling signals in leaf assimilates to reach twig tissues (Gessler et al. 2009).

Linear models have been used for testing the effects of time, soil moisture (i.e. control dry vs. irrigated), needle age (i.e. old vs. new needles), twig tissue (i.e. twig phloem vs. xylem), and species (i.e. new pine needles vs. mistletoe leaves) and their interactions on carbon and oxygen isotopic compositions in water and assimilates. One-way ANOVA and a Post-hoc Fisher-LSD test were used for comparing isotope ratios among plant organs. R version 3.4.4 was used for all statistical analyses (R Core Team 2019).

Results

Uptake of ^{18}O -label into water of pine and mistletoe tissues

The average $\Delta\delta^{18}\text{O}$ of water vapour ($\Delta\delta^{18}\text{O}_{\text{Vapour}}$) was calculated as the difference between the average $\delta^{18}\text{O}$ value in the chamber ($440.3 \pm 36.9\text{‰}$) and that under ambient conditions during the experimental period ($-18.3 \pm 2.4\text{‰}$, mean $\pm$ SD) following Eqn. 1. The $\Delta\delta^{18}\text{O}_{\text{Vapour}}$ value of 458.6‰ reflects full isotopic equilibration between the ^{18}O -labelled water source and leaf water and is thus used as a reference for this study (Lehmann et al. 2018). $\Delta\delta^{18}\text{O}$ values of water ($\Delta\delta^{18}\text{O}_{\text{Water}}$) in all pine tissues clearly showed H_2^{18}O uptake, with maximum values at the end of the labelling period that were similar to the $\Delta\delta^{18}\text{O}_{\text{Vapour}}$ reference (Fig. 2; Table 1). After the labelling period, $\Delta\delta^{18}\text{O}_{\text{Water}}$ values quickly decreased in all pine tissues and reached pre-labelling values after 48 h ($P < 0.001$ for time, Table 2). $\Delta\delta^{18}\text{O}_{\text{Water}}$ values in pine needles of both age classes showed no difference, while twig phloem and xylem water were clearly lower compared to needles by more than a factor of 10 and 20 ($P < 0.001$ for tissue, Table 2), respectively. However, soil moisture had no significant effect on $\Delta\delta^{18}\text{O}_{\text{Water}}$ values in any pine tissue ($P >$

0.05) and no interaction between soil moisture and time was observed ($P > 0.05$, Table 2). Similar to pine needles, $\Delta\delta^{18}\text{O}_{\text{Water}}$ values in mistletoe leaves increased during the labelling period ($P < 0.001$ for time, Tables 1 & 3), were similar to the $\Delta\delta^{18}\text{O}_{\text{Vapour}}$ reference at 4 h, and were not affected by soil moisture or interactions (all $P > 0.05$, Tables 1 & 3).

Incorporation of ^{13}C - and ^{18}O -label into assimilates of pine and mistletoe tissues

$\Delta\delta^{18}\text{O}$ values of assimilates in form of water-soluble compounds ($\Delta\delta^{18}\text{O}_{\text{WSC}}$) in new and old pine needles and in mistletoe leaves followed a similar temporal pattern as $\Delta\delta^{18}\text{O}_{\text{Water}}$ values, although with a much slower turn-over rate, showing that the ^{18}O -label was incorporated into assimilates (Fig. 3, Table 1). Temporal variation in $\Delta\delta^{18}\text{O}_{\text{WSC}}$ values was dependent on pine tissues ($P < 0.001$ for the interaction between time and pine tissue, Table 2). $\Delta\delta^{18}\text{O}_{\text{WSC}}$ values in both needle age classes were at maximum at the end of the 4 h labelling period and decreased until pre-labelling conditions were nearly reached after 192 h. $\Delta\delta^{18}\text{O}_{\text{WSC}}$ values in twig tissues were more than two to three times lower compared to needles, with peak values after 10 to 24 h. However, soil moisture had no significant effect on $\Delta\delta^{18}\text{O}_{\text{WSC}}$ in any pine tissues ($P > 0.05$, Table 2) and also no interaction between soil moisture and time was observed ($P > 0.05$, Table 2). $\Delta\delta^{18}\text{O}_{\text{WSC}}$ values in mistletoe leaves also increased during the labelling period ($P < 0.001$ for time, Tables 1 & 3), but were about two times lower compared to those in pine needles ($P < 0.01$ for species). Soil moisture or interactions between soil moisture and time had no effect on $\Delta\delta^{18}\text{O}_{\text{WSC}}$ values in mistletoe leaves (both $P > 0.05$, Table 3).

Also, the ^{13}C label from CO_2 was clearly incorporated into plant assimilates as indicated by $\Delta\delta^{13}\text{C}$ values of water-soluble compounds ($\Delta\delta^{13}\text{C}_{\text{WSC}}$) in pine needles and mistletoe leaves (Fig. 4, Table 1). $\Delta\delta^{13}\text{C}_{\text{WSC}}$ values in both needle age classes were at maximum at the end of the 4 h labelling period and decreased until pre-labelling conditions were nearly reached after 192 h. Temporal variation was also observed for $\Delta\delta^{13}\text{C}_{\text{WSC}}$ values in twig tissues, with peak values after 7 to 24 h. However, in contrast to oxygen isotopes, $\Delta\delta^{13}\text{C}_{\text{WSC}}$ values in pine tissues were significantly higher under irrigated than under control dry soil moisture conditions, with the extent of the treatment effect dependent on pine tissue and time ($P < 0.01$ for both interactions, Table 2). $\Delta\delta^{13}\text{C}_{\text{WSC}}$ values of mistletoe leaves also increased during the labelling period ($P < 0.001$ for time, Tables 1 & 3), were higher under irrigated than under control conditions ($P < 0.05$

for soil moisture, Table 3), but about two times lower compared to those in pine needles ($P < 0.03$ for species, Tables 1 & 3).

^{13}C - and ^{18}O -label allocation in water and assimilates of pine tissues

Furthermore, we determined the allocation of both tracers in response to the irrigation treatment by estimating the leaf-to-twigg transfer (LTT) of ^{13}C - and ^{18}O -label for water and assimilates (Eqn. 2, Table 4). For water, the LTT of ^{18}O -label at the end of the labelling period was two times higher to the twig phloem (ca. 8.3%) than to the twig xylem (ca. 3.5%, $P < 0.001$ for twig tissue, Table 5). The LTT of ^{13}C - and ^{18}O -label was not different between twig tissues ($P > 0.05$, Table 5), although a tendency of higher LTT values for phloem assimilates was observed. Needle age and soil moisture showed no effect on any LTT values for water and assimilates ($P > 0.05$). Moreover, we calculated the average mean residence time (MRT; Table S2) for ^{13}C and ^{18}O in water (2.2 to 5.9 h) and assimilates (31.5 to 114.6 h) of pine needles following Lehmann et al. (2020). MRT of both tracers in water and assimilates was neither affected by needle age nor soil moisture, and MRT of both tracers was not different in assimilates (Tables S3).

Discussion

The ^{18}O -label uptake into plant water is not affected by soil moisture, needle age, or species

The ^{18}O -signal was clearly visible in the water of pine needles and mistletoe leaves already within the first two hours after start of the labelling (Fig. 2), with maximum $\Delta\delta^{18}\text{O}_{\text{Water}}$ values similar to $\Delta\delta^{18}\text{O}_{\text{Vapour}}$ (ca. 458.6‰) at the end of the labelling period. The result goes along with previous studies showing that for many species it takes about 5 h until full isotopic equilibration between the ^{18}O -labelled water source and leaf water is reached (Roden and Ehleringer 1999; Kim and Lee 2011; Lehmann et al. 2020). The soil moisture conditions did not influence $\Delta\delta^{18}\text{O}_{\text{Water}}$ in pine and mistletoes, consistent with recent findings, where the ^{18}O -label uptake from vapour into leaf water was similar between watered and drought-stressed oak saplings after a fog event (Lehmann et al. 2018). This means that also known differences in needle morphology between the control and irrigated site (Dobbertin et al. 2010) have no effect on the equilibration process. Moreover, needle age has recently been observed to influence the leaf water enrichment due to suberization of the needle tissues (Roden et al. 2015). However, we observed no difference in ^{18}O -label uptake between needle age classes (Table 2), indicating that potential anatomical or

morphological differences along with aging of needles have also no direct effect on the bi-directional isotopic equilibration process. Besides that, ^{18}O -label uptake into leaf water was not different between pine and mistletoe leaves, suggesting that differences in leaf morphology were not large enough for an effect. After 48 h, no more traces of the ^{18}O -label were observed in water of any pine tissue under both soil moisture conditions (Fig. 2), assuming efficient dilution by the transpiration stream and equilibration with ambient (unlabelled) water vapour. We can therefore demonstrate that the mixing and equilibration process between water molecules in atmospheric vapour and leaf water is not or only slightly influenced by soil moisture, needle age, and species.

Several reasons may explain the non-responsive ^{18}O -label uptake into leaf water. Goldsmith et al. (2017) recently showed that the ^{18}O -label uptake is independent of net foliar water uptake (FWU), i.e. a positive net flux of water that enters the leaf (Dawson and Goldsmith 2018). With respect to isotopes, this means that ^{18}O -label uptake into leaf water alone cannot be indicative of FWU processes. Furthermore, the authors conclude that the ^{18}O -label uptake is mainly based on a bidirectional exchange of water molecules inside and outside the leaves, which occurs independently of net uptake of water. It should be also noted that even under conditions where no net water uptake from the atmosphere to the leaves takes place and thus plants transpire, there is still a gross influx from the atmosphere to the leaf that exceeds the gross water flux from the roots to the leaf (Farquhar and Cernusak 2005). The pathways for FWU are still under debate (Berry et al. 2018), but gaseous water vapour uptake through stomata seems to be the main entrance point (Lehmann et al. 2018). However, also other non-stomatal pathways are known such as water uptake through the cuticle (Riederer and Schreiber 2001) or in form of liquid water along the stomatal chamber and guard cells (Burkhardt et al. 2012; Burkhardt and Hunsche 2013). Such pathways could theoretically bypass the stomatal response to soil moisture, particularly when leaves are physically wet during labelling conditions as performed in this study. Moreover, the isotopic equilibration process between water vapour or liquid water on the leaves and leaf water is a very fast process. Differences in ^{18}O -label uptake due to different stomatal responses to soil moisture can therefore likely only be observed very early during the equilibration process or not at all. Thus, studies with high-temporal resolution are needed to disentangle the relative contribution of stomatal and non-stomatal pathways to ^{18}O -label uptake via bidirectional exchange and/or potential foliar water uptake.

Higher diffusion of leaf water into twig phloem than xylem under high humidity

The ^{18}O -label was also observed in the twig water (i.e. phloem and xylem) of pine trees, although to a much lower extent compared to needles (Fig. 2, Table 4). The uptake of ^{18}O -labelled water into both twig tissues is most likely explained by the diffusion of leaf water into main veins and twigs under highly reduced net transpiration and thus a low xylem water flux due to high humidity (Studer et al. 2015; Lehmann et al. 2018) and potentially via lenticels (Groh et al. 2002). Interestingly, there were remarkable ^{18}O -differences between phloem and xylem. The leaf-to-twig transfer of ^{18}O -label was about twice as high for phloem as for xylem during the first 24 h (Tables 4 & 5). The ^{18}O -label in xylem water might have been diluted by a higher water content or by a small transpirational flow when air humidity was not fully saturated. The higher ^{18}O -label in phloem water was most likely influenced by the osmotic pressure that leads to an additional influx of leaf water during loading of assimilates into the phloem. Besides, the ^{18}O -label was already observed in phloem water after 2 h of labelling and thus before it was detected in the xylem water (Fig. 2). We speculate that leaf water is first transferred into the phloem before it partially mixes with xylem water along the translocation pathway (Münch counterflow) ((Münch counterflow; Hölttä et al. 2006). A prioritized back-diffusion of ^{18}O -enriched leaf water via translocation pathways into twig phloem is also supported by studies observing an ^{18}O -enrichment of phloem water compared to xylem water at natural isotope abundances (Cernusak et al. 2003).

In contrast to plant water, we found no clear indication that the allocation of both tracer via assimilates from leaves to twigs was affected by the soil moisture conditions, needle age, or different twig tissues (Tables 4 & 5). This demonstrates that the C and O follow very similar allocation strategies and that the carbon and oxygen isotopic composition of sink tissues are likely produced from assimilate sources of the same age.

On the mechanisms causing differences in ^{13}C - and ^{18}O -label incorporation into assimilates

The irrigation treatment conducted in the natural dry coniferous forest did affect the ^{13}C -label uptake and incorporation, but surprisingly not the ^{18}O -label incorporation into assimilates in any pine or mistletoe tissue (Figs. 3 & 4, Tables 1 & 2). But what are the mechanisms causing the differences in ^{13}C and ^{18}O -label incorporation into plant assimilates?

The increased soil moisture conditions in our pine forest changed the crown structure and leaf morphology (Dobbertin et al. 2010), positively affected plant water status and needle mass of pine trees (Schönbeck et al. 2018), and caused higher stem growth rates of irrigated vs. control trees (Timofeeva et al. 2017). The higher water availability likely goes along with plant physiological changes such as increased carbon assimilation and stomatal conductance (Flexas et al. 2002). The differences in ^{13}C -label incorporation into assimilates might therefore be explained by differences in the stomatal responses controlling the diffusional pathways of CO_2 through the stomata cavity towards the site of CO_2 assimilation in mesophyll (Farquhar et al. 1989). Irrigated plants may have opened their stomata more rapidly than the drought exposed control plants in response to the high humidity conditions during labelling, causing a higher CO_2 diffusion into the leaves and thus a higher ^{13}C -label incorporation. On the other hand, the ^{13}C -label incorporation pattern is likely explained by increased carbon assimilation rates due to the increased water supply, but also due to the higher demand for assimilates for the increased stem growth of irrigated trees (Eilmann et al. 2011). Differences in leaf assimilate pool sizes that could affect the ^{13}C -label incorporation can be excluded, given that concentrations of the non-structural carbohydrates in the needles of the studied pine trees were not affected by the long-term irrigation treatment (Schönbeck et al. 2018). However, one should note that the gas-exchange of the wetted leaves during labelling conditions might have been different compared to natural conditions (Dawson and Goldsmith 2018), given that wetting of leaves and high humidity can affect carbon assimilation and stomatal behavior (Aparecido et al. 2017; Gerlein-Safdi et al. 2018b; Berry and Goldsmith 2020). Thus, the ^{13}C -label incorporation pattern in this study are likely caused by the dualism of photosynthetic and stomatal responses to soil moisture and to the applied labelling conditions (wet leaves & high humidity).

The ^{18}O -label incorporation pattern is surprising given that we expected higher incorporation under irrigated conditions due to higher carbon assimilation rates, which would be in accordance with the ^{13}C -label incorporation pattern (Figs. 3 & 4, Tables 1 & 2). We thus assume that the ^{18}O -label incorporation into assimilates could have been additionally influenced by non-photosynthetic reactions (Sternberg et al. 1986), which is not possible for ^{13}C -label incorporation. Given that the ^{18}O -label uptake into leaf water and its distribution via veins is a very rapid process (in minutes), the ^{18}O -label is widespread available throughout the leaf for incorporation

processes already shortly after labelling start (Gerlein-Safdi et al. 2018a). In the open linear form during mutarotation, carbonyl groups of hexoses can exchange O atoms with surrounding water, a process which is especially fast for smaller triose phosphates molecules (Schmidt et al. 2001). Besides, hydrolysis reactions in metabolic pathways lead to an additional integration of oxygen into sugars (Lehmann et al. 2017). A relatively high contribution of such non-photosynthetic reactions to the ^{18}O -label incorporation could mask prevalent differences in CO_2 diffusion and carbon assimilation processes in response to soil moisture conditions. We speculate that also other compounds such as amino or organic acids could integrate the ^{18}O -label, but also compounds that have been synthesized at a different day. If true, this might have consequences for our understanding of how variations in leaf water oxygen isotope composition are incorporated into organic matter. For instance, isotopic variations in leaf water could be integrated also under low photosynthetic activity or in the absence of photosynthetic activity at nighttime, e.g. during starch breakdown (Gessler et al. 2007). Nevertheless, we do not assume that photosynthetic assimilation rates can be neglected in the ^{18}O -label incorporation processes. Recent studies demonstrated that the ^{18}O -label uptake into individual sugars and starch are clearly related to the net CO_2 assimilation rates and also influenced by photosynthetic modes (i.e. C_3 , C_4 , CAM) (Lehmann et al. 2018; Lehmann et al. 2020). Mistletoes as hemiparasites are known for their lower photosynthetic activities compared to their hosts (Matsubara et al. 2002; Mathiasen et al. 2008), and indeed incorporation of both tracers into assimilates were lower compared to pine needles in our study (Figs. 2 & 3, Table 3); yet, the detachment of the mistletoe leaves may have also contributed to lower assimilation rates. Future studies should therefore clarify the relative contribution of photosynthetic vs non-photosynthetic pathways for the isotopic signal transfer from water to organics.

Conclusions

Our multi-isotope pulse-labelling with $^{13}\text{CO}_2$ and H_2^{18}O provides a novel approach to infer on C and O uptake, incorporation, and allocation processes in plant foliage. This approach can be particularly helpful at high humidity conditions when gas-exchange measurements are challenging. We found no indications that morphological leaf traits and soil moisture affected the ^{18}O -label uptake into leaf water. We speculate that the result could be different if the processes would have been studied in a higher temporal resolution. The increased ^{13}C -label incorporation

into assimilates under irrigated vs. control dry conditions is likely explained by the interacting effects of stomatal responses and carbon assimilation to soil moisture and labelling conditions (wet leaves & high humidity). On the other hand, the absence of a soil moisture effect on ^{18}O -label incorporation into assimilates highlights the relevance of non-photosynthetic processes for the oxygen isotopic signal transfer from water to plant assimilates. Our results are particularly important for climate reconstructions using tree ring analyses. It is essential to determine which of the oxygen isotope signal are reflected in the $\delta^{18}\text{O}$ of the organic matter: is it predominantly a result of a) the photosynthetic incorporation, which would store $\delta^{18}\text{O}$ values of the ambient water vapor modulated by leaf water enrichment, or is it the non-photosynthetic incorporation, which would predominantly reflect the soil/source water and potentially the precipitation water. A careful data analysis in combination with specific experiments will be key for this question.

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Figure 1: Environmental conditions during the experiment. (a) Temperature and relative humidity and (b) precipitation (vertical lines) and soil water potential at 10 cm depth under control dry and irrigated conditions. The dual-isotope pulse-labelling was conducted on three consecutive days (grey bars).

Figure 2: Uptake of ^{18}O -label into plant tissue water. $\Delta\delta^{18}\text{O}$ values of water ($\Delta\delta^{18}\text{O}_{\text{Water}}$) in different pine tissues and mistletoe leaves under control dry and irrigated conditions during and after exposure to a 4 h labelling event (shaded area) with ^{18}O -enriched water and ^{13}C -enriched CO_2 are given. $\Delta\delta^{18}\text{O}$ value of water vapour ($\Delta\delta^{18}\text{O}_{\text{Vapour}}$) reflects a reference for full isotopic equilibration between the ^{18}O -labelled water source and leaf water. Please note the X-axis break, as well as the change in Y-axis scale between upper and lower panel. Mean ± 1 SE ($n = 2\text{--}3$ individuals per treatment).

Figure 3: Incorporation of ^{18}O -label into assimilates. $\Delta\delta^{18}\text{O}$ values of water-soluble compounds ($\Delta\delta^{18}\text{O}_{\text{WSC}}$) in different pine tissues and mistletoe leaves under control dry and irrigated conditions during and after exposure to a 4 h labelling event (shaded area) with ^{18}O -enriched water and ^{13}C -enriched CO_2 are given. Please note the X-axis break, as well as the change in Y-axis scale between upper and lower panel. Mean ± 1 SE (n = 2-3 individuals per treatment).

Figure 4: Incorporation of ^{13}C -label into assimilates. $\Delta\delta^{13}\text{C}$ values of water-soluble compounds ($\Delta\delta^{13}\text{C}_{\text{WSC}}$) in different pine tissues and mistletoe leaves under control dry and irrigated conditions during and after exposure to a 4 h labelling event (shaded area) with ^{18}O -enriched water and ^{13}C -enriched CO_2 are given. Please note the X-axis break, as well as the change in Y-axis scale between upper and lower panel. Mean ± 1 SE (n = 2-3 individuals per treatment).

Table 1: Comparison of ^{13}C - and ^{18}O -label uptake and incorporation into water and assimilates among species and tissues at the end of the 4 h labelling period. The $\Delta\delta^{18}\text{O}$ values in water ($\Delta\delta^{18}\text{O}_{\text{water}}$), as well as the $\Delta\delta^{18}\text{O}$ and $\Delta\delta^{13}\text{C}$ values of water-soluble compounds ($\Delta\delta^{13}\text{C}_{\text{WSC}}$, $\Delta\delta^{18}\text{O}_{\text{WSC}}$) in different pine tissues and mistletoe leaves under control dry and irrigated conditions are given (n = 2-3 individuals). Different capital letters indicate significant differences within each soil moisture treatment (One-way ANOVA and Fisher-LSD post-hoc). Mean $\pm$ 1 SE.

Tissue	Soil moisture	$\Delta\delta^{18}\text{O}_{\text{water}}$ (‰)	$\Delta\delta^{18}\text{O}_{\text{WSC}}$ (‰)	$\Delta\delta^{13}\text{C}_{\text{WSC}}$ (‰)
New needle	Control	429.7 $\pm$ 59.9 ^A	28.1 $\pm$ 1.7 ^A	14.6 $\pm$ 5.1 ^A
Old needle	Control	431.2 $\pm$ 136.1 ^A	26.9 $\pm$ 7.8 ^A	7.2 $\pm$ 3.2 ^{AB}
Mistletoe leaf	Control	467.2 $\pm$ 77.0 ^A	17.6 $\pm$ 4.9 ^{AB}	3.8 $\pm$ 1.9 ^B
Twig phloem	Control	40.1 $\pm$ 19.7 ^B	5.4 $\pm$ 2.7 ^B	1.3 $\pm$ 3.3 ^B
Twig xylem	Control	18.2 $\pm$ 6.2 ^B	5.4 $\pm$ 2.6 ^B	3.9 $\pm$ 1.8 ^B
New needle	Irrigated	398.0 $\pm$ 114.9 ^A	23.3 $\pm$ 2.1 ^{AB}	26.7 $\pm$ 8.8 ^A
Old needle	Irrigated	395.1 $\pm$ 103.7 ^A	28.7 $\pm$ 6.5 ^A	32.0 $\pm$ 11.3 ^A
Mistletoe leaf	Irrigated	561.5 $\pm$ 74.4 ^A	13.7 $\pm$ 0.7 ^{BC}	12.5 $\pm$ 1.7 ^{AB}
Twig phloem	Irrigated	35.28 $\pm$ 15.1 ^B	5.08 $\pm$ 1.3 ^C	5.6 $\pm$ 2.4 ^B
Twig xylem	Irrigated	12.67 $\pm$ 4.4 ^B	6.60 $\pm$ 3.3 ^C	6.2 $\pm$ 1.8 ^B

Table 2: Results of linear models for $\Delta\delta^{18}\text{O}$ values in water ($\Delta\delta^{18}\text{O}_{\text{Water}}$) and for $\Delta\delta^{18}\text{O}$ and $\Delta\delta^{13}\text{C}$ values in water-soluble compounds ($\Delta\delta^{13}\text{C}_{\text{WSC}}$, $\Delta\delta^{18}\text{O}_{\text{WSC}}$) for pine. P-values are given for soil moisture treatment (i.e. control/irrigated), pine tissue (i.e. new and old needles, twig phloem and xylem), time (i.e. 0 to 192 h after start of labelling), and their interactions.

Factors	$\Delta\delta^{18}\text{O}_{\text{Water}}$	$\Delta\delta^{18}\text{O}_{\text{WSC}}$	$\Delta\delta^{13}\text{C}_{\text{WSC}}$
Soil moisture	0.93	0.73	<0.001***
Pine tissue	<0.001***	<0.001***	<0.001***
Time	<0.001***	<0.001***	<0.001***
Soil moisture * Pine tissue	0.99	0.54	0.01**
Soil moisture * Time	0.96	0.08	0.01**
Pine tissue * Time	0.63	<0.001***	0.32

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3: Results of linear models for $\Delta\delta^{18}\text{O}$ values in water ($\Delta\delta^{18}\text{O}_{\text{Water}}$) and for $\Delta\delta^{18}\text{O}$ and $\Delta\delta^{13}\text{C}$ values in water-soluble compounds ($\Delta\delta^{13}\text{C}_{\text{WSC}}$, $\Delta\delta^{18}\text{O}_{\text{WSC}}$) in pine needles and mistletoe leaves. P-values are given for soil moisture treatment (i.e. control/irrigated), species (i.e. new pine needles/ mistletoe leaves), time (i.e. 0 to 4 h after start of labelling), and their interactions.

Factors	$\Delta\delta^{18}\text{O}_{\text{Water}}$	$\Delta\delta^{18}\text{O}_{\text{WSC}}$	$\Delta\delta^{13}\text{C}_{\text{WSC}}$
Soil moisture	0.82	0.19	0.05*
Species	0.06	0.01**	0.03*
Time	<0.001***	<0.001***	<0.001***
Soil moisture * Species	0.62	0.96	0.73
Soil moisture * Time	0.96	0.61	0.36
Species * Time	0.38	0.07	0.27

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 4: Leaf-to-twigg transfer (LTT, %) of ^{13}C - and ^{18}O -label in pine water and assimilates under control dry and irrigated conditions. Note that a delay of 24 h was assumed for leaf assimilates to reach twig tissues, but not for water (see LTT calculations for more details). Mean $\pm$ 1 SE (n = 2-3 individuals per treatment).

	LTT (%) - Control		LTT (%) - Irrigated	
	Phloem	Xylem	Phloem	Xylem
Leaf/twig tissue				
New needles - $\Delta\delta^{18}\text{O}_{\text{Water}}$	8.5 $\pm$ 3.3	4.0 $\pm$ 1.0	8.1 $\pm$ 1.3	3.1 $\pm$ 0.2
Old needles - $\Delta\delta^{18}\text{O}_{\text{Water}}$	8.4 $\pm$ 1.9	4.1 $\pm$ 0.3	8.2 $\pm$ 1.6	3.1 $\pm$ 0.3
New needles - $\Delta\delta^{18}\text{O}_{\text{WSC}}$	32.7 $\pm$ 6.4	31.5 $\pm$ 3.4	47.7 $\pm$ 5.6	27.6 $\pm$ 14.0
Old needles - $\Delta\delta^{18}\text{O}_{\text{WSC}}$	48.2 $\pm$ 25.0	42.5 $\pm$ 16.2	42.0 $\pm$ 8.6	20.9 $\pm$ 8.1
New needles - $\Delta\delta^{13}\text{C}_{\text{WSC}}$	n.a.	53.8 $\pm$ 7.2	47.0 $\pm$ 9.6	29.5 $\pm$ 15.5
Old needles - $\Delta\delta^{13}\text{C}_{\text{WSC}}$	n.a.	n.a.	42.5 $\pm$ 10.0	23.2 $\pm$ 11.2

n.a., not available due to low increase in $\Delta\delta^{13}\text{C}_{\text{WSC}}$ values of old needles or twig phloem under control conditions

Table 5: Results of linear models for leaf-to-twigg transfer (LTT) of ^{18}O -label in water ($\text{LTT}_{\text{Water-O}}$) and of ^{13}C - and ^{18}O -label in assimilates ($\text{LTT}_{\text{WSC-C}}$, $\text{LTT}_{\text{WSC-O}}$) for pine. P-values are given for soil moisture treatment (i.e. control/irrigated), twig tissue (i.e. phloem/xylem), needle age (i.e. new and old needles), and their interactions.

Factors	$\text{LTT}_{\text{Water-O}}$	$\text{LTT}_{\text{WSC-O}}$	$\text{LTT}_{\text{WSC-C}}$
Soil moisture	0.56	0.66	n.a.
Twig tissue	<0.001***	0.18	0.16
Needle age	0.99	0.71	0.66
Soil moisture * Twig tissue	0.74	0.32	n.a.
Soil moisture * Needle age	0.87	0.34	n.a.
Twig tissue * Needle age	0.97	0.97	0.67

n.a., not available due to low increase in $\Delta\delta^{13}\text{C}_{\text{WSC}}$ values of old needles or twig phloem under control conditions

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$