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Summary

- The long-standing hypothesis that the isotopic composition of plant stem water reflects that of source water is being challenged by studies reporting bulk water from woody stems with an isotopic composition that cannot be attributed to any potential water source. The mechanism behind such source–stem water isotopic offsets is still poorly understood.
- Using a novel technique to extract selectively sap water from xylem conduits, we show that, in cut stems and potted plants, the isotopic composition of sap water reflects that of irrigation water, demonstrating unambiguously that no isotopic fractionation occurs during root water uptake or sap water extraction. By contrast, water in nonconductive xylem tissues is always depleted in deuterium compared with sap water, irrespective of wood anatomy.
- Previous studies have shown that isotopic heterogeneity also exists in soils at the pore scale in which water adsorbed onto soil particles is more depleted in deuterium than unbound water. Data collected at a riparian forest indicated that sap water matches best unbound soil water from depth below −70 cm, while bulk stem and soil water differ markedly.
- We conclude that source–stem isotopic offsets can be explained by micrometre-scale heterogeneity in the isotope ratios of water within woody stems and soil micro-pores.

Introduction

In the xylem of woody plants, sap water flows from roots to leaves through the apoplastic network of vessels or tracheids in stems and branches (Pickard, 1981). Based on early evidence showing that the isotopic composition of source water in hydroponically plants grown is not modified by root water uptake (Washburn & Smith, 1934; Zimmermann *et al.*, 1967), it was concluded that the isotopic composition of bulk stem water should reflect that of source water, at least in plants with suberised stems that prevent bulk stem water evaporative loss and isotopic enrichment (Dawson & Ehleringer, 1993). However, bulk stem water is not only 'sap' water. Living cells from the parenchyma, phloem and growing xylem cells ('symplastic' water), as well as the apoplastic intercellular spaces between xylem cells ('capillary' water) also contain water, and potentially provide some to the xylem conduits (Tyree & Yang, 1990; Jupa *et al.*, 2016). A relatively large amount of water is also present within cell walls ('fibre' water), bound through hydrogen bonds to cellulose, hemicellulose and, to a lesser extent, lignin (Berry & Roderick, 2005). Only the symplastic water pool is held inside a biological membrane.

Current techniques to collect water from woody stems do not allow the separation of these four different water pools. Most commonly, water is extracted from excised stems placed in a vacuum line and heated to release water vapour that is distilled and cryogenically trapped (cryogenic vacuum distillation, CVD). With the conditions of pressure (< 10 Pa) and temperature (60–80°C) typically used, CVD collects from all four stem water pools. There is, however, growing evidence that stem water collected this way (i.e. bulk stem water) is not a good indicator of plant source water as its isotopic composition is often depleted in ^{2}H compared with any potential water source (Zhao *et al.*, 2016; Barbeta *et al.*, 2019, 2020; Poca *et al.*, 2019). This source–stem isotopic offset is illustrated in Fig. 1(a,b) where bulk stem water from a riparian *Fagus sylvatica* L. forest displays δ^2H values that are more negative than δ^2H values of soil water from top and deep horizons, or ground and stream water. Similar offsets from the same riparian forest have been reported previously for an entire growing season and also shown to occur in another species (*Quercus robur* L.) and trees of different stature (Barbeta *et al.*, 2019). More recently, we showed that such δ^2H offsets between bulk stem and soil water can also be found in potted *F. sylvatica* saplings grown in semicontrolled glasshouse environments

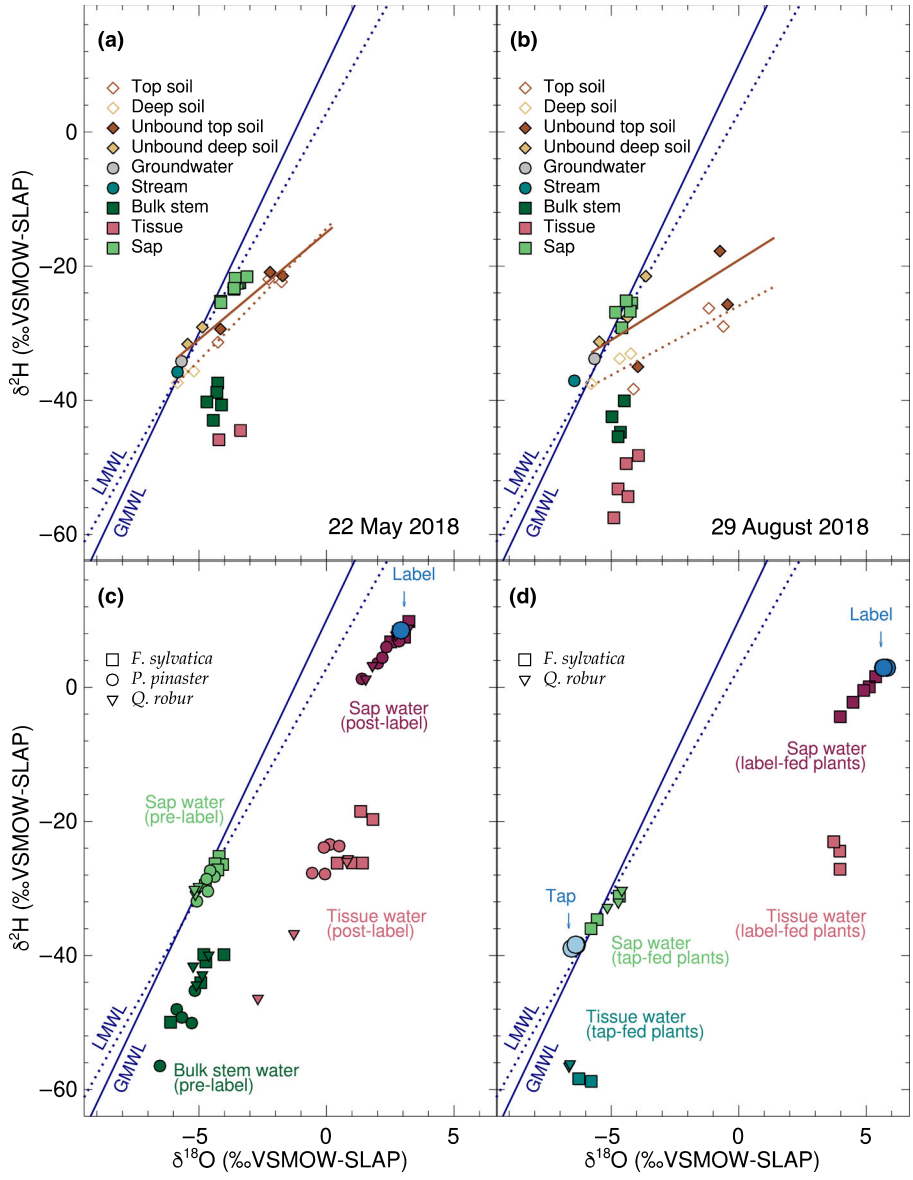

Fig. 1 Dual isotope representation of different water pools. Panels (a, b) show the isotopic composition of bulk stem water, sap water and residual tissue water from branches of adult *Fagus sylvatica* trees in the Ciron riparian forest and their potential water sources (top and deep soil, groundwater and stream water) at two dates during the 2018 growing season. Open symbols for top and deep soil correspond to bulk soil water samples, whereas closed symbols for soil water correspond to the 'unbound' soil water (see text for details on how unbound soil water was estimated). Panel (c) shows the isotopic separation between bulk stem water and sap water in cut branches sampled from the same field site and the good agreement of sap water with meteoric water (in prelabelled branches from the field) and with label water (in the same branches after flushing them with label water). Panel (d) shows the good agreement of irrigation water with sap water extracted from the main stem of potted saplings irrigated for several days with either tap or label water. GMWL, global meteoric water line; LMWL, local meteoric water line. Brown dotted and solid lines in panels (a) and (b) indicate soil evaporation lines from bulk or unbound soil water, respectively.

(Barbeta *et al.*, 2020). Many other studies have reported isotopic offsets of similar magnitude between soil and stem water, using different water extraction and isotopic determination techniques (Lin & da Sternberg, 1993; Ellsworth & Williams, 2007; Geris *et al.*, 2015; Vargas *et al.*, 2017; Wang *et al.*, 2017; Barbeta *et al.*, 2019; Brum *et al.*, 2019; Poca *et al.*, 2019).

Several hypotheses have been advanced, and often rejected by follow-up studies, to explain why bulk stem water would be isotopically depleted in deuterium compared with soil water (or any other potential water source). Because soil–stem isotopic offsets were initially considered specific to halophytic and xerophytic plants, a first hypothesis proposed that the suberised root endodermis and developed Casparian strip of these plants caused an isotopic fractionation during water uptake because water was forced to flow through the symplastic route, and caused a more pronounced isotopic fractionation than if water moved through roots via the apoplast (Lin & da Sternberg, 1993; Ellsworth & Williams, 2007; Poca *et al.*, 2019). This first hypothesis was challenged by several studies reporting soil–root isotopic offsets in plant species in which root water uptake through the apoplastic

route should not be impeded (Zhao *et al.*, 2016; Vargas *et al.*, 2017; Barbeta *et al.*, 2019), and rejected recently by indirect evidence from a semicontrolled experiment on *F. sylvatica* saplings, indicating no fractionation by root water uptake despite strong soil–stem water isotope offsets (Barbeta *et al.*, 2020).

An alternative hypothesis was proposed based on a single study on potted *Persea americana* saplings. It was suggested that within-pore soil water evaporation, followed by vapour transport and condensation on the root tips, may have been responsible for the observed isotopic offsets (Vargas *et al.*, 2017). However, as long as the condensed water at the root tip maintains thermodynamic and isotopic equilibrium with water vapour in soil pores (i.e. in most if not all situations), such a chain of reactions cannot create any soil–stem isotopic offsets of several per mil amplitude displacement (Barbeta *et al.*, 2020). Furthermore, the proposed chain of reactions and associated isotopic fractionations cannot explain why large soil–stem isotopic offsets are found even in well watered situations in which liquid–vapour isotopic equilibration are most expected (Barbeta *et al.*, 2020).

It was proposed recently that bulk stem water has an isotopic composition that differs significantly from that of sap (and therefore source) water. Support for this hypothesis was obtained by Zhao *et al.* (2016), who extracted sap water using a syringe inserted in the sapwood of riparian *Populus* trees. They found that water collected this way was enriched in ^{2}H compared with bulk stem water and coincided well with the isotopic composition of groundwater, the only plausible water source for these riparian desert trees. Isotopic heterogeneity in stem water pools (i.e. between long-transport conduits and other tissues) was also proposed recently as the most plausible hypothesis to explain isotopic offsets between soil and stem water in potted *F. sylvatica* saplings (Barbeta *et al.*, 2020). More recently, Chen *et al.* (2020) showed in nine plant species from diverse habitats that transpired water vapour (a proxy of sap water at isotopic steady state) had the same isotopic composition as source water, demonstrating the absence of fractionation during root water uptake already evidenced (Zhao *et al.*, 2016; Barbeta *et al.*, 2020), even in species from xeric and saline habitats. Chen *et al.* (2020) also confirmed that bulk stem water extracted using CVD was systematically more depleted in ^{2}H than sap water, and proposed that this depletion was caused by H exchange between organic compounds (mostly cellulose) and water during water extraction, rather than by isotopic heterogeneity in bulk stem water. They based their argument on the fact that stem–sap isotopic offsets increased with stem relative water content, and therefore extraction times.

Irrespective of whether such offsets are caused by extraction artefacts or by naturally occurring isotopic heterogeneities between stem water pools, these studies clearly showed that determination of the isotopic composition of sap water, not bulk stem water, is needed for identifying plant source water. Unfortunately, the isotopic steady-state gas exchange technique proposed by Chen *et al.* (2020) is difficult to deploy in the field, and the sap water collection technique used by Zhao *et al.* (2016) is only possible with tree species that experience positive root pressure and with a risk of contamination with water from other stem tissue regions. Here, we used a flow-rotor centrifuge originally designed to study the hydraulic embolism resistance of woody stems (Cochard, 2002) and later improved with a custom-made water collector (Pivovaroff *et al.*, 2016; Peng *et al.*, 2019). Using staining methods, it was recently shown that this particular centrifuge technique, from this point forwards called the 'cavitron' technique, should mainly extract sap water from the lumina of xylem conduits (Peng *et al.*, 2019). We applied this sap water extraction technique on cut branches of adult trees over an entire growing season. We also applied the technique to branches that had been refilled with water of a known isotopic composition and also to stems of irrigated potted saplings. We then analysed and compared the isotopic composition of (1) bulk stem water, that is all the water in woody stems collected using CVD; (2) sap water, that is the water only present in xylem conduits (either tracheids or vessels) as extracted with the 'cavitron' technique; and (3) 'residual tissue' water (or more simply 'tissue' water), that is the water remaining in woody stems after the extraction of sap water, obtained using cryogenic extraction.

Materials and Methods

Field site

The study site was a mixed riparian forest on the karstic canyon formed by the Ciron, a tributary of the Garonne river in southwest (SW) France (44°23′N, 0°18′W; 60 m above sea level (asl)). Soil texture ranged from coarse sand at the surface to loamy coarse sand in the deeper horizons and a distinguishable carbonate-rich C horizon (Supporting Information Table S1). This riparian forest is dominated by deciduous species including *F. sylvatica* and *Q. robur*. The site has a temperate oceanic climate (Cfb in the Köppen–Geiger classification; Peel *et al.*, 2007). Over the period 1897–2015, the mean annual temperature was 12.9°C and the mean annual precipitation was 813 mm yr^{-1}, distributed rather evenly over the seasons.

Stem, soil and water sampling

For the present study, we selected one of the plots sampled in a previous study (Barbeta *et al.*, 2019), in which a more detailed description of the plots is available. In 2018, we conducted sampling campaigns each month over the entire growing season. Five dominant *F. sylvatica* trees were chosen for collecting different stem water pools. From long (> 1 m length), relatively straight branches, a subsection, a few cm in length, was cut and used for bulk stem water. After removing the bark and phloem in the field, the subsection was immediately placed in an airtight Exetainer® sealed with Parafilm® and kept in a cool box until storage in the laboratory at 4°C. Leaves belonging to that subbranch were also collected. The central stem of each branch was re-cut to a segment of *c.* 60 cm in length and, without peeling off the bark, the open cuts were covered with Parafilm®. These longer stem segments were sealed in plastic bags and also stored at 4°C once in the laboratory to minimise stem evaporation. Sap water was always extracted from those longer stems within 24 h of their collection in the field. On a few occasions, the same sampling

procedure was used on *Q. robur* and *P. pinaster* branches to test the novel extraction method (see below). Additionally, for each species, a subset of five of these stem samples were flushed with isotopically labelled water ($\delta^{18}O = 2.9 \pm 0.2‰$; $\delta^{2}H = 8.5 \pm 0.6‰$). The flushing was conducted by attaching sterile silicone tubes (Novosil; Fisherbrand, Fisher Scientific, Göteborg, Sweden) to one extreme of each stem with airtight and watertight connections. The flushing time lasted for at least 2 h at a pressure of 1.8 bar for vessel-bearing species (*F. sylvatica* and *Q. robur*). For tracheid-bearing species (*P. pinaster*) samples were flushed over 18 h at 0.1 bar to prevent the torus sealing against the pit aperture. In addition to plant material, three soil cores amongst the sampled trees were extracted with a soil auger. Each soil core was split into topsoil (0–10 cm) and deep soil (from 70–80 to 110–120 cm depending on the depth of the bedrock). Soil samples were placed in 20 ml vials with positive insert screw-top caps, sealed with Parafilm® and kept in a cool box until they were stored in the laboratory at 4°C.

Samples of stream water, groundwater (from a well at *c.* 50 m from the river) and fog and rain water (from collectors installed in a small open area *c.* 100 m away from the sampling plot) were also collected for each campaign. Details on the rain collector can be found in a previous paper (Barbeta *et al.*, 2019).

Experiment on potted plants

To further test the proposed methodology to extract sap water from woody stems, we also grew potted saplings of *F. sylvatica* and *Q. robur*. Over 18 d, 7–10 l pots containing peat soils and saplings (height 1–1.5 m) were placed in a climate-controlled growth chamber with a 16 h : 8 h, day : night cycle (photosynthetically active radiation: 700 μmol m^{-2} s^{-1}; day temperature; 24°C; night temperature; 22°C; relative humidity: 40–50%). Each pot was placed on an automated balance to monitor water losses and irrigated daily with tap water ($\delta^{18}O = -38.19 \pm 0.32‰$; $\delta^{2}H = -6.43 \pm 0.10‰$) until field capacity. We covered the soil with aluminium foil to prevent soil evaporation and isotopic heterogeneity with depth. During the last 3 d of the experiment, we switched irrigation water from tap to labelled water ($\delta^{18}O = 2.91 \pm 0.01‰$; $\delta^{2}H = 5.72 \pm 0.08‰$) for three of the seven saplings (label-fed plants), and increased the irrigation rate (3 l d^{-1}) to ensure near-complete replacement of soil water with labelled water. The other four pots were irrigated at the same rate but with tap water (tap-fed plants). Transpiration rates ranged from 0.18 to 0.32 l d^{-1} and were similar in both tap-fed and label-fed plants. On the last day of the irrigation period, at midday, trees were harvested and the main stem cut and prepared for immediate centrifugation and cryogenic extraction, following the same procedure as that used for branches from the field (see above). Soil gravimetric water content was also measured and averaged *c.* 0.7 g g^{-1} with no difference between treatments or species.

Water extraction methods

The extraction of water from soils, stems and leaves was performed using CVD using a design and methodology adapted from Orlowski *et al.* (2013), as described in Jones *et al.* (2017). Up to 24 samples kept in sampling glass vials were de-capped and immediately inserted into larger extraction glass vials connected to a vacuum extraction line, and immersed in liquid nitrogen (LN_2) baths to prevent evaporation from the samples. Glass wool was placed in the extraction vials to prevent solid particles from entering the vacuum line. The latter was then evacuated down to a (static) pressure of < 10 Pa, and the liquid nitrogen (LN_2) baths were moved to another part of the vacuum line with glass U-tubes. Extraction glass vials were immediately re-immersed in water baths at ambient temperature, then gradually heated to 80°C (within 1 h) to start the distillation process. Pressure in the extraction line was continuously monitored with sub-atmospheric pressure sensors (APG100 Active Pirani Vacuum Gauges; Edwards, Burgess Hill, UK) to check that the extraction vials and vacuum line remained leak-tight throughout the entire extraction and that the water extraction had ended, typically within 2.5 h. Stem residual tissue water was extracted using the same methodology from subsections of the longer branches used to extract sap water (see below) and after removing the phloem and the bark as done in the field on the stem subsections used for bulk stem water isotopic determination. Gravimetric water content and extraction yields were assessed for each soil and plant sample by weighing the sample before and after water extraction, and the extracted water. We also checked that the water extraction had been completed by oven drying all samples at 105°C for 24 h and re-weighing them.

Sap water was extracted using a cavitron flow-rotor originally designed to study hydraulic embolism resistance of woody stems (Cochard, 2002). The rotor was fitted on a high-speed centrifuge and equipped with custom-made sap water collectors made of a plastic reservoir and a watertight resin lid, enabling the insertion of the woody stem in the reservoir while preventing the extracted water from escaping from the bottom of the reservoir once spinning had stopped. Branches with a length of 40 cm were spun first at 2190 ***g*** force for 120 s, leading to a minimum negative pressure in the middle of the branch of *c.* −2 MPa. Rotation was then stopped, and the liquid extracted from the branch was removed from both sap water collectors and stored in glass vials at 4°C until isotopic analysis. The collectors and the sample were then placed back in the rotor and spun at 6233 ***g*** force for another 120 s, corresponding to a minimum pressure of *c.* −6 MPa. Rotation was stopped again, and the liquid extracted from the branch was collected similarly. A section *c.* 10 cm long was then cut from the centre of the woody stem to extract residual tissue water remaining in the sample using the cryogenic vacuum extraction line described above.

As with any other sampling technique to measure the isotopic composition of water, partial evaporation of the water between field collection and water analysis must be avoided, as it can create strong biases on the isotopic results. Best care was taken to prevent stem evaporation during collection and transportation of the samples (see 'Stem, soil and water sampling' in the Materials and Methods section) but evaporation during sap water extraction could not be avoided. However, with a similar experimental setup, it has been shown that evaporation

of the collected water during spinning at 4427 ***g*** force operated at a rate of *c.* 0.5 µl min^{-1} (Peng *et al.*, 2019). After 2 min of spinning, such an evaporation rate would correspond to *c.* 0.1% of the water volume collected (typically 1 ml), which should affect only marginally the isotopic composition of the collected water. Although stem evaporation during collection, transportation and storage was not quantified, our results from both field and glasshouse experiments (see the Results section) clearly indicated that our protocol was sufficient to prevent any major stem evaporative artefacts.

Stable isotope analyses

The isotopic composition (δ^2H and $\delta^{18}O$) of the different waters was measured with an off-axis integrated cavity optical spectrometer (TIWA-45EP; Los Gatos Research, San Jose, CA, USA) coupled to an autosampler (PAL LC-xt; CTC Analytics AG, Zwingen, Switzerland). Details on the precision of the instrument and calibration can be found in previous studies (Barbeta *et al.*, 2019, 2020). All isotopic data reported here are expressed on the VSMOW-SLAP scale.

Because the presence of organic compounds (ethanol, methanol and/or other biogenic volatile compounds) in water samples can lead to large isotopic discrepancies in laser-based analyses (Wassenaar *et al.*, 2008; Martín-Gómez *et al.*, 2015), we developed a postcorrection algorithm for the presence of organic compounds based on the narrowband (for methanol) and broadband (for ethanol) metrics of the absorption spectra (Schultz *et al.*, 2011; Leen *et al.*, 2012). Postcorrections relating how these metrics affected the isotopic composition of deionised water samples contaminated with known amounts of ethanol and methanol were developed specifically for our instrument. Only plant water samples presented signs of contamination by organic compounds. For sap water, 19% of the samples were flagged as contaminated, resulting in a mean correction of −0.71‰ (δ^2H) and −0.35‰ ($\delta^{18}O$) for samples containing organic compounds. Amongst cryogenically extracted stem water samples, 78% were flagged as contaminated with mean corrections of −1.5‰ (δ^2H) and −0.85‰ ($\delta^{18}O$). Leaves presented the highest amounts of organic compounds, as 90% of the samples were flagged and suffered mean corrections of −4.28‰ (δ^2H) and −2.61‰ ($\delta^{18}O$). For a subset of our data, isotopic measurements of plant water and its sources obtained with our instrument (OA-ICOS) were also compared with those obtained with analysers using isotope ratio mass spectrometry and infrared laser cavity ring-down spectroscopy (Methods S1). This comparison confirmed that the observed isotopic patterns were not caused by the isotopic determination method (Fig. S1).

Finally, we aimed to quantify the potential effect of the CVD artefacts revealed by Chen *et al.* (2020), who proposed a correction method for such artefacts based on a linear relationship between stem water content and bulk stem water δ^2H offset to sap water. We applied this correction on δ^2H data of CVD-extracted water samples and compared the results with δ^2H data of sap water.

Estimating the isotopic composition of unbound soil water

Pore-scale isotopic heterogeneity in soil water is being increasingly recognised (Oerter *et al.*, 2019; Bowers *et al.*, 2020; Orlowski & Breuer, 2020). A recent study by Chen *et al.* (2016) developed an empirical formulation to estimate isotopic offsets between bulk and unbound soil water. Because the soil water pools accessed by roots are preferentially those not bound to soil particles, we used this empirical formulation to improve our estimates of the isotopic composition of plant-accessible soil water (called mobile or unbound water from this point forwards). By doing so, we assumed that unbound soil water *sensu* Chen *et al.* (2016) corresponded closely to the soil water held above the permanent wilting point at −1.5 MPa. Because the organic fraction of our soil samples was negligible to significantly influence the mean density of soil particles, the empirical formulation simplified to:

$$\delta^{18}O_{unbound} = \delta^{18}O_{bulk} + \frac{1 - f_{sand}}{2.65\, W_{bulk}} \times 0.906‰ \quad \text{Eqn 1a}$$

$$\delta^{2}H_{unbound} = \delta^{2}H_{bulk} + \frac{1 - f_{sand}}{2.65\, W_{bulk}} \times 17.75‰ \quad \text{Eqn 1b}$$

where f_{sand} and W_{bulk} represent the sand fraction and gravimetric water content of the soil sample, respectively. The sand fraction was subtracted because only finer minerals are considered to create the isotopic offset due to their greater ability to attract large amounts of adsorbed water (Chen *et al.*, 2016). An average sand fraction of 0.92 was used based on texture analysis of the different soil horizons (Table S1).

Data analyses

Statistical differences between the isotopic composition of bulk stem water, sap water and residual tissue water from field samples, as well as between those of labelled residual tissue and vessel water were assessed using Generalised Linear Mixed Models (GLMMs) from the package LMER (Bates *et al.*, 2015) in R (R Core Team, 2019). The same models were also used to assess statistical differences between bulk and residual tissue gravimetric water contents, or between the isotopic composition of waters extracted at −2 and −6 MPa. These GLMMs allow us to use the date of sampling and/or tree individual as random factors, when necessary.

Results

Absence of isotopic fractionation during centrifugation of cut branches from the field

Sap water extracted from cut branches of *F. sylvatica*, *Q. robur* and *P. pinaster* collected from the field (i.e. 'prelabel') using the 'cavitron' technique, fell on the local meteoric water line (LMWL; the regression line between $\delta^{18}O$ and δ^2H of local rainfall water) (Fig. 1a–c). Bulk stem water (as obtained using CVD)

from these 'prelabel' branches was more depleted in ^{2}H than sap water, by 16‰ on average ($P < 0.0001$) while differences in the $\delta^{18}O$ values were less significant ($P = 0.075$). To verify the absence of fractionation during centrifugation we flushed the then-embolised stems with labelled water of known isotopic composition and re-extracted sap water from the same stems using the 'cavitron' technique (see the Materials and Methods section). The $\delta^{18}O$ and $\delta^{2}H$ of sap water re-extracted after flushing (i.e. 'postlabel') was not significantly different from those of the labelled water (Fig. 1c) and fell on a mixing line between prelabel sap water values and the labelled water, indicative of an incomplete replacement of the former. These results were observed on three tree species of different wood anatomies and no interspecific difference ($P > 0.05$) in the isotopic composition of sap or bulk stem water pools was found (Fig. 1c).

Water from fresh samples was extracted at two different rotation speeds to distinguish between water released from open conduits and intercellular spaces (capillary water extracted at less than −2 MPa), and water from filled vessels and tracheids (cavitation water extracted between −2 and −6 MPa when xylem conduits embolised). The threshold of −2 MPa was chosen according to dehydration isotherms previously described for *F. sylvatica* branches (Jupa *et al.*, 2016). In some samples, extractions at −2 MPa did not yield sufficient water (< 0.3 ml) for isotopic analysis. For all the other samples, there was no significant difference in $\delta^{18}O$ or $\delta^{2}H$ between water extracted at −2 MPa and −6 MPa ($P = 0.20$ for $\delta^{18}O$ and $P = 0.54$ for $\delta^{2}H$), indicating that the two water pools are well connected.

Absence of sap-source water isotopic offsets using the 'cavitron' technique

To verify that the water extracted with the 'cavitron' technique was a good indicator of plant source water (and therefore sap water) we also performed experiments on potted *F. sylvatica* and *Q. robur* saplings irrigated daily with tap water for 18 d, except for a subset of three plants that was abundantly irrigated with labelled water for the last 3 d before sampling (see the Materials and Methods section). Results from this experiment showed that, for both species, the isotopic composition of sap water extracted with the 'cavitron' technique (from the main stems of the saplings) matched very closely that of irrigation water, with some small but significant ($P < 0.05$) variations (Fig. 1d). In tap-fed plants, the small variations in sap water were aligned along what resembles an evaporation line originating from tap water. In label-fed plants, the narrow distribution of sap water again followed a mixing line between new labelled water and old tap water (Fig. 1d), consistent with observations on postlabel cut branches from the field (Fig. 1c).

Bulk and residual tissue water contents

Bulk stem water content in *F. sylvatica* branches from the field decreased mostly in spring and early summer (Fig. 2a). Residual tissue water content followed the same pattern, leading to a nearly constant tissue-to-bulk water content ratio over the growing season (78 ± 5.6% on average, ranging from 70% to 92%; see Fig. 2b). Interestingly summertime tissue water content remained close to the range of values expected for the fibre saturation point (Berry & Roderick, 2005) of *F. sylvatica* wood (indicated by the hatched area in Fig. 2a).

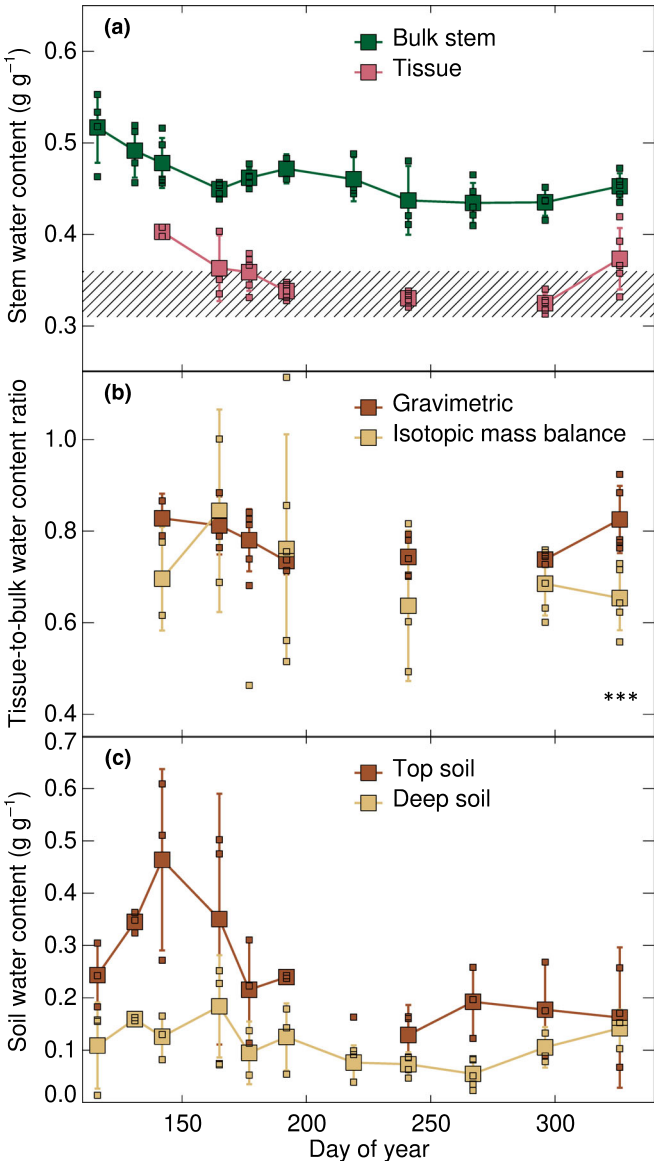

Fig. 2 Field gravimetric water contents. Temporal variations of gravimetric water contents and their ratios during the 2018 growing season at the study site, a riparian forest in the southwest (SW) of France. (a) Bulk stem water and stem tissue water from *Fagus sylvatica* branches. Hatched area represents the expected range of wood fibre saturation point for *F. sylvatica* wood (Barkas, 1936; Berry & Roderick, 2005). (b) Tissue-to-bulk water content ratios of the same branches, estimated either by gravimetric measurements or by isotopic data and isotopic mass balance. Tissue-to-bulk water content ratios derived from isotopic mass balance were estimated for each individual branch as $(\delta_{bulk} - \delta_{sap})/(\delta_{tissue} - \delta_{sap})$ using both $\delta^{18}O$ and $\delta^{2}H$ data and a Bayesian optimisation approach (with a prior value of the ratio of 0.5 ± 0.5) to account for the measurement uncertainty on the isotopic data. Significant differences between the two ratios are indicated ***, $P < 0.01$. (c) Deep and top soil water. In (a–c) larger symbols represent the mean ± standard deviation (n =3–5).

Isotopic composition of sap and residual tissue water in the field

Over the growing season, there were notable differences in the isotopic composition of bulk stem water, sap water and residual tissue water of *F. sylvatica* branches. In the dual isotope space, sap water was always close to the LMWL, whereas the δ^2H of residual tissue water was more negative than that of rain, stream water, ground water or bulk soil water (Figs 1, 3). Bulk stem water exhibited intermediate δ^2H values between sap and residual tissue water, suggesting that it represented a mixture of both pools. Sap water was always enriched in ^2H over bulk stem water (+16.6‰, $P < 0.0001$), whereas residual tissue water was always depleted in ^2H over bulk stem water, but to a lesser extent (−8.8‰, $P < 0.0001$). This latter result is expected by isotopic mass balance, because residual tissue water constitutes a larger proportion of total bulk water (Fig. 2b). Unlike δ^2H, the $\delta^{18}O$ of sap and residual tissue water was significantly more enriched than that of bulk stem water (+1.1‰, $P < 0.0001$ and +0.4‰, $P < 0.001$, respectively). However, when uncertainties were accounted for, the residual tissue-to-bulk water content ratio deduced from the *combined* δ^2H and $\delta^{18}O$ isotopic mass balances was always consistent with the residual tissue-to-bulk water content ratio measured gravimetrically, except for the last date in November when the isotopic mass balance resulted in significantly ($P < 0.01$) smaller ratios compared with gravimetric data (Fig. 2b).

We found interspecific differences in the isotopic offsets ($\Delta^{18}O$ and Δ^2H) between bulk stem water and sap water (Fig. S2). For Δ^2H, significant differences were found between all three species: *Q. robur* had the most negative Δ^2H (−22.3 ± 5.4‰), followed by *F. sylvatica* (−17.6 ± 4.6‰) and *P. pinaster* (−12.7 ± 1.7‰). For $\Delta^{18}O$, a marginally significant difference was found only between *Q. robur* and *P. pinaster* but the interspecific order was the same: *Q. robur* had the most negative $\Delta^{18}O$ (−22121.5 ± 1.05‰), followed by *F. sylvatica* (−1.1 ± 0.8‰) and *P. pinaster* (−0.5 ± 0.9‰).

Plant water sources

The fact that residual tissue water content was always close to the fibre saturation point (Fig. 2a) is an indication that fibre-bound water must represent a large part of this stem water pool. It is therefore not surprising to observe strong hydrogen isotope effects on this water pool compared with other stem water pools, knowing that fibre-bound water is attached to cell walls through hydrogen bonds (Berry & Roderick, 2005). Similar surface isotopic effects have also been found for water films adsorbed onto mineral or organic surfaces (Chen *et al.*, 2016; Lin & Horita, 2016; Lin *et al.*, 2018). These studies implied that the isotopic composition of soil water accessible to plants should differ from that of bulk soil water. Using the empirical formulation proposed by Chen and colleagues to quantify the isotopic offset between bulk soil water and 'unbound' soil water (see the Materials and Methods section), we found that unbound water in the deep soil layer had an isotopic composition that resembled that of rain, as it plotted on the LMWL (Figs 1a,b, 4). A similar result had been

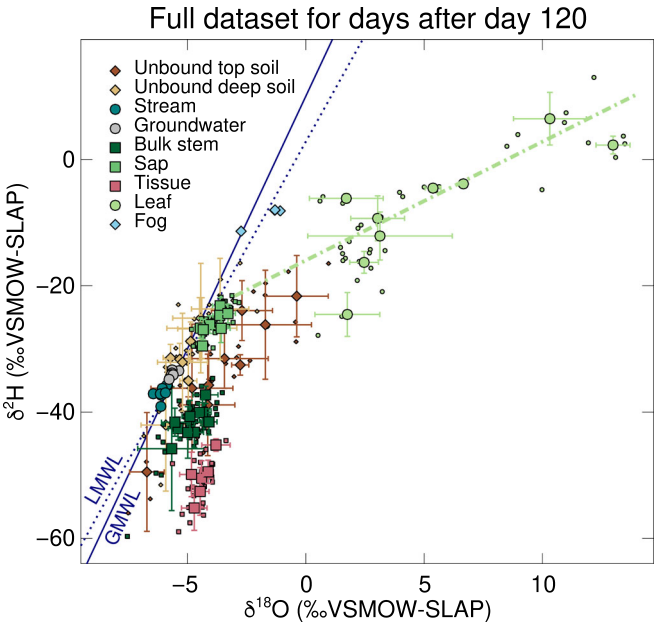

Fig. 3 Dual isotope representation of field water pools. Isotopic data for the 2018 growing season at the study site, a riparian *Fagus sylvatica* forest in the southwest of France. Larger symbols represent daily means ± standard deviations for each sampling campaign, while smaller symbols are individual points ($n = 3$–5). GMWL, global meteoric water line; LMWL, local meteoric water line. The dot–dashed green line represents the 'growing-season' average leaf water evaporation line (see 'Plant water sources' in the Results section).

observed in a soil pasture in Germany (Chen *et al.*, 2016). Unbound topsoil water was more variable, following similar seasonal variations as bulk topsoil water. More interestingly, the soil evaporation line exhibited by bulk soil water was still present for unbound soil water and nearly overlapped with sap water, while bulk stem water was taken even further away from it (Fig. 1a,b). Indeed, for each field campaign, bulk stem water $\delta^{18}O$ and δ^2H values rarely overlapped with those of 'unbound' soil water (Fig. 3). By contrast, for both δ^2H and $\delta^{18}O$, sap water followed more closely variations in unbound water from deep soil layers.

Only on a few rare occasions the isotopic composition of sap water became more enriched in both δ^2H and $\delta^{18}O$ than top and deep soil water: at the beginning of July (day 192), after a few days of heavy rain (> 35 mm d^{-1}) that depleted top soil water below that of deep soil water, and at the end of the growing season, just before and after leaf fall (days 296 and 326). In both situations, sap water δ^2H and $\delta^{18}O$ remained close to the values they had at the previous sampling campaign (Fig. 3). In the former case, this is consistent with the idea that the heavy rains in July displaced the unbound top soil water (enriched in both O and H isotopes; Fig. 2b) to deeper intermediate horizons (located between the top and bottom sampling depths where it was still accessible to trees). This explanation is strongly supported by the enrichment of unbound water in the deepest soil layers in the subsequent sampling date. By contrast, the latter case is most likely to have been caused by a reduction of transpiration accompanying leaf senescence that resulted in a slower isotopic turnover

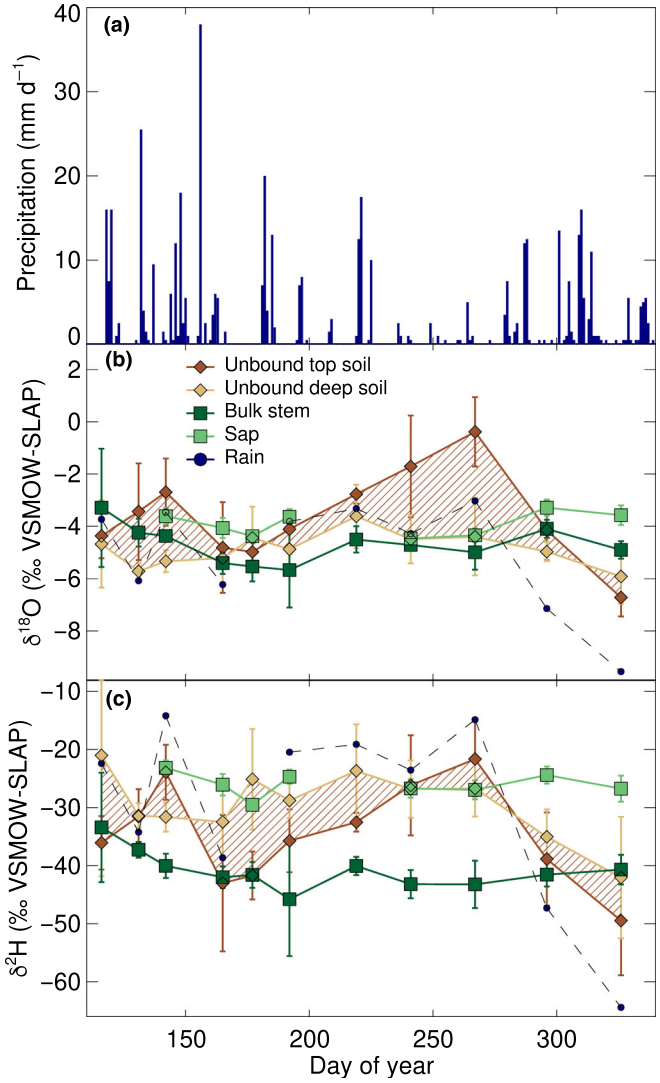

Fig. 4 Seasonal variations of daily precipitation and 'unbound' top and deep soil water, bulk stem and 'sap' water, and rain water during the 2018 growing season. (a) Precipitation. (b) Oxygen isotope signals. (c) Hydrogen isotope signals. In (b, c), the hatched area is here to better visualise the range of values in the soil, taking top and deep soil as end members.

of sap water at the end of the season. At other dates over the growing season, our results indicated that trees mostly accessed water from deep soil layers (Fig. 3).

The isotopic stability of sap water and bulk stem water over the growing season is quite remarkable (Fig. 3) and is an indication that the depth of root water uptake did not suffer strong shifts over the growing season. Therefore, topsoil water, whose isotopic composition varies strongly over time and space, is unlikely to be a significant source of water for these riparian *F. sylvatica* trees. This isotopic stability of sap and bulk stem water pools also implies that the origin of leaf water should be temporally conservative as well. In these conditions, a linear regression in the dual isotope space of all the isotopic data from leaf water can be interpreted as a 'growing season' leaf evaporation line, whose intersection with the LMWL should correspond approximately to the source of leaf water. When performing such a regression, sap water is found as the most probable source of leaf water, in contrast with bulk stem water or residual tissue water that is too depleted in deuterium (Fig. 4).

Discussion

Which stem water pool is extracted with the 'cavitron' technique?

The isotopic difference between sap water and bulk stem water (16.6‰ in δ^2H) is of similar magnitude to the soil–stem isotopic offsets previously reported in glasshouse and field studies (Vargas *et al.*, 2017; Barbeta *et al.*, 2019, 2020; Poca *et al.*, 2019). Our findings bring strong evidence that isotopic compartmentalisation within woody stems underpins these observed soil–stem isotopic offsets, and are larger than those expected solely from CVD-related artefacts (Fig. S3). But can we be sure that water collected with the cavitron apparatus is more representative of the water being taken up by roots and transpired by leaves as opposed to bulk stem water obtained by CVD? Three lines of evidence support this assertion.

Firstly, water extracted with the cavitron apparatus overlapped in the dual isotope space with unbound soil water from the deep horizons (Figs 3, 4), after accounting for isotopic fractionation processes occurring at the soil pore level (i.e. the isotopic offset with bulk soil water; Fig. S4). Unbound soil water, sometimes referred to as mobile soil water, is the water pool that is more likely to have been accessed by roots (Bowling *et al.*, 2017) and, therefore, its isotopic composition should be similar to that of sap water when it is available. Secondly, the growing-season average leaf water evaporative enrichment line had its origin in 'cavitron-based' sap water values, whereas both bulk stem water and residual tissue water were too depleted in ^{2}H to be identified as the source of water for the leaves (Fig. 4). Thirdly, water extracted with the cavitron apparatus from potted saplings coincided well with irrigation water for both tap-fed and label-fed saplings (Fig. 1d). Small deviations between irrigation and 'cavitron-based' sap water could be explained by a slight evaporative enrichment of soil water or cut stem water (for tap-fed plants) and additional mixing of old and new sap water (for label-fed plants). In any case, the deviation from irrigation water was much smaller than the offset between sap and bulk stem water, demonstrating that the 'cavitron' technique was suitable to characterise the isotopic composition of sap water separately from that of bulk stem water.

The fact that the water collected with the 'cavitron' technique has an isotopic composition that matches that of sap water does not necessarily mean that the collected water is *only* sap water. It is generally thought that, during centrifugation, a cut branch would first release capillary water and water from open or large conduits, then from elastic living tissues and finally from smaller vessels and tracheids (Tyree & Yang, 1990). Following this principle, as most of the smaller conduits from *F. sylvatica* branches are embolised at −6 MPa (Stojnić *et al.*, 2018), the water from living cells should have also been extracted alongside sap water from capillaries, vessels and tracheids. However, if this was the

case, we would expect to have co-extracted large amounts of organic compounds that would have affected the performance of the isotopic determination of sap water (Martín-Gómez *et al.*, 2015), but this was not the case (Fig. S1). In fact, recent studies using X-ray computed microtomography (microCT) imaging showed that water held in fibres and living cells surrounding the conduits is released only after severe cavitation events (e.g. Knipfer *et al.*, 2019). This pattern has been observed in all microCT studies of which we are aware, encompassing 13 woody species, on either intact plants (Cochard *et al.*, 2015; Knipfer *et al.*, 2015; Charrier *et al.*, 2016; Choat *et al.*, 2016; Li *et al.*, 2020) or cut samples (Dalla-salda *et al.*, 2014; Torres-Ruiz *et al.*, 2015; Knipfer *et al.*, 2019). This implies that the water extracted with the cavitron apparatus is mainly drawn from intercellular spaces and xylem conduits (our results on water extraction at two different rotation speeds indicate that they are hydraulically connected), leaving behind a large part of symplastic water from living cells (mainly parenchyma) and water within cell walls. The fact that the stem water content after centrifugation always remains close to the fibre saturation point (Fig. 2) is not in contradiction with the idea that symplastic water from living cells is still present. The fibre saturation point is usually measured on dead wood samples (i.e. without viable cells) but also under an atmosphere saturated in water vapour (usually > 99% relative humidity, see (Berry & Roderick, 2005)). In our study, stem water contents were measured in room (and therefore relatively dry) air. In this situation cell walls should not be saturated with water, while living cells should still contain some water.

What causes the isotopic depletion of bulk stem water compared with sap water?

If a significant part of the 'residual tissue' water left in the sample after cavitation extraction is made up of water held in living parenchyma cells, surface isotopic effects during adsorption of water onto cellulosic fibres or other hydrophilic organic substances (Chen *et al.*, 2016) may not be the only cause for the lower δ^2H of bulk stem water compared with sap water. In particular, the elastic fraction of symplastic water that continuously exchanges with xylem conduits *via* aquaporins (Pfautsch *et al.*, 2015; Secchi *et al.*, 2017) may also contribute to the overall isotopic depletion of residual tissue water compared with sap water, assuming that this water exchange through aquaporins is a mass dependent fractionating process favouring the exchange of the light isotopologue, as previously postulated (Zhao *et al.*, 2016; Poca *et al.*, 2019). However, there is only indirect evidence that aquaporin-mediated transport may be a fractionating process during plant water transport (Mamonov *et al.*, 2007). In fact, root water uptake does not necessarily entail isotopic fractionation (Barbeta *et al.*, 2020), whereas it involves water channelling through aquaporins in the symplastic pathway (Chaumont & Tyerman, 2017). By contrast, surface effects on the isotopic composition of adsorbed water have already been demonstrated (Richard *et al.*, 2007; Chen *et al.*, 2016, 2021; Lin *et al.*, 2018) and are currently a more plausible explanation for water isotopic heterogeneity in woody stems.

Chen *et al.* (2020) proposed that the systematic isotopic offset (Δ^2H) between bulk stem water extracted using CVD and the transpiration (sap) water that they observed was caused by H exchange between organic compounds (mostly cellulose) and water during water extraction, rather than by isotopic heterogeneity in bulk stem water. Using their relationship between Δ^2H offsets and stem relative water content, we quantified that Δ^2H offsets produced by H exchange during CVD could be, on average, −8.5‰ for bulk stem water and −9.9‰ for tissue water, values that are much smaller than the observed Δ^2H offsets reported in the present study (−15.7 ± 1.0‰ for bulk stem water and −24.2 ± 2.6‰ for tissue water; Fig. S3). As in Chen *et al.* (2020) we also observed a relationship between Δ^2H offsets and stem water relative content, but it was not significant when analysing bulk stem water and tissue water separately (Fig. S3). Furthermore, compared with Chen *et al.* (2020), the slope of the relationship was much steeper (0.745 vs 0.144), demonstrating that stem water content was not the only factor affecting Δ^2H offsets.

Similarly to Chen *et al.* (2020) we also observed interspecific differences in Δ^2H (Fig. S2) that seemed linked to interspecific wood anatomical features. We found larger isotopic offsets between bulk stem water and sap water with higher volume fractions of cell wall (and therefore higher wood density) and parenchyma cells. The smallest Δ^2H were found in *P. pinaster*, the species with the lowest wood density (0.41 g cm^{-3}) and parenchyma cell volume fraction (7.4%) compared with the angiosperm species (0.57 and 0.59 g cm^{-3} and 18% and 21% for *F. sylvatica* and *Q. robur*, respectively) (Zanne *et al.*, 2010; Morris *et al.*, 2016). Coniferous wood is also known to contain higher fractions of lignin, a more hydrophobic cell wall compound compared with cellulose and hemicellulose; this could explain the smaller isotopic offsets found in *P. pinaster*.

In short, although our results are qualitatively coherent with those in Chen *et al.* (2020), they are not quantitatively. Also, it is still unclear whether these bulk stem water Δ^2H offsets are caused by H exchange between wood material and water during CVD (as proposed by Chen *et al.*, 2020) or by aquaporin- or surface-mediated within-stem water isotope heterogeneity (as proposed by Zhao *et al.*, 2016 or Barbeta *et al.*, 2020). This is because it is very difficult to disentangle the two, as both are related to the amount of H bonds between water and wood material, and therefore to the stem water content. However, what is clear from all these studies is that the isotopic composition of CVD-extracted bulk stem water deviates significantly from sap water.

Implications of stem water isotopic heterogeneity for plant water source identification

The isotopic composition of bulk stem water has been extensively used to trace water fluxes in the soil–plant–atmosphere continuum and therefore has been instrumental for elaborating hydrological and ecological theories of plant water use. Our results demonstrated that the δ^2H of bulk stem water is different from the δ^2H of water in the transpiration stream. In this respect, the analysis of the isotopic composition of sap water may have led to significantly different conclusions in previous studies that

attributed plant water sources. For example, it is likely that isotope-based estimations of plant groundwater use (Barbeta & Peñuelas, 2017; Evaristo & McDonnell, 2017) are slightly overestimated when using bulk stem water instead of true sap water. The soil–stem water offset may cause commonly applied isotope mixing models to underestimate the contribution of soil water to plant water use, while overestimating the contribution of isotopically depleted ground water. Also, the isotopic differences between meteoric and runoff water and bulk stem water at the origin of the two water worlds hypothesis (Brooks *et al.*, 2010; McDonnell, 2014) may have been much smaller if sap water had been used instead of bulk stem water, because its isotopic composition is much closer to that of the meteoric water line (Figs 1, 4) and, therefore, to stream and ground water. Therefore the basis for the conceptual separation of belowground water pools into green water, accessed by plants, and blue water, contributing to groundwater and runoff now needs to be revisited. Finally, it should be highlighted that $\delta^{18}O$ was fairly similar among soil and stem water, regardless of the pool. Our results, therefore, confirmed the reliability of $\delta^{18}O$ as a tracer of the water movement along the soil (source)–plant continuum (Barbeta *et al.*, 2019).

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Author contributions

AB, JO and RB designed research; AB and ND performed field sampling; PM and RB performed potted plant experiments; RB, AB and PM performed sap water extractions; BF and ND performed bulk stem water extractions; ND, PM, JCD, AB, JO and LW performed stable isotope determination and analyses; AB and JO performed the data analysis and wrote the manuscript, with contributions from all authors.

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Data availability

Data gathered in this study are now available upon request to the authors and will be made publicly available through a future data paper (together with data from other studies belonging to the same project) and databases such as the one projected in the COST action CA19120 – WATer isotopeS in the critical zONe: from groundwater recharge to plant transpiration.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Interlaboratory comparison of water isotope analyses.

Fig. S2 Interspecific differences in the bulk-sap isotopic offsets.

Fig. S3 Quantification of potential artefacts caused by H exchange during CVD extraction.

Fig. S4 Dual isotope representation of field water pools (soil water not corrected for surface effects).

Methods S1 Interlaboratory comparison of water isotope analyses.

Table S1 Soil properties at the study site.

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