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Progressive enrichment in ¹⁸O and ²H in xylem water along sap flow paths in *Fagus sylvatica* trees

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Abstract

Isotopic signatures of xylem water in different tree compartments such as roots, boles and branches, differ due to physiological and physical processes occurring inside trees. Accordingly, we hypothesised that the extent of such differences among the isotopic signatures of tree compartments is coherent with the distance travelled by the water inside trees and to its residence time. To test this, we compared the O-H isotopic composition of xylem water collected using an in-situ water extraction method from roots, boles and branches of Fagus sylvatica trees growing on three geomorphological units of the Weierbach experimental catchment, Luxembourg. There was progressive ¹⁸O and ²H enrichment in xylem waters along the root-branch flow path for all the studied trees. Three explanations could be considered for this progressive enrichment: internal fractionation by xylem-phloem water exchange, chemical reactions of metabolic pathways and variable ages of water retained in the xylem, reflecting historical variation in isotopic composition of uptake water. Support for the hypothesis of isotopic fractionation linked to xylem-phloem water exchange and chemical reactions is that enrichment was generally consistent with the distance travelled by the water and to its residence time inside the trees. However, the relative enrichment of ²H and ¹⁸O was not consistent along the flow path, with Δ^2 H/ Δ^{18} O \sim 7.5 from the soil into the roots and bole and \sim 4.7–6.5 for pathways that included smaller branches. This contrast suggests different processes controlling aboveground isotopic enrichment. In particular, the slope of \sim 7.5 in the lower tree is also consistent with variation in tree water uptake varying along the local meteoric water line, with water in the roots being closer to the composition of rainfall close to the time of sampling and water in the bole being closer to the composition of rainfall from the previous summer. The timing of root and bole sampling in early spring, just before leaf-out, means sap flow was very slow and makes it plausible that varyingage water was present in the tree at that time. We also compared the O-H isotopic composition of those samples with the ones of the potential water sources to identify the origin of the water uptaken. The latter varied during the 3 years of sampling, with a preferential uptake from near-surface waters. Our results suggest multiple

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes. © 2023 The Authors. *Ecohydrology* published by John Wiley & Sons Ltd. biochemical, physiological and physical processes may play fundamental roles in the isotopic composition of xylem water within trees.

KEYWORDS

beech, tree water extraction, water isotopes, Weierbach experimental catchment, xylem

1 | INTRODUCTION

Water availability in forest ecosystems controls tree physiological processes such as transpiration, photosynthesis, and growth (Bréda et al., 2006). Brinkmann et al. (2019) showed that the water uptake depth of deciduous temperate trees varies according to soil water availability and that species differ in root system plasticity in response to water accessibility in the subsurface. When water availability is reduced during the growing season—as is common in temperate forests and intensified during droughts—it affects water transfer along the soil-tree-atmosphere continuum, restricting growth and transpiration and thus affecting tree adaptation strategies and survival. Despite the number of research projects focusing on this topic, a full understanding of where trees uptake water remains elusive.

Most ecohydrological studies to determine tree water sources rely on water stable isotopes, which have been used since the 1960s. The principle behind their hydrological application is that different water pools are often characterised by distinctive H and O isotopic compositions. These so-called 'isotopic signatures' allow tracing water through various hydrological processes such as groundwater recharge (Adomako et al., 2015; Joshi et al., 2018; Liu & Yamanaka, 2012; Muir & Coplen, 1981) and water dynamics in the vadose zone (Barbecot et al., 2018; Barnes & Allison, 1988; Dawson, 1993; Gascuel-Odoux & Mérot, 1986; Rodriguez et al., 2018) and in urban environments (Ehleringer et al., 2016; Houhou et al., 2010; Wilcox et al., 2004). They can also be used to reconstruct past environmental conditions (Dawson & Ehleringer, 1993; Yakir, 1992a), both in terrestrial and aquatic environments (Deniro & Epstein, 1981), relying on the isotopic composition of wood cellulose, since this derives from the source water at the time of biosynthesis.

Despite the extensive use of water stable isotopes to study treewater interactions, such as transpiration and tree water uptake (Brooks et al., 2010; Burgess et al., 2000; Ehleringer & Dawson, 1992; Fabiani et al., 2021; Farrington et al., 1996; Schlesinger & Jasechko, 2014; Wang & Yakir, 2000; Wilm et al., 1944), the effect that tree physiology and the related biochemical processes have on O-H isotopic composition of xylem water is still poorly understood because most studies have focused on the isotopic composition of bulk water in trees rather than specifically on the most mobile xylem sap in the young sapwood.

Water in trees consists of several pools, each of which is characterised by a distinct isotopic signature (Barbeta et al., 2022, and reference therein). This phenomenon, known as water compartmentation (Yakir, 1992b), can be linked to a multitude of factors including differences in the residence times of the water distributed among the tree tissues, biochemical interactions along metabolic pathways and physical processes involving water. Water participates in several chemical reactions in trees, modifying its isotopic composition via O and H isotopic exchange processes. Cormier et al. (2018), for instance, showed H isotopic exchange between water and organic molecules during plant carbohydrate and lipid biosynthesis, resulting in ²H enrichment for the water and depletion for the organic compounds. Photosynthetic and post-photosynthetic metabolism, indeed, play a role in the O-H isotopic modification of water and organic molecules. Organic matter produced during photosynthesis exhibits a lighter isotopic signature than the available water (Estep & Hoering, 1980) and, when transported through the tree, exchanges isotopes with water (Yakir, 1992a; Yakir & DeNiro, 1990). A typical example is cellulose biosynthesis, which involves isotopic exchange between water and organic precursors influencing $\delta^2 H$ mutually (Hill et al., 1995; Sternberg, 2009; Yakir, 1992a). More recently, Nehemy et al. (2022) reported that light water molecules are more prone to flow from xylem to phloem vessels, leading to an enrichment in heavy water isotopes in the xylem and a depletion in the phloem, supporting what was already assumed in previous studies (Ziegler, 1989). Most ecohydrological studies that used water isotopes have not considered that these multiple isotopic exchange processes inside trees-both biochemical and physical-make water isotopic signatures in the root-branch pathway non-conservative. The impact that these multiple processes may have on the isotopic signature of xylem water along the root-branch flow path needs to be investigated in more detail. A better understanding of the net effect of multiple isotopic fractionations is needed to improve the utility of isotopic tracers for evaluating the plant-water interactions in forest ecosystems.

This study aims to provide evidence that the isotopic signature of xylem water changes during longitudinal flow from roots to branches and to emphasise potential physical and biochemical processes that may act in this way and that should be considered when studying the isotopic composition of xylem waters for ecohydrological purposes. We hypothesised that isotopic signatures of xylem water extracted from different tree compartments such as roots, boles and branches differ due to continuous changes on the originally uptaken source. Moreover, the intensity of such changes is coherent with the distance travelled by the water and its residence time inside trees. Assuming that no isotopic fractionation occurs during the water uptake (Dawson & Ehleringer, 1991; Ehleringer & Dawson, 1992;

White et al., 1985), the hypothesis entails that the O-H isotopic composition of root sap is the closest to that of water sources and the longer the distance travelled by the water and/or the longer the residence time inside trees, the longer the sap is exposed to physical and metabolic processes and the greater is the change in its O-H isotopic composition. We base the investigation on liquid water extracted at low vacuum and ambient temperature from xylem, avoiding methodological biases associated with the typical cryogenic distillation technique (Allen & Kirchner, 2022). We focused our study on Fagus sylvatica trees during the beginning of the growing season when trees start photosynthesis. The latter is an important phase for the recovery of the hydraulic conductivity of trees after winter embolism, during which biochemical processes-such as the ones promoting the formation of a new xylem ring (e.g. cellulose biosynthesis)-may alter the isotopic composition of xylem water (Améglio et al., 2002; Cochard et al., 2001). The choice of F. sylvatica as a candidate for this study is linked to the fact that this species is one of the most widespread in Europe and one of the most sensitive to drought (Lindner et al., 2014). Furthermore, its importance resides in its dominance in regional ecosystems and in its economic utility (Houston Durrant et al., 2016). Therefore, understanding the water uptake and storage dynamics of F. sylvatica is crucial for preventing climate change from threatening its geographical and ecological distribution (Fang & Lechowicz, 2006; Martinez del Castillo et al., 2022).

2 | MATERIALS AND METHODS

2.1 | Study site

The study was conducted in the Weierbach experimental catchment (Hissler et al., 2021), which is a 0.45 km^2 forested headwater catchment of the Attert River Basin, in the Luxembourg Ardennes Massif at 512 m a.s.l. The Attert River Basin has been a focus area for

eco-hydrological research since 2010 (Antonelli et al., 2017; Fabiani et al., 2021 and 2022; Glaser et al., 2016; Hoek van Dijke et al., 2019; Schoppach et al., 2021). The most abundant tree species are European beech (*F. sylvatica* L.) and oak (*Quercus petraea*), together covering 75% of the catchment, while Norway spruce (*Picea abies*) and Douglas fir (*Pseudotsuga menziesii*) cover the remaining 25%.

As geomorphology and hydrological processes vary within the catchment (Martínez-Carreras et al., 2016), three sites were chosen to identify the potential variation of water sources available to tree uptake across the different morphological units: a plateau, a hillslope and a riparian area (Figure 1).

2.2 | Sampling campaigns

Three sampling campaigns were carried out in 3 years with varying methods and hydro-climatic conditions (Figure 2) over the beginning of the growing season. The campaigns included sap collection from different structural parts of the trees (roots, bole and branches) and different potential water sources (streamwater, soil solutions and shallow and deep groundwaters). From March to May 2017, we collected sap samples only from roots and branches of the plateau trees, soil solution at 20 and 60 cm depths, shallow groundwater (220 cm depth) and deep groundwater (720 cm depth). In March 2020, a 1 day sampling campaign was carried out on the three sites to collect root sap; branch sap; soil solutions at 20, 40 and 60 cm depths and groundwaters and streamwater. In July and August of the same year, we collected branch sap, groundwaters and streamwater, but no soil solutions or shallow groundwater because the soil and well were dry in summer. In March 2021, sampling was carried out at the three morphological units to collect branch sap, root sap, bole sap, soil solutions, groundwaters and streamwater.

For all sampling campaigns, three to four beech trees in each experimental site were selected. All sampled trees in the plateau and hillslope were large-diameter individuals (50–75 cm diameter), while in the riparian area, only trees with smaller diameters (25–50 cm)



FIGURE 1 Aerial map of the Weierbach experimental catchment and its relative position in Luxembourg.



FIGURE 2 Measured hydrological parameters (Hissler et al., 2021) and measured sap velocity (Fabiani et al., 2021) and calculated VPD (according to Fabiani et al., 2021) at the Weierbach experimental catchment during the tree sampling campaigns in 2017, 2020 and 2021. White dots indicate the specific dates of sampling. Groundwater level relates to the depth below the surface; sap velocity and temperature are the daily means derived by hourly measurements.

were available. The hydro-climatological conditions and the sap velocity at the time of the sampling were recorded for a better understanding of water stable isotope dynamics in trees (Figure 2).

Waters sampling, tree sap extraction 2.3 techniques and sample analyses

Precipitation was collected using a 3 L Palmex rain sampler (Gröning et al., 2012), which minimises gas transfer between the bottle headspace and the open atmosphere to reduce evaporation and therefore minimise isotopic fractionation. The average isotopic composition of the summer and winter precipitations was computed using the online database of the Weierbach experimental catchment (Hissler et al., 2021). The soil solution was collected at 20, 40 and 60 cm depths using Teflon/quartz suction cups (SDEC, Reignac-sur-Indre, France) connected to 2 L Nalgene bottles under a vacuum of 80 kPa. Shallow and deep groundwaters were collected from wells using a peristaltic pump, while the streamwater was directly gathered into polyethylene bottles.

Sap from upper tap roots, boles and second- and third-order branches was directly extracted as liquid in the field using in-situ extraction techniques. Two in-situ vacuum extraction (ISVE) and one overpressure technique were applied to collect sap according to the targeted tree compartment and to the season, as sap accessibility greatly differs between the beginning of the growing season in spring (March to May) and the transpiration period in summer (July to August). These extraction techniques were all designed to collect sap flowing in the xylem at the moment of sampling, so we considered all sap samples as equal.

The first ISVE technique was used in spring for roots and boles. Cores from roots and boles were removed (data not shown in this study) with an increment borer, and the sap was collected by connecting the resulting cavity in the tree to a modified version of the portable device developed by Regulski and Peterson (1982) and applying vacuum. The apparatus consists of a vacuum system (Figure 3a,b) composed of a Duran[®] 100 mL glass bottle capped by a two-hole KIMBLE[®] filtration adapter cap and a metal-free 50 mL centrifuge tube for sap conveyance. A vacuum of 0.80 kPa was applied using a field vacuum pump (SDEC, model PAV 2000). This system allows for the collection of 10 to 15 mL/min of sap. The second ISVE technique was applied in spring for sap collection from non-suberised and second- and third-order branches. After cutting away the extremity (~1 cm), a 60 mL polypropylene syringe (BD Plastipak[™]) was connected via a rubber tube to the end of the branch and the sap was extracted by pulling and blocking the syringe piston (Figure 3c). To obtain representative samples, 10 syringes were installed on each tree in all radial directions and at branch heights from 2 to 5 m. All sampling replicates were pooled into one liquid sample per tree.

The overpressure technique was used to obtain the sap during the growing season, from July to August. During this period, the highly negative water pressure in the xylem did not allow the extraction of sap with the low vacuum techniques used in spring, so extraction of water was performed on second-order branch chunks of 10-15 cm length (Figure 3d) by extrusion using a peristaltic pump (Solinst, peristaltic pump model 410). Once a chunk of branch was cut, the bark at each chunk extremity was removed to avoid contamination from phloem. A rubber tube was then used to connect one end of the chunk and the peristaltic pump. The peristaltic pump was then activated to push air into the chunk, forcing the sap out from the

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FIGURE 3 Apparatus for in-situ vacuum extraction of sap from (a) roots and (b) boles prior to leaf out, (c) syringe system for branch sap collection prior to leaf-out and (d) branch sap extraction system used during transpiration.

other extremity directly into a 5 mL syringe equipped with a 0.45 μ m filtering membrane. To avoid isotopic fractionation due to the contact with the atmosphere, sap samples collected in this way were immediately expressed into 2 mL vials and sealed. With this technique, the volume of water extracted over time was lower than that of *ISVE* methods and 10 to 15 chunks were needed to collect 1 mL of sap in ca. 5 min. We applied the minimum pressure necessary to push the water out of the branch (the pressure applied was not measured).

All sap and water samples were filtered at 0.45 μ m, transferred to 2 mL glass vials and stored at 4°C before analysis. Precipitation, soil solutions, groundwater and streamwater were analysed using a Los Gatos Research Triple Isotopic Water Analyser (LGR TIWA-45-EP) coupled with an automatic sampler (PAL System). The analytical precision of the instrument is $\pm 0.50\%$ for δ^2 H and $\pm 0.15\%$ for δ^{18} O. The data obtained were processed with LWIA Post Analysis software. All sap samples were analysed via Picarro Cavity Ring Down Spectrometer (CRDS) (L2140-i, Picarro Inc., USA) coupled with an automatic sampler and a Micro-Combustion Module[™] to eliminate organic compounds within the water samples. The presence of remaining organic contaminants was monitored with ChemCorrect[™] software (Picarro Inc., USA). The analytical precision of the instrument is ±0.7‰ for δ^2 H and ±0.05‰ for δ^{18} O. The potential memory effects during the analyses with LGR and Picarro were minimised by measuring the isotopic composition 9 and 10 times for each sample and, among those, using only the last five values as the results (Penna et al., 2012).

3 | RESULTS

3.1 | Variability of the O-H isotope composition of rainfall, regolith waters and sap

The historical database of the O and H isotopic composition of the rainfall (Hissler et al., 2021–and subsequent isotopic measurements) collected in the Weierbach experimental catchment revealed

 δ^2 H ranging between -128.36% and -14.37%, and δ^{18} O between -16.86% and -2.38% yielding the following local meteoric water line (LMWL):

$$\delta^2 H = 7.4^* \,\delta^{18} O + 6.8. \tag{1}$$

The rain collected during the summer and the winter before the sampling was enriched and depleted, respectively, in heavy isotopes in comparison with rain collected during the sampling campaigns (Figure 4). Despite the different locations, spatial and temporal variations of the O and H isotopic composition of the groundwaters were low, while the variation of soil solution was the highest among all potential tree water sources (Table 1). Based on the depth and sampling dates, the isotopic composition of water sources (groundwater, soil solutions and streamwater) collected at the plateau and hillslope sites varied spatially and temporally, whereas isotopic composition of all water types collected in the riparian area was quite consistent. Such lack of large heterogeneity is probably linked to the fact that groundwater, streamwater and riparian soil solution mix in this location (Bonanno et al., 2021).

Spatial variability of isotopic composition of root sap was lower in 2017 and 2020 than in 2021. The variability of the branch sap collected by *ISVE* in summer 2020 was low and its isotopic composition was lighter than that of samples collected in March of the same year.

The isotopic signatures of *ISVE*-extracted sap fell onto the LMWL (Figure 4) for all sampling sites regardless of their geological morphology, hydrological conditions, sampling date or tree size.

Among all the trees and sampling campaigns, Ic-excess (deviation in observed δ^2 H from the LMWL; Landwehr & Coplen, 2006) was stable for ISVE root sap and bole sap (lc-excess_{RS} = 1.34 ± 0.91 and lc-excess_{TS} = 1.14 ± 0.83, respectively) and close to that of the groundwaters, streamwater and soil solutions $(lc-excess_{GW} = 1.78 \pm 0.61,$ lc-excess_{SW} = 2.08 ± 0.08 and lc-excess_{SS} = 1.24 ± 0.93, respectively) (Figure 5). On the other hand, branch sap collected with syringes and peristaltic pump showed the



FIGURE 4 Dual isotope diagrams for all the collected waters in this study, sorted by year of sampling and morphological units. BS, branch sap; GW, groundwater; RS, root sap; SS20,40,60, soil solutions at 20, 40 and 60 cm depth; SW, streamwater; TS, bole sap.

highest variability and deviation from the LMWL and from the potential water sources (lc-excess_{BS} = -5.12 ± 1.56 in 2017, lc-excess_{PP} = -2.52 ± 2.03 in 2020 and lc-excess_{BS} = -11.97 ± 2.77 in 2021), with the only exception of March 2020 (lc-excess_{BS} = 0.89 ± 0.99).

3.2 | Progressive ¹⁸O and ²H enrichment in xylem water along the root-branch flow path

For all trees and all sampling dates at all locations, the trend of the O-H isotope signature of the sap collected by *IS*VE was a systematic enrichment in heavy isotopes from the roots, through the bole, to the branches (Figure 6). The sap collected from the branches plotted below the meteoric water line (Figure 4), so the lc-excess of these samples was negative (Figure 5).

The gradual enrichment in heavy isotopes has been quantified for both elements as increments (Δ):

$$\Delta^{18} O = \delta^{18} O_b - \delta^{18} O_a, \tag{2}$$

$$\Delta^2 \mathsf{H} = \delta^2 \mathsf{H}_b - \delta^2 \mathsf{H}_a, \tag{3}$$

where specific tree compartments are denoted *b* and *a* (e.g. bole and roots). Δ^2 H and Δ^{18} O for root-bole, bole-branch and root-branch were significantly correlated across all trees, sites, and dates (Figure 7). The slope of this relationship is the ratio of the net fractionation of ²H to ¹⁸O along the sap flow path. Generally, tight fits of data to sampling-event lines indicate that the ratio was consistent in time and space. The morphological characteristics of the sampling locations did not play a role in the slope of the relationship in the for soil-root or root-bole comparisons, whereas the Δ^2 H and Δ^{18} O relationship from root to branch was specific for each year, varying from 6.5 to 5.0 among the three sampling campaigns (Figure 7). The slope of Δ^2 H/ Δ^{18} O for the root-bole segment was 7.5, which matched the slope of the LMWL, but the lower slopes in branch samples are consistent with an evaporation-like trend.

TABLE 1 Mean $\delta^2 H$ and $\delta^{18} O$ and standard deviation for all samples.

			2017		2020		2021	
Method	Sample	Site	δ ² Η (‰)	δ ¹⁸ Ο (‰)	δ²Η (‰)	δ ¹⁸ Ο (‰)	δ ² Η (‰)	δ ¹⁸ Ο (‰)
Peristaltic pump from wells	GW	P7	-52.35 ± 0.33	-8.21 ± 0.07	-55.17 ± 0.21	-8.61 ± 0.12	-56.30 ± 0.29	-8.80 ± 0.03
		P9	-	-	-54.72	-8.61	-56.10	-8.83
		RP	-	-	-53.99	-8.40	-55.05	-8.70
Teflon/quartz suction cups	SS	P7	-56.53 ± 5.15	-8.75 ± 0.75	-53.11 ± 3.12	-8.39 ± 0.39	-65.58 ± 7.63	-9.85 ± 0.97
		P9	-	-	-52.59 ± 6.82	-8.21 ± 1.00	-62.95 ± 7.09	-9.39 ± 0.83
		RP	-	-	-53.43 ± 0.18	-8.34 ± 0.06	-55.87 ± 0.45	-8.74 ± 0.06
Hand-collection with polyethylene bottles	SW	RP	-	-	-54.02	-8.51	-55.39	-8.67
ISVE with syringes or vacuum pump	RS	P7	-47.77 ± 2.15	-7.51 ± 0.28	-49.47 ± 2.18	-7.91 ± 0.29	-59.99 ± 2.77	-9.06 ± 0.38
		P9	-	-	-47.39 ± 1.47	-7.62 ± 0.16	-55.97 ± 2.67	-8.67 ± 0.35
		RP	-	-	-51.30 ± 0.05	-8.15 ± 0.00	-52.70 ± 3.30	-8.07 ± 0.47
	BS	P7	-36.83 ± 1.37	-5.20 ± 0.36	-34.97 ± 0.74	-5.70 ± 0.16	-28.20 ± 8.89	-2.86 ± 1.75
		P9	-	-	-34.97 ± 0.41	-5.84 ± 0.13	-32.16 ± 3.62	-3.69 ± 0.73
		RP	-	-	-34.71 ± 1.19	-5.73 ± 0.06	-26.80 ± 6.46	-3.13 ± 0.98
	TS	P7	-	-	-	-	-53.13 ± 4.83	-8-27 ± 0.74
		P9	-	-	-	-	-49.81 ± 3.93	-7.85 ± 0.53
		RP	-	-	-	-	-48.86 ± 3.91	-7.61 ± 0.38
Overpressure with peristaltic pump	BS PP	P7	-	-	-45.66 ± 2.03	-6.73 ± 0.51	-	-
		P9	-	-	-46.87 ± 1.20	-6.88 ± 0.15	-	-
		RP	-	-	-42.55 ± 3.66	-6.41 ± 0.78	-	-

Abbreviations: BS, branch sap; GW, groundwater; ISVE, in-situ vacuum extraction from trees; ISVE PP, in-situ vacuum extraction from branch chunks; P7, plateau; P9, hillslope; RP, riparian area; RS, root sap; SS, soil solution; SW, streamwater; TS, bole sap.

4 | DISCUSSION

4.1 | Progressive 2 H and 18 O enrichment in the xylem water

Our results show progressive heavy isotope enrichment in xylem water, which appears to be consistent with the distance travelled from roots towards the upper tree compartments. However, the results also indicate that this progressive enrichment seems to be controlled by different mechanisms according to the tree compartment where it occurs. The enrichment observed from the soil to the bole stayed coherent between both O and H elements and is located along the meteoric water line, whereas the enrichment that appears between the bole and the branch presents a more evaporation-like trend.

4.1.1 | ²H and ¹⁸O enrichment from root to bole

The most reasonable way to explain the enrichment between the roots and the bole is to consider the mixing of water of different ages. It is likely that the sap collected from the above-ground parts of the trees is characterised by a mixing between the recently uptaken water and water stored in the tree. This mixing mechanism is consistent with

the evolution of the root-to-bole isotopic signature of the xylem water along the LMWL: The farther the water flows upward, the closer its isotopic composition was with the rain collected the previous summer. Low transpiration and evaporation of *F. sylvatica* during winter might have kept the isotopic signature of this storage water unchanged until spring, when the mixing likely began due to the uptake of fresh water for embolism recovery and for the development of new twigs and buds. This is corroborated by the isotopic composition of xylem sap collected in this study, which is always located on the LMWL between the summer and the winter rainwater (Figure 4). Unfortunately, there is no water available in this soil type in summer, making a direct comparison between the soil water and the root sap in winter and summer impossible.

In relation to a recent publication by Nehemy et al. (2022), another crucial factor to consider regarding the enrichment of sap isotopic composition between the roots and the bole is the process of xylem-phloem water exchange. They reported a lighter isotopic signature of phloem water in comparison to xylem water, attributing it to fractionation during water flow from xylem to phloem through aquaporins. If the extent of water exchange between xylem and phloem increases with the distance and/or time of exposure of the xylem sap to such a process, it would result in the progressive enrichment we observed (Figure 6).

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δ²H



FIGURE 6 Progressive enrichment of the isotope ratios from the groundwater to the branches in the three experimental plots. For tree waters, circles indicate ISVE (syringe-vacuum pump) samples collected in spring, while squares indicate summer samples collected with the peristaltic pump. BS, branch sap; GW, deep groundwater; RS, root sap; sh-GW, shallow groundwater; SS, soil solution; SW, streamwater; TS, bole sap.



δ¹⁸O









Moreover, water participates in biochemical reactions linked to DeNiro, 1990). Some of these metabolic pathways involving xylem the biosynthesis of many molecules, undergoing biochemical isotope water may be responsible for the gradual enrichment observed, due to difference in chemical reaction rates between light and heavy fractionation (da Silveira Lobo Sternberg, 1988; Yakir &

FIGURE 7 Δ^2 H vs. Δ^{18} O relationship for paired sap samples collected in this study.



water molecules. Indeed, lighter isotopes preferentially participate in chemical reactions due to the kinetic isotope effect, according to which the reaction rate of lighter isotopes is greater than that of heavier isotopes since the former are characterised by a lower bond dissociation energy (Costa Filho et al., 2013; Ouellette & David Rawn, 2018). This kinetic isotope effect might have an important role in the isotopic exchange between metabolic intermediates and water during post-photosynthetic metabolism, creating fractionation. This type of isotope exchange during plant biosynthetic pathways involves a preferential fractionation of light O-H isotopes into organic molecules and the consequent enrichment of heavy isotopes in the water, as shown for several biochemical processes such as lipid and carbohydrates biosynthesis (Cormier et al., 2018), including cellulose (Hermoso de Mendoza et al., 2022; Kagawa & Battipaglia, 2022; Sternberg et al., 1986). Post-photosynthetic O-H isotopic exchange mainly occurs via carbonyl hydration, which is a good candidate reaction to explain xylem transformations, as it involves isotopic exchange between xylem water and metabolites during cellulose biosynthesis (Cheesman & Cernusak, 2017; Hill et al., 1995; Samuel & Silver, 1965; Sternberg & Deniro, 1983). Considering the kinetic isotope effect, it is likely that light water isotopologues preferentially participate in this reaction, systematically 'depriving' xylem water of the lighter isotopes and leading to a progressive enrichment of xylem water due to the exposure of sap water to the biochemical interactions.

Sap velocity might play a role in the seasonal variation of enrichment. In summer, when the flow is faster due to transpiration, the residence time of xylem water within the tree is reduced. If water exchanges and chemical reactions drive the isotopic enrichment of xylem water, the degree of contact (i.e. distance travelled and residence time) likely drives the extent of isotopic modification along the flow path within the tree. This hypothesis is supported by observations of less-enriched branch sap in July and August compared to March (Figure 4b,d,f), when sap flux is near zero (Fabiani et al., 2021; Figure 2). However, the extent of isotopic enrichment linked to the aforementioned mechanisms remains highly uncertain, and further investigations are needed to elucidate the impact of the relationship between water masses, sap velocity and isotope exchange reactions. Further studies are also needed to elucidate, for example, whether the isotopic enrichment along the sap flow path is a species-specific pattern or is recurrent also in other tree species, or if an isotopic gradient in the cellulose along the tree exists, since the composition of this latter is affected by the isotopic signature of the xylem water at the time of biosynthesis.

4.1.2 | Evaporation trend in the isotopic signature of branch sap

The branch xylem water, in 2017 and 2021, plotted below the LMWL (Figure 5) and to the right of the other xylem waters (Figure 4), in a characteristic pattern of evaporation. This pattern is consistent with bark evaporative enrichment. Martín-Gómez et al. (2017) studied the effect that the reduction in the sap flow rate has on the isotopic composition of twigs water by a series of experiments based on simulating hydraulic failure and reduced leaf transpiration. They showed that the O and H isotopic composition of the resulted tree water is enriched in heavy isotopes following the typical evaporation line. This led to the conclusion that, in reduced sap flow conditions, the bark evaporative enrichment plays a key role in the isotopic signature of sap water, even in short time lapse (<1 h). Moreover, considering that root sap and bole sap showed regolith source-like lc-excess values, the marked changes observed in branch sap samples can be attributed to

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processes occurring at the branch level and not below. The total absence of evaporative enrichment in the sap of roots and bole, even in low sap flow conditions, is likely linked to the presence of hydrophobic suberised bark, and by a higher volume of water in those compartments in comparison with finer twigs. The absence of suberised tissues in twigs likely enhances this evaporative effect, due to the lack of hydrophobic properties of non-suberised bark (Franceschi et al., 2005; Krokene, 2015). The evaporative effect was indeed stronger in water extracted from young non-suberised twigs than in that collected from suberised branches. The fact that the sap extracted from the branches in summer had a lower evaporative effect than that of the water collected from the finer twigs in spring, is consistent with this reasoning (Figure 4). Moreover, higher temperatures contribute to reducing the difference in evaporation rate between heavy and light water isotopologues (Mook, 2005), potentially reducing the evaporative enrichment at the branch level in summer. But why was the evaporative enrichment in branch sap isotopic composition not observed in March 2020? The most likely answer is that varied local meteorological conditions, such as air temperature variations, among the sampling campaigns might have affected the bark evaporation process. In spring 2020, when the evaporative-like enrichment was smaller, the temperature was around 4°C, while in 2017, summer 2020 and spring 2021, the temperature was near or above 10°C (Figure 2). The Δ^2 H and Δ^{18} O relationship slope evolved accordingly (Figure 7). In the case of our sampling dates, the air temperature was different, which might matter for a range of eco-physiological and physical processes governing isotopic fractionation in twigs. However, further investigations on branch sap under different meteorological and environmental conditions are needed to elucidate and guantify their effect on the isotopic composition of the branch sap and therefore the lc-excess values.

4.2 | *IS*VE and overpressure: Advantages, limitations, type of water extracted and comparison with other extraction techniques

A guestion arises for ISVE techniques: What water pool(s) are we collecting? Our experimental design did not allow us to answer this question directly, but previous works aid in the interpretation. Zhao et al. (2016) extracted xylem sap by exudation through a needle, taking advantage of positive xylem pressure during the absence of transpiration, and successfully matched the isotopic composition of the water source to that of the exuded sap. Barbeta et al. (2022) extracted water from cut twigs by centrifugation and obtained water that plotted close to the expected water sources, while parallel samples obtained by cryogenic vacuum distillation showed the typical offset in ²H. Root sap extracted by *ISVE* gave similar results to extraction by needle or centrifuge: isotopic composition coherent with the potential water sources. Indeed, ISVE waters did not result in the bias in ²H that is typical of cryogenic extraction and, more specifically, isotopic signatures of ISVE root and bole sap samples fell onto the LMWL along with potential water sources (Figure 4). However, the fact that the three extraction techniques reported in this manuscript were not compared to determine whether or not they extract the same sap fraction, or saps having the same isotopic composition, represents a limitation of this study and further investigations are needed to shed light on the matter.

The success of the ISVE sampling technique lies in its exceptional clarity when identifying the water sources used by trees. The isotopic signature of root sap collected in plateau and hillslope was close to that of the soil solutions in 2017 and 2020, suggesting a preferential uptake of shallow soil water (Figure 4), which is in line with what already suggested by Fabiani et al. (2021) for the same site. In 2021 instead, sap isotopic signature seems to be resulting from a mixing of soil solutions and groundwater. Results from the cryogenic extraction presented by the above-mentioned authors-who made also use of sap flow and groundwater table measurements, showed that groundwater does not significantly contribute to the tree water uptake. However, capillary processes and the resulting capillary fringe were not considered in both studies, leaving an open question regarding the origin of the water uptaken in summer, when the soil is dry. Concerning the riparian area, it was not possible to identify the origin of the water uptaken, due to the similar isotopic signature shown by the available water sources in the experimental plot. However, our data suggest that root sap always presented a closer isotopic composition to the available water sources and that it had not encountered isotopic modification by metabolic processes, transpiration, mixing or other transformations that were more important in the above-ground parts of the tree.

Apart from the large volume of water extracted, which represents a great advantage for hydrological and geochemical studies, *IS*VE methods illustrated are cheap and easy to apply. Pumps are the only expensive instruments, but we assume that any lab working on field experiments already owns them. All the used *IS*VE equipment (vacuum chamber, syringes and tubings) can be cleaned and re-used indefinitely, lowering even more the cost of these techniques in the long term. Moreover, tree waters extracted with *IS*VE resulted clean and transparent and the post-processing showed low or absence of organic interferences during the analysis of these samples (data not shown). This suggests that methodological biases linked to organic interferences, usually resulting from more common methods (Millar et al., 2018) were avoided. Thus, *IS*VE appears to be a simple and viable alternative to extraction by needle, centrifugation or cryogenic techniques.

The main limitation of the *IS*VE techniques is that they can be applied only until the early stage of leaf development. After leaf out, transpiration reduces the pressure inside the xylem so that extraction with weak vacuum techniques is more difficult. In this sense, an ISVE extraction with a more powerful vacuum pump can be tested in order to overcome the negative water pressure in the tree. However, such a high pressure may disrupt or alter the hydro-connectivity of the water compartments inside trees—by, for example, destroying vessels and creating internal water mixing—delivering a sample of water that is a mix of different pools and potentially harming the tree.

A viable solution to the transpiration problem is offered by the overpressure technique that allows to collect water, albeit in a much

smaller volume, even during the growing season. The principle behind the overpressure technique is the same as the Scholander pressure chamber (SPC-Scholander et al., 1965), where a leafy twig is sealed inside a vacuum chamber with an end exposed in the outside and pressure is applied through a gas tank, pressurising the chamber and pushing the water out from the twig's outside end. The volume of water collected from each chunk with the overpressure method was low (not measured) but potentially higher, in the unit of time, in comparison with the SPC. Zuecco et al. (2022), for instance, collected less than 200 µL of water from a single twig in less than 10 min with SPC, while with the overpressure method, 10 to 15 chunks were used to collect about 1 mL of water in ca. 5 min. Therefore, in comparison with the SPC, the overpressure appears as a more efficient technique to extract water from branches. However, a comparative approach applying both techniques on the same species and under the same environmental conditions is needed to avoid potential biases in the comparison as the volume of water collectible with these techniques may vary seasonally (Rennenberg et al., 1996). Finally, the overpressure method does not require any other specific equipment (such as gas tank or vacuum chamber) making this technique a viable alternative to the SPC, also in terms of costs and field user comfortability. The main limitation of the overpressure is related to concerns that may arise with its application on roots. The significant number of roots needed to collect a sufficient volume of water and the destructiveness of the method, make the overpressure technique potentially harmful not only to the tree but also to the soil, whose stratification and hydraulic properties would be altered. This is why the overpressure technique was not applied to roots in this study.

5 | CONCLUSION

We focused on understanding how physical and biochemical processes may lead to changes in the isotopic composition of xylem sap along the root-branch flow path of *F. sylvatica* at the beginning of the growing season. We used an in-situ water extraction method on beech trees to obtain xylem water from roots, boles and branches. We then were able to directly compare the isotopic composition of sap liquid samples with potential water sources.

Our results showed that there was a vertical gradient in the isotopic composition of xylem water, characterised by a progressive ¹⁸O and ²H enrichment along the root-branch flow path and an evaporation-like trend in the branch sap. The extent of the enrichment of heavy isotopes in xylem water appeared to be consistent with the distance travelled by the water and its residence time inside the trees. Thus, water extracted by *IS*VE from roots appears to represent the recently uptaken water, while water extracted above the stems is progressively affected by various fractionating processes. The fractionation is likely attributable to a combination of the following: (i) mixing of waters of different ages inside the trees, (ii) xylemphloem water exchange, (iii) bark evaporative enrichment and (iv) kinetic isotope effect on carbonyl hydration reactions involving xylem water during cellulose biosynthesis. These dominant processes may vary by location within the tree. The mixing water of different ages between the root and the bole and the bark evaporative enrichment from the bole to the branches seem to be the predominant processes in our experiment because of the early spring bias to our sampling. Therefore, a more precise experimental approach is now needed to identify the contribution of the different physical and biochemical impacts on the xylem water isotopic composition on a seasonal basis. However, our results clearly indicate that isotopic transformations within trees are multiple and complex.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Zenodo at https://doi.org/10.5281/zenodo.7783983, reference number 10.5281/zenodo.7783983.

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