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Cryogenic vacuum distillation vs Cavitron methods in ecohydrology: Extraction protocol effects on plant water isotopic values

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ABSTRACT

Stable isotope ratios of hydrogen and oxygen in plant water are widely used for water tracing in ecohydrology studies. In this approach, plant water is extracted for isotopic analysis of δ^2H and $\delta^{18}O$. Among the extraction methods, cryogenic vacuum distillation (CVD) has been the most popular, but its impact on the isotopic composition of plant water is currently under debate. Newer Cavitron method have been proposed to replace CVD method for xylem water extraction. These recommendations have been largely based on comparisons between Cavitron-xylem water with low temperatures CVD-xylem water. However, the CVD protocol (extraction temperature and time) varies widely across laboratories, and no direct, systematic comparison has yet been made between extraction temperature and time protocols vs. the Cavitron approach. Here we compared the isotopic values of xylem water from the same tree obtained through CVD extraction at 60 °C (60, 120, 240, 360 min), 100 °C (30, 60, 120, 240 min), 140 °C (15, 30, 60, 120, 240 min), and 200 °C (15, 30 min). Subsequently, we compared the results of CVD-xylem water with Cavitron-xylem water. Our data show that isotopic values for CVD-xylem water became more positive with increasing extraction time under the same extraction temperature. Such extractions also became more isotopically positive with increasing extraction temperature for the same extraction time. When total extraction efficiency exceeded 98 %, there was no $\delta^{18}O$ difference in CVD-xylem water among any of the different protocols ($p > 0.05$). However, lower extraction temperatures resulted in more negative δ^2H when compared to higher temperature extraction ($p < 0.05$). Cavitron-xylem water was close to CVD-xylem water (average difference of 3 ‰ for δ^2H and 0.5 ‰ for $\delta^{18}O$, $n = 79$) when total extraction efficiency for CVD was below 98 %. But for extraction efficiencies beyond 98 %, the Cavitron-xylem water was more negative in δ^2H (17.3 ‰, $n = 70$) and $\delta^{18}O$ (1.7 ‰, $n = 70$) than CVD-xylem water. Compared to Cavitron-xylem water, CVD-xylem water at 200 °C with extraction efficiency > 98 % was closer to the soil water. Further study is necessary to conduct a complete cross-comparison between Cavitron and CVD at different temperatures with different species at various water and salt stress conditions. But our results suggest that abandoning CVD for plant water maybe premature until such complete comparison work is done.

1. Introduction

Stable isotopes of hydrogen and oxygen have been widely used for tracing plant water (Barbeta and Peñuelas 2017; Brooks et al., 2010; Dawson and Ehleringer 1991; Sprenger et al., 2018), including the origin, spatial source, and age of transpiration (Evaristo et al., 2019; Mennekes et al., 2021; Nehemy et al., 2022b). Traditionally, this tracing approach involves sampling and extracting water from plants. The most

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sampled plant tissue for source investigation is the xylem – the vascular tissue that conducts water from roots to leaves (Brooks et al., 2010; Brunel et al., 1995; Zhao et al., 2024). Xylem water can be sampled in roots, stems, branches, and leaves (Benettin et al., 2021; Peters and Yakir 2008; Song and Barbour 2016; Vega-Grau et al., 2021; Zhao et al., 2016), and other tissues (e.g., the phloem) are also sampled for water dynamics of the entire plants (Nehemy et al., 2022a; Treydte et al., 2021). Although in situ sampling of xylem water is possible and provides data in higher temporal resolution (Kühnhammer et al., 2022; Marshall et al., 2020; Volkmann et al., 2016), there are still significant limitations with it being widely applied across sites (e.g., access to power at the site, high cost of dedicated field-based laser spectroscopy analyzer) and plant growth forms (i.e., it is limited to mature plants and straight branches) (Duvert et al., 2024). Therefore, the traditional destructive sampling methods are still the most widely used. In these approaches, the collected plant materials are stored in vials in the field, and then water is extracted subsequently from the samples in laboratory.

Several techniques are available to extract water from vascular tissue in the laboratory, such as cryogenic vacuum distillation (CVD) (Koeniger et al., 2011; Orlowski et al., 2013), azeotropic distillation (Revesz and Woods 1990), centrifugation (Bowers et al., 2020; Edmunds and Bath 1976), centrifugation using Cavitron (Barbeta et al., 2022), and high-pressure mechanical squeezing (Mazurek et al., 2015). All of these techniques were developed for soil pore water extraction and then adapted to plant water extraction, except the Cavitron method (Barbeta et al., 2022), which is specifically tailored to plant tissue. As for soils, most studies still use the CVD extraction method to extract water from plants, given the ability to collect and store samples for later analysis, which enables research in remote field sites. Several protocols are reported in the literature for CVD extraction method (e.g., Table S1) but still without a clear, unified approach (Millar et al., 2022).

The early study by Araguás-Araguás et al. (1995) investigating CVD extraction of soil under distinct extraction times and temperatures showed that different protocols yielded accurate isotope results for sandy soils when more than 98 % of water was extracted but not for soils with higher clay content. They recommended clayey soil extraction approach to be tailored to the research question. Later, West et al. (2006) investigated CVD extraction time protocols for soil and plant samples using the same temperature (boiling water; ~100 °C) and showed that the isotopic value of extracted water increased with extraction time until reaching a certain threshold depending on the investigated material. Following Araguás-Araguás et al. (1995), an extraction efficiency of 98 % has been used as a standard threshold during the cryogenic extraction of plant samples, which means that 98 % of the water in the sample (bulk xylem water) is extracted, assuming that no fractionation is introduced during the extraction process. We simply do not know if these sorts of findings now made for soil and their CVD extraction applies to plants in terms of its impact on the isotopic results for xylem water following different CVD protocols (i.e., extraction temperature and time, Table S1).

The CVD method for plant water extractions has indeed been questioned recently regarding the possible bias it introduces to the observed plant water isotopic composition (Chen et al., 2020), and correction protocols have been discussed (Allen and Kirchner 2022; He et al., 2023). Additionally, while cryogenic techniques aim for higher extraction efficiency, it is possible that the bulk water in the xylem may not represent the water actively moving within conduits and contributing to transpiration. In contrast, other techniques, such as centrifugation and Cavitron, do not prioritize high extraction efficiency; rather, they specifically target the more mobile water within xylem (Barbeta et al., 2022; Duvert et al., 2024; He et al., 2023; Wen et al., 2023). Theoretically, the Cavitron approach is thought to extract water that would participate in the transpiration stream by applying a centrifugation force equivalent to –2 MPa to –6 MPa, without extracting potentially intracellular water like the CVD method. The applicability of Cavitron to extract water for plant water source analysis has been shown experimentally in controlled conditions (Barbeta et al., 2022; Wen et al., 2023) and in the field (Duvert et al., 2024; He et al., 2023). Barbeta et al. (2022) showed that isotopic values of Cavitron-xylem water were similar to labeled water used in pot irrigation experiments. Wen et al. (2022) demonstrated that Cavitron-xylem water closely matched the water used to rehydrate wood stems. Duvert et al. (2024) and He et al. (2023) showed Cavitron-xylem water was located on the source water line. While confirming the applicability of Cavitron, they also pointed out that CVD-xylem water was more depleted in deuterium compared to the reference water (source water). However, no studies that we are aware of have compared the different CVD extraction protocols and Cavitron methods aimed at determining whether their performance differs due to different CVD protocols.

Here, we investigate the isotopic signature of xylem water using various CVD protocols to determine the effect of the CVD extraction protocol on the comparison with the Cavitron method. The specific research questions were thus: i) what is the effect of CVD extraction protocol (in terms of extraction temperature, time, and efficiency) on plant water isotopic values? and ii) how do the results obtained from the Cavitron method compare to those waters extracted via CVD, considering varying extraction protocols?

2. Material and methods

2.1. Site description

The field sampling was conducted in Changwu Tableland, located in the southeastern part of the Chinese Loess Plateau. The site has an elevation of 1200 m above mean sea level and a continental monsoon climate. The mean temperature of the site is 9.4 °C, with annual precipitation of 585 mm, and potential evapotranspiration of 897 mm per year. Approximately 53 % of the annual precipitation occurs between July and September. Grasslands and steppe forests dominated the Loess Plateau during the Holocene periods (Shang and Li 2010). However, intensive farming during the last 2000 years severely depleted grasslands and forests (Wang et al., 2006). To tackle the ecosystem degradation issue, the “Grain to Green” program was implemented by the Government of China in 1999, and apple orchards became the predominant land use type in this region. We selected a healthy apple tree in a 21-year-old apple orchard, planted in 2001, to conduct our experiment. The tree was 3.2 m tall and 23.4 cm in diameter at 130 cm height.

2.2. Sampling

We commenced the experiment by collecting soil samples at a distance of 1.5 m from the tree stem on July 29th, 2022. The soil sampling was conducted using a hollow-stem hand auger with increments of 20 cm to a depth of 10 m. Upon approaching the desired depth, we immediately sampled the soil by filling three-quarters of each 12 mL glass vial (737 W, Labco Ltd., UK). Following the initial background sampling, we irrigated the apple tree daily at evening until August 6th, 2022, totaling an amount equivalent of 160 mm. Irrigation was provided using tap water (–11.04 ‰ for $\delta^{18}O$ and –78.40 ‰ for $\delta^{2}H$, originally pumped from groundwater), and a drip irrigation system was placed around the stem of the apple tree. During the experiment, one 9 mm precipitation event occurred on August 3, 2022 with isotopic values of −4.67 ‰ and −27.17 ‰ for $\delta^{18}O$ and $\delta^{2}H$, respectively. On August 7th, 2022, we repeated collection procedures for soil samples following the identical protocol mentioned above and we collected branch samples from the south-facing side of the selected tree after sunset at about 7 pm to prevent potential evaporation during sampling processes. We collected straight branches that were ~ 1 cm in diameter and longer than 28 cm. The branches used for cryogenic extraction were quickly processed in the field by removing the inner barks, cutting the xylem into small pieces, and placing them into 12 mL glass vials (737 W, Labco Ltd., UK; n = 10 per protocol). We also collected an additional set of 10

branches adjacent to the sampled branch for Cavitron analysis (referred to as Cavitron samples hereafter), which were wrapped with parafilm and stored in a cooler with ice packs following the procedures of He et al. (2023) and Wen et al. (2022). Subsequently, the Cavitron samples were transported to the laboratory where water was extracted within 24 h. The vials with samples were securely sealed with parafilm before being stored in a cooler with ice packs in the field and transferred to a refrigerator at 4 °C in laboratory on the same day.

2.3. Laboratory analysis

We used a capillary and vial-style CVD extraction system (Koeniger et al., 2011) to extract xylem and soil water. Briefly, the sample vials were frozen using liquid nitrogen and then connected to another empty vial (collection vial) through a capillary tubing, depressurized to less than 0.01 mbar, and then heated at a predetermined temperature for a specific duration time. Within this closed system, the water evaporated from the sample vials and condensed into collection vials. The extracted water was transferred into a sealed 2 mL screw glass vial using a cap and parafilm for storage. The extracted sample vials were oven-dried at 105 °C for 24 h to assess extraction efficiencies. Fifteen distinct CVD protocols (Table 1), with 10 replications per treatment, were used to extract the plant samples. For soil samples, we used 200 °C and 30 min for the CVD extraction.

We used a Cavitron (H2100R; Xiang Yi Lab Instr. Co., Ltd.) for water extraction. In the laboratory, we trimmed the branches to 27.4 cm by removing small portions from both ends. Then, we peeled off the bark from around 4.5 cm on both ends, inserted the bark-free ends into cuvettes, and wrapped the cuvettes and branches with parafilm. Subsequently, we set the rotation speed in the Cavitron to 7636 rpm, which is equivalent to a centrifugal force of −6 MPa (Alder et al., 1997; Cochard 2002; Du et al., 2019), set the temperature to 10 °C, and used a rotation time of 2 min to collect enough water (Duvert et al., 2024; He et al., 2023). On average, 0.4 g water was obtained via Cavitron. The extracted water was collected into two cuvettes and immediately transferred to a 2 mL screw-capped glass vial for isotopic analysis.

2.4. Extraction efficiency

For the CVD method, we weighed empty sample vials, sample vials with samples, and collection vials both before and after xylem water extraction. The sample vials were weighed again after oven-drying the extracted samples. The weight difference between a collection vial before and after water extraction represents the amount of water actually collected. The weight difference between the sample vial pre- and post-extractions indicates the amount of water extracted from the sample (i.e., sample-extracted water). The difference in weights between a sample at pre-extraction and post-oven-drying reflects the total water present in the sample. Typically, extraction efficiency is calculated by dividing the sample-extracted water by the total water. However, we calculated extraction efficiency by dividing the collected water by the total water. This approach provides a more realistic measure termed 'total extraction efficiency' hereafter.

Table 1
The temperatures and times used for cryogenic vacuum distillation extraction.

Temperature (°C)	Time (min)
60	60, 120, 240, 360
100	30, 60, 120, 240
140	15, 30, 60, 120, 240
200	15, 30

2.5. Isotope measurements and data analysis

Plant-water samples were analyzed using a mass spectrometer (253 Plus; Thermo Fisher Scientific Inc., Bremen, Germany) attached to an elemental analyzer (EA Isolink; Thermo Fisher Scientif Inc.) at Ludong University, China. Soil-water, irrigation, and precipitation water samples were measured using a laser spectrometer (IWA-45EP; Los Gatos Research, Inc., San Jose, CA, USA) at Northwest A & F University, China. The measured isotopic values of hydrogen and oxygen were normalized to the Vienna Standard Mean Ocean Water and Standard Light Antarctic Precipitation (VSMOW-SLAP) scale via the co-measured standards. The isotopic compositions were expressed in per mil (Equation (1).

$$\delta(2Hor18O) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1000‰ \quad (1)$$

where R_{sample} and $R_{standard}$ denote the ratio of number of heavy isotopes to that of the light isotopes in the sample water and standard water, respectively.

To compare the difference of Cavitron-xylem water and CVD-xylem water to the source water, following previous approaches that compare Cavitron and CVD, we calculated the deuterium offset, Equation (2), based on the soil water line (De la Casa et al., 2022; Duvert et al., 2024).

$$\delta^2H_{offset} = \delta^2H_{Cavitron-orCVD-xylemwater} - \left(a\delta^{18}O_{Cavitron-orCVD-xylemwater} + b\right) \quad (2)$$

where a and b are the slope and intercept of the soil water line, respectively.

We used the Kruskal-Wallis test (Kruskal and Wallis 1952) and post hoc Dunn's test (Dunn 1964) to assess statistical differences in isotopic composition among different CVD extraction protocols and to compare the deuterium offset of Cavitron-xylem water and CVD-xylem water with zero, the offset to the soil water line. Non-parametric statistical tests were used due to the non-normal distribution of the isotope data. The normalities were evaluated with the quantile–quantile plots, histograms, and Shapiro–Wilk test. We then compared the cryogenic isotope results with those obtained from Cavitron-xylem water.

We calculated the relative water content of stem samples by dividing the total amount of water by the weight of wet material, which is the difference in weights between the empty sample vial and the pre-extraction sample-filled vial. We used the relative water content and total water amount to address isotopic bias caused by CVD that was reported by Chen et al. (2020) due to H-exchange with cellular during the extraction and by Diao et al. (2022) due to the water amount issue for the extraction.

3. Results

3.1. Comparison of cryogenic extraction across different protocols

Generally, when comparing extraction approaches under the same temperature, we found that longer extraction time produced more positive isotopic values (Fig. 1). When comparing extraction approaches under the same extraction time, higher temperatures produced more positive isotopic values (Fig. 1). At 100 °C, the isotopic values became fairly stable when extraction time was longer than 60 min, implying that there was no isotopic difference between xylem waters for CVD-100–60 (approach-temperature–time), CVD-100–120, and CVD-100–240 ($p > 0.05$). At 140 °C, the isotopic values of xylem water became fairly stable when extraction time was longer than 30 min, implying that there was no isotopic difference between xylem water for CVD-140–30, CVD-140–60, CVD-140–120, and CVD-140–240 ($p > 0.05$).

Xylem water δ^2H and $\delta^{18}O$ extracted at CVD-200–30 showed no difference with those extracted at 100 °C with extraction time longer than 60 min (Figure S1) and those extracted at 140 °C with extraction time longer than 30 min ($p > 0.05$). Generally, the isotope values of

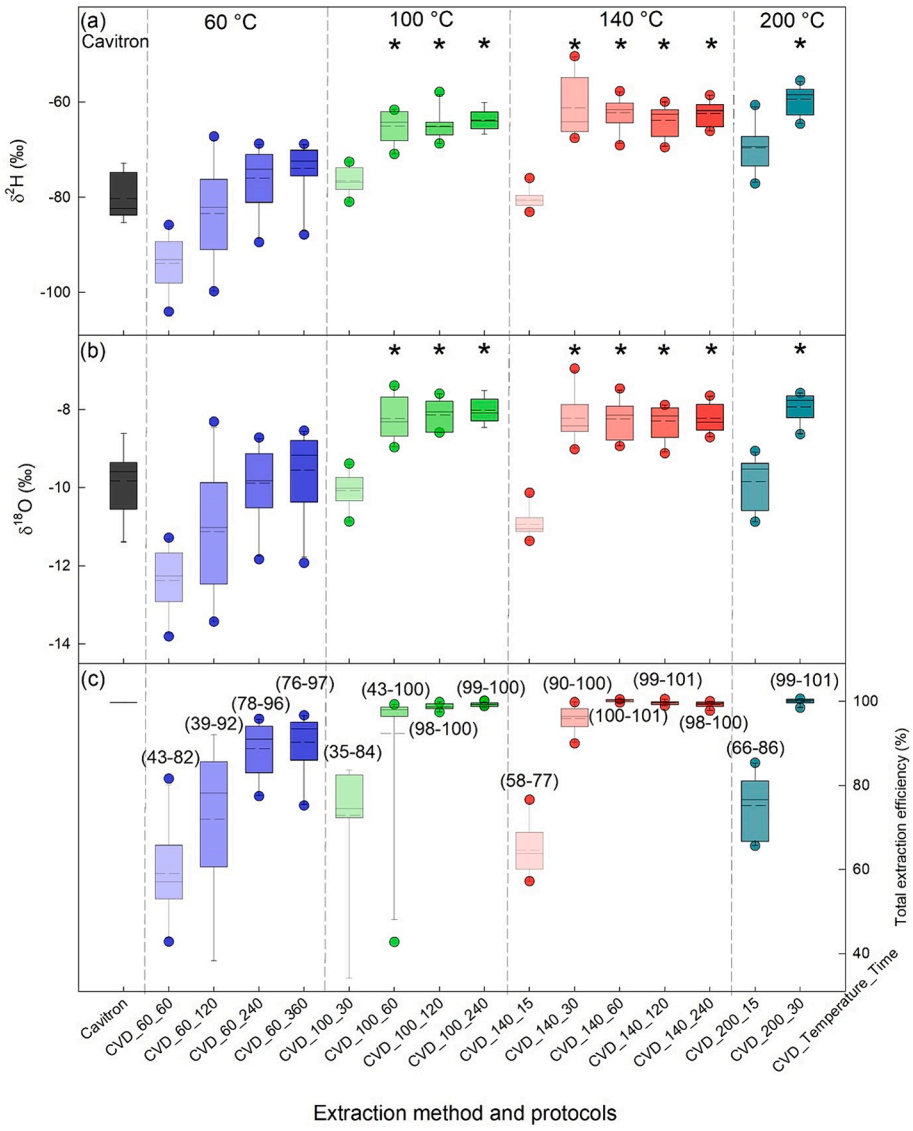

Fig. 1. Box plot for δ^2H (a) and $\delta^{18}O$ (b), and total extraction efficiency (c) of Cavitron-extracted xylem water (black), CVD-extracted xylem water (CVD_Temperature_Time; Blue for 60 °C, green for 100 °C, red for 140 °C, cyan for 200 °C) with 10 replicates per protocol. The dashed lines in the box indicated mean value; the solid lines in the box indicated medium value; stars on the top of the boxes indicated a significant difference of CVD-extracted xylem water from Cavitron-extracted xylem water ($p < 0.05$); numbers on the top of the boxes indicated the range of total extraction efficiency. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

xylem water extracted under shorter extraction time protocols (e.g., CVD-100–30, CVD-140–15, and CVD-200–15) were not noticeably different from xylem water extracted at 60 °C ($p > 0.05$). Conversely, the isotope values of xylem water extracted at temperature ≥ 60 °C with shorter extraction times (i.e., CVD-100–30, CVD-140–15, and CVD-200–15) were significantly different from those extracted at higher temperatures with longer extraction times ($p < 0.05$). However, a few samples did not follow this general pattern; for example, δ^2H of xylem water extracted at CVD-200–15 showed a significant difference from that extracted at CVD-60–60 ($p < 0.05$) but no difference from the other protocols ($p > 0.05$). Additionally, δ^2H of xylem water extracted at CVD-140–15 showed no difference from that extracted at CVD-100–240 ($p > 0.05$).

3.2. *Xylem water signature as influenced by CVD extraction temperature and extraction efficiency*

Total extraction efficiency varied across the methods compared (Fig. 1c); higher temperatures and longer extraction times provide higher total extraction efficiency. Generally, the higher the temperature, the lower the extraction time needed to reach the total extraction efficiency above 98 %. For 60 °C extraction temperature, extraction efficiency was below 98 % at all extraction times investigated. For 100 °C extraction temperature, total extraction efficiency was also below 98 % for extraction time of 30 min, while for 60 min extraction time, total extraction efficiency showed some variability. Extraction times longer than 60 min at 100 °C extraction temperature resulted in total extraction efficiency ≥ 98 %. For 140 °C extraction temperature, total extraction efficiency was below 98 % for 15 min extraction time, fluctuated close to 98 % for 30 min extraction time, and exceeded 98 % for 60 min or longer extraction time. For 200 °C extraction temperature, the extraction efficiency was below 98 % for 15 min extraction time but was above 98 % for longer extraction times (Fig. 1c).

Xylem water $\delta^{18}O$ and δ^2H values were more positive when total extraction efficiency exceeded 98 % compared to lower than 98 % (Fig. 2). Xylem water $\delta^{18}O$ ranged from –103.9 ‰ to –50.3 ‰, and δ^2H ranged from –13.8 ‰ to –7.4 ‰ with < 98 % total extraction efficiency. Total extraction efficiency > 98 % yielded xylem water isotopes ranging

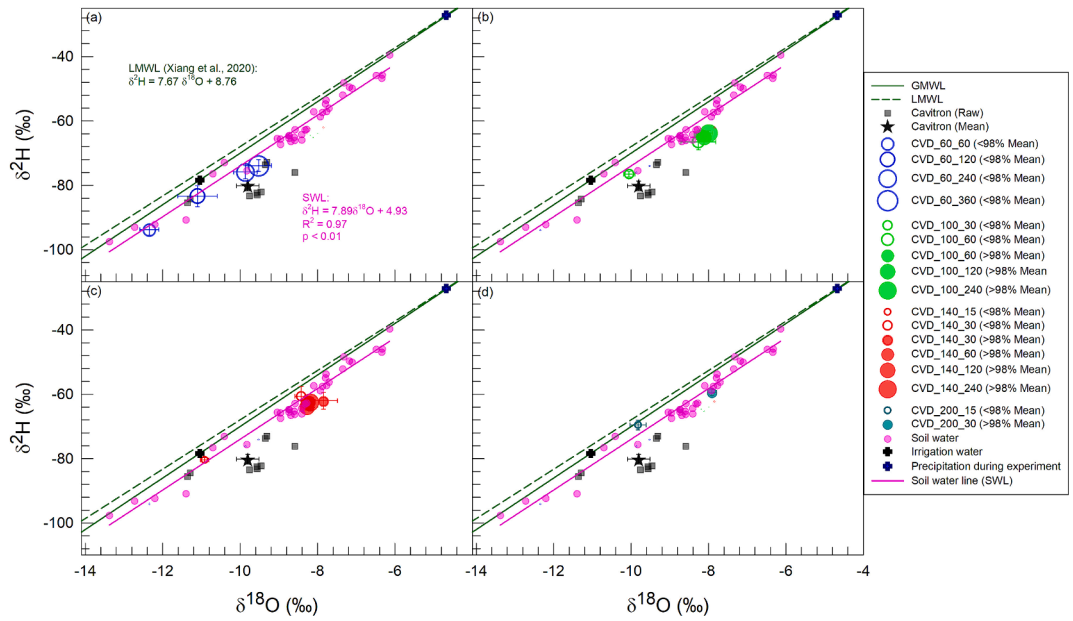

Fig. 2. The dual-isotope plot of soil water (pink circles), irrigation (black cross), precipitation (blue cross), and xylem water extracted by Cavitron (black) and cryogenic vacuum distillation with different protocols (CVD_Temperature_Time). Squares indicates the raw data, and circles represented the average ± standard error. Hollow symbols indicated the total extraction efficiency was below 98 % and solid symbols represents the total extraction efficiency was above 98 % for CVD. Colors indicates extraction temperatures (blue for 60 °C, green for 100 °C, red for 140 °C, and cyan for 200 °C), and sizes showed extraction time (the size increased with the increasing time). The solid green line indicates the Global Meteoric Water Line (GMWL), the dashed green line indicates the Local Meteoric Water Line (LMWL), and the pink line indicates the soil water line (SWL). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from –70.3 ‰ to –55.5 ‰ for δ^2H and from –9.1 ‰ to –6.9 ‰ for $\delta^{18}O$. With total extraction efficiency exceeding 98 %, longer extraction time did not yield an isotopic difference compared to shorter times at the same temperature (Fig. 3). However, after combining the data from different extraction times at the same temperature, we found that low extraction temperatures were significantly more depleted in deuterium ($p < 0.05$) than those resulting at higher extraction temperatures with a mean δ^2H value of –64.4 ‰, −62.7 ‰, and –59.3 ‰ for 100 °C, 140 °C,

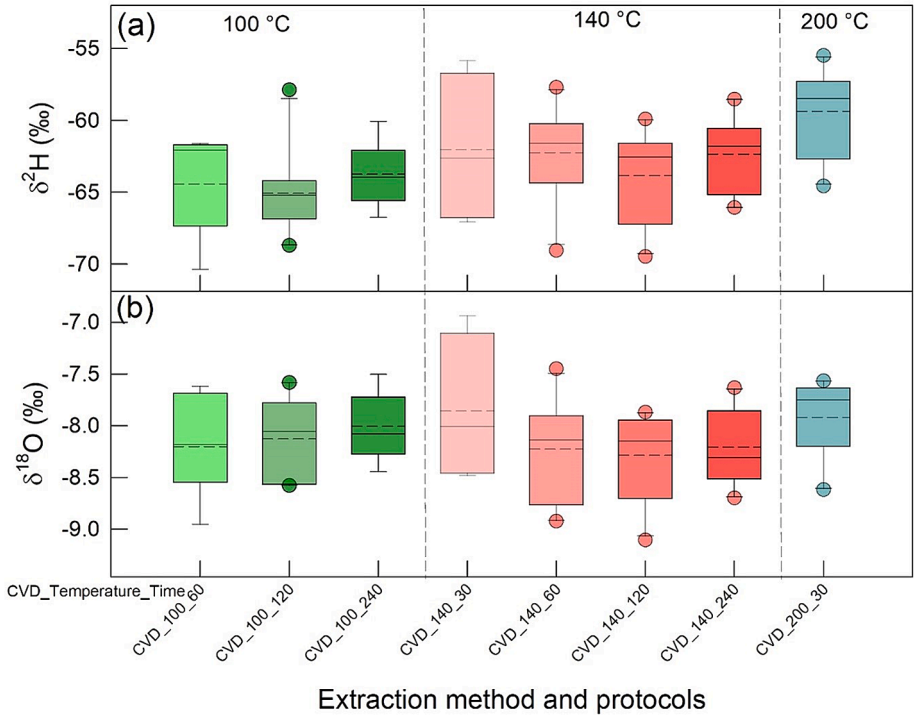

Fig. 3. Box plots for δ^2H (a) and $\delta^{18}O$ (b) of xylem water extracted by cryogenic vacuum distillation using different protocols (CVD_Temperature_Time) with all samples having total extraction efficiency above 98 %. Green for 100 °C, red for 140 °C, and cyan for 200 °C. The dashed lines in the box indicated the mean value; the solid lines in the box indicated the medium value. There was no significant isotopic difference between the different protocols ($p > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and 200 °C, respectively (Fig. 4). Nevertheless, this trend was not observed for $\delta^{18}O$ with a mean value of −8.1 ‰, −8.2 ‰, and −7.9 ‰ for 100 °C, 140 °C, and 200 °C, respectively.

There was a significant linear relationship between $\delta^{2}H$ of CVD-extracted water and total extraction efficiency at each extraction temperature ($p < 0.01$) (Fig. 5). The slope of the regression line decreased with the increase in extraction temperature and was 0.57, 0.53, 0.51, and 0.37 for 60 °C, 100 °C, 140 °C, and 200 °C extraction temperature, respectively. In contrast, the intercept of the regression line increased with increasing extraction temperature; the intercept was −126.87, –116.90. –113.01, and –97.38 for 60 °C, 100 °C, 140 °C, and 200 °C extraction temperature, respectively. $\delta^{18}O$ of CVD-extracted water exhibited a similar pattern with $\delta^{2}H$.

3.3. Comparison between Cavitron-xylem water and CVD-xylem water

The total amount of CVD-extracted xylem water sample varied from 1.15 g to 2.67 g, with an average of 1.99 g. The relative water content of CVD-xylem sample varied from 44.8 % to 62.3 %, averaging 51.7 %. The total amount of xylem water obtained with Cavitron was 0.4 g. On average, Cavitron-xylem water was more depleted in heavy isotopes when compared to CVD-xylem water, with total extraction efficiency > 98 % (Fig. 1). Cavitron overlapped with xylem water extracted by CVD with extraction efficiency < 98 %. Cavitron-xylem $\delta^{2}H$ and $\delta^{18}O$ were statistically distinct from CVD-xylem water extracted (Fig. 1) at 100 °C with extraction time > 60 min, and also from 140 °C and 200 °C extraction temperatures with extraction time > 15 min ($p < 0.05$); but Cavitron-xylem water was statistically similar to CVD-xylem water extracted at 60 °C for all extraction times, 100 °C for 30 min extraction time, 140 °C for 15 min extraction time, and 200 °C for 15 min extraction time. In dual-isotope space, the mean Cavitron-xylem water plotted below the LMWL, soil water line, and CVD-xylem water (Fig. 2); although, some cavitron samples plotted within the soil water distribution. Furthermore, mean Cavitron-xylem water showed significant differences from the source water (Fig. 6), i.e., the offset from the soil water line was significantly different from zero ($p < 0.05$), with the extraction efficiency above 98 %, the isotopic values of CVD-xylem water were closer to that of source water with the increasing extraction temperatures and showed no difference from the source water at 200 °C ($p > 0.05$).

4. Discussion

4.1. Influence of CVD protocols on xylem water isotopic composition

Our results revealed that isotope values of CVD-xylem water obtained by using protocols that provided high extraction efficiency (>98 %) were statistically higher than those that provided low extraction efficiency (<98 %) ($p < 0.05$). At the same temperature, longer extraction time yielded more positive isotopic values with increasing extraction efficiency. Once extraction efficiency reached 98 %, there was no isotopic difference with longer extraction times ($p > 0.05$). With extraction efficiency > 98 %, lower extraction temperature resulted in deuterium depletion when compared to higher temperature protocols ($p < 0.05$), but not for $\delta^{18}O$. Thus, studies using different extraction temperatures and times in CVD (e.g., Table S1) are comparable for $\delta^{18}O$ when extraction efficiency is > 98 %. But studies that use lower extraction temperatures (e.g., the “glass and manifold CVD system” and its derivatives) might show generally lower $\delta^{2}H$ values. Note that not all studies in the literature report extraction efficiency. In some studies, extracted samples were pre- and post-extraction weighted, but the adopted extraction efficiency was not always reported.

Our observations for xylem water are similar to what Araguás-Araguás et al. (1995) first reported for sandy soils, where extraction efficiency < 98 % resulted in extracted water more depleted in heavy isotopes, and those were statistically different from water obtained with extraction efficiency > 98 %. This result indicates that xylem water extraction follows the Rayleigh distillation process (Gat 1996), where the extracted water is depleted in heavy isotopes compared to the remaining bulk water, and the isotopic difference between extracted water and bulk water decreases with the increasing percentage of extracted water (with increasing extraction efficiency).

During Rayleigh distillation process, another key factor is temperature, which controls isotopic fractionation (Gat 1996). We also observed temperature differences in the slopes and intercepts of regression lines between extraction efficiency and isotopic composition. Higher temperatures produced a smaller isotopic difference of extracted water from bulk water. Therefore, under the same extraction efficiency, lower temperature yielded higher depletion in heavy isotopes than higher temperature extraction protocols. During the cryogenic extraction of xylem samples, an additional process takes place beyond Rayleigh distillation that could explain the isotopic observations, i.e., deuterium depletion with lower temperatures compared to higher temperatures with extraction efficiency > 98 %. In xylem tissues, there is a large quantity of exchangeable hydrogen on the hydroxyl groups of wood

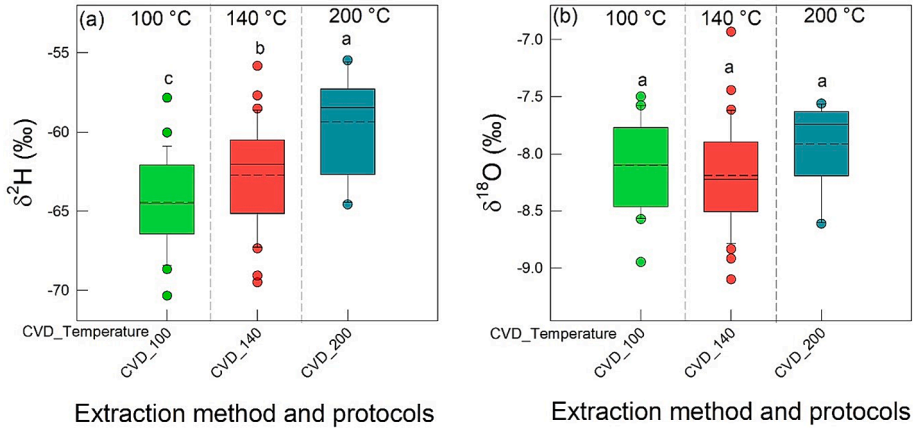

Fig. 4. Box plots for $\delta^{2}H$ (a) and $\delta^{18}O$ (b) of xylem water extracted by cryogenic vacuum distillation at different temperatures (CVD_Temperature), with all samples having total extraction efficiency above 98 %. Green for 100 °C, red for 140 °C, and cyan for 200 °C. The dashed lines in the box indicated the mean value; the solid lines in the box indicated the medium value; different letters on the top of the boxes indicated significant differences between groups ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

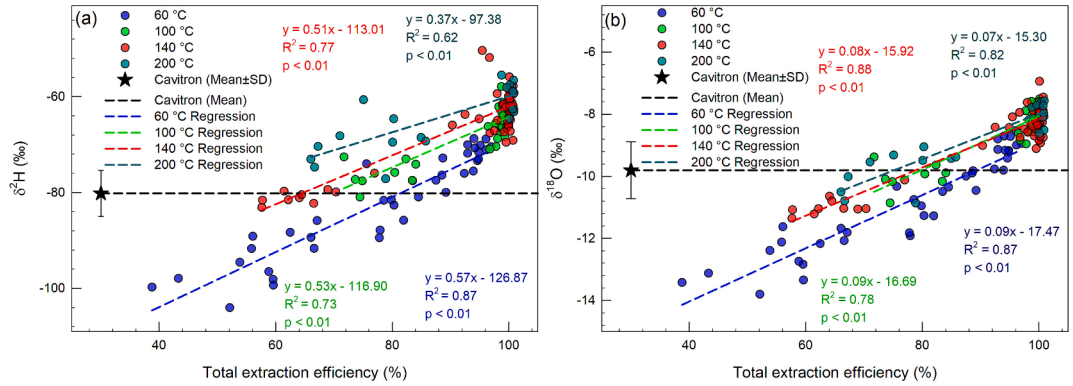

Fig. 5. The correlation of δ^2H (a) and $\delta^{18}O$ (b) of xylem water extracted by cryogenic vacuum distillation (CVD) with total extraction efficiency and isotopic comparison between Cavitron-extracted xylem water and CVD-extracted xylem water. The extraction efficiency of Cavitron is ~ 2 %. For easier plotting, we added the Cavitron at location on the plot having 30 % total extraction efficiency.

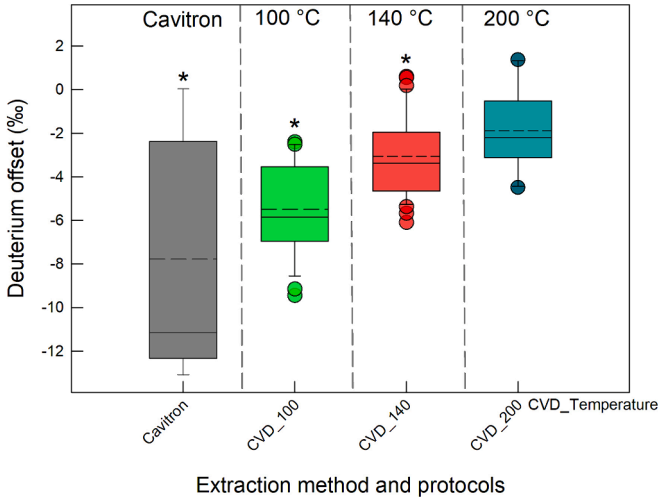

Fig. 6. Box plots for deuterium offset of xylem water extracted by Cavitron and cryogenic vacuum distillation at different temperatures (CVD_Temperature), with all samples having total extraction efficiency above 98 %. Green for 100 °C, red for 140 °C, and cyan for 200 °C. The dashed lines in the box indicated the mean value; the solid lines in the box indicated the medium value; stars on the top of the boxes indicated significant differences from zero ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cellulose and hemicellulose (Feng et al., 1993; Pettersen, 1984), which can rapidly exchange with xylem water during extraction (Frilette et al., 1948). During the extraction, water with lighter isotopes preferentially evaporates from the sample, and water with heavier isotopes stays behind in the sample. The exchangeable hydrogen actively equilibrates with water remaining in the xylem. Therefore, at the end of extraction, xylem tissues contain more heavy hydrogen compared to pre-extraction xylem tissues. According to isotope mass balance, the extracted xylem water would be more depleted in deuterium than the "real" xylem water. The heavy isotope depletion effect can be reduced by increasing the extraction temperature (Younger et al., 2024). With higher extraction temperature, the isotopic difference between evaporated water and remaining water becomes smaller (Horita and Wesolowski 1994) and eventually decreasing the isotopic difference between xylem tissue and extracted xylem water. Higher temperature led to an enrichment of heavy isotopes in extracted water from clayey soils (Araguás-Araguás et al., 1995) and wheat samples (Millar et al., 2018). Higher temperature extraction may co-extract volatile organic compounds from plant samples (West et al., 2010), which affects the subsequent isotopic analysis, especially for isotope ratio infrared spectrometry (Brand et al., 2009; Cui et al., 2021; Martín-Gómez et al., 2015). However, in our study, isotope ratio mass spectrometry was employed. The possibility of organic contamination producing the positive isotopic values for higher temperatures is small. Generally, it is believed that oxygen on hydroxyl sites in xylem tissue does not exchange with oxygen in water (Cohn and Urey 1938), which our results support as there was no $\delta^{18}O$ difference among various extraction temperatures with extraction efficiency > 98 %.

Recently, several studies reported deuterium depletion in xylem water caused by CVD extraction (Chen et al., 2020; Wen et al., 2022) and acknowledged the water amount issue during CVD extraction (Diao et al., 2022). For the water amount issue, the xylem water amount in our study was always above 1.15 mL, which is higher than the threshold of 0.6 mL indicated by Diao et al. (2022). Therefore, our results were not affected by the water amount issue. Moreover, the studies reporting a deuterium depletion employed CVD systems using low extraction temperatures, ranging from 60 °C to 105 °C (Chen et al., 2020; Barbeta et al., 2019; Wen et al., 2023; He et al., 2023). Based on our data, the deuterium depletion caused by CVD may be due to employing lower temperature protocols. Employing higher temperatures for CVD could decrease the δ^2H bias and make the δ^2H within acceptable ranges. Recent research showed that δ^2H bias can be eliminated by increasing CVD temperature to values near 170 °C and potentially completely eliminated when increasing it to 229 °C (Younger et al., 2024).

4.2. CVD-xylem water differs from Cavitron-xylem water

Cavitron has been proposed as an alternative to using CVD systems when extracting plant water for water source investigations (Barbeta et al., 2022; He et al., 2023; Wen et al., 2023). While we do not disagree that Cavitron is a useful approach, the reality is that many studies might still continue to use CVD given its accessibility. And perhaps with some adjustment, CVD could indeed be used effectively going forward with revised protocols for extraction. Our data showed that Cavitron-xylem water was more depleted in heavy isotopes than CVD-xylem water with > 98 % total extraction efficiency. But, Cavitron-xylem water was statistically similar to all CVD-xylem water with < 98 % total extraction efficiency. Cavitron-xylem water showed similar δ^2H values to CVD that followed 60 °C for 240 min and 100 °C for 30 min protocols. CVD-xylem water was slightly more depleted in deuterium than Cavitron-xylem water in two scenarios only, with extraction temperature of 60 °C and extraction time of 60 min and 120 min. Compared with Cavitron-xylem water, the CVD-xylem water extracted at 200 °C with extraction efficiency > 98 % was closer to the source water (i.e., soil water line). Our results differ from all previous Cavitron studies, i.e., Barbeta et al. (2022), Duvert et al. (2024), Wen et al. (2023), and He et al. (2023). Their studies showed CVD-xylem water was more depleted in deuterium

than Cavitron-xylem water. While their Cavitron system and its applied protocol are similar to our studies, their reported CVD protocols used lower extraction temperatures. Their employed protocols were: 80 °C for 150 min, 100 °C for 90 min, 100 °C for 120 min, and 105 °C for 180 min for Barbeta et al. (2022), Duvert et al. (2024), Wen et al. (2023), and He et al. (2023), respectively. Barbeta et al. (2022) and Duvert et al. (2024) used a cryogenic system described by Orlowski et al. (2013), while other investigators have used the capillary and vial system of Koeniger et al. (2011). Barbeta et al. (2022) and Duvert et al. (2024) did not report extraction efficiency, but Wen et al. (2023) and He et al. (2023) used 98 % extraction efficiency. As their reported $\delta^{18}O$ was similar between Cavitron-xylem water and CVD-xylem water, we speculate that the deuterium depletion was not caused by lower extraction efficiency but by the lower temperature used by the earlier studies.

Moreover, in our study, Cavitron-xylem water was not only different from CVD-xylem water in $\delta^{2}H$ but also in $\delta^{18}O$. In dual-isotope space, our Cavitron-xylem water was located below the CVD-xylem water with total extraction efficiency above 98 %. Cavitron and CVD exhibit notable differences in their water extraction approach. Because of the methodological design employed during Cavitron, both the outer and inner bark are maintained in the branch sample (Barbeta et al., 2022; Wen et al., 2023) to avoid evaporation during centrifugation. There is potential extraction of water from elastic tissues, such as the phloem. This phenomenon can be understood through two distinct mechanisms. First, the xylem and inner bark are radially connected through parenchyma rays and during extraction when negative pressure is applied through centrifugation, phloem water can be released into the xylem, mimicking the transpiration process with the release of water of elastic storage at lower xylem water potentials (Du et al., 2019; Pfautsch et al., 2015; Venturas et al., 2017) as the potentials become extremely low (–6 MPa) during Cavitron centrifugation. Second, the phloem is exposed on the ends of the branch with the transversal cut and water can be easily extracted along with xylem water since the sieve cells are exposed. Recent studies have shown that phloem water can be significantly more depleted in deuterium than xylem water (Nehemy et al., 2022a; Treydte et al., 2021), but not always (Nehemy et al., 2022a).

Our offset of Cavitron-xylem water from the source water (i.e., soil water line) is at odds with the published Cavitron-related papers, i.e., Barbeta et al. (2022), Duvert et al. (2024), Wen et al. (2022), Wen et al. (2023), and He et al. (2023). There are a few reasons that could explain this. One reason could be the definition of the true source, such as irrigation water signatures or bulk soil water lines used in previous studies. While we heavily irrigated the tree and sampled xylem water after estimating its travel time, xylem signatures did not reflect this new source. Thus, we used the bulk soil water line as others (De la Casa et al., 2022; Duvert et al., 2024; He et al., 2023). However, this soil water line isotopic signatures can also be impacted by its own extraction protocols (e.g., temperature). Another possibility is the time of sampling that might impact xylem water isotopic signatures. He et al. (2023) did their experiments on the same field site as us, and we followed the same operational procedures. The main difference is the sampling time, i.e., after sunset in our study, and in the afternoon for He et al (2023). Sampling the branches after sunset reduced the risk of evaporation during the highly evaporative time of day; however, in the evening, the reduced transpiration rates and trees refilling of internal water storages could have influenced xylem water isotopic composition due to the potential internal fractionation process during phloem refilling (Nehemy et al., 2022a). The difference could also be a result of other physiological processes linked to bark conductance that might lead to evaporative enrichment depending on the conditions (i.e. relative humidity and temperature) related to the time of the day sampled. We acknowledge that there might be other reasons for these differences as well. Clearly, more research is needed to tease out these potential effects.

In total, the $\delta^{2}H$ discrepancy when comparing CVD and Cavitron-xylem water observed in this study and others (Barbeta et al., 2022; Wen et al., 2023; He et al., 2023) might be due to an effect of lower extraction temperature used in CVD in previous studies and, the potential influence of phloem water during the extraction that could be more depleted (Nehemy et al., 2022a; Treydte et al., 2021) or more enriched (Cernusak et al., 2005) in heavy isotopes than xylem water, and the different sampling times that might influence other physiological processes. We understand that some laboratories are currently updating the Cavitron protocol to decrease the extension of the bark and phloem covering the branch prior to extraction and several papers are being prepared. However, the results reported in the literature still reflect the previous practice of using the branch with bark and phloem. Clearly, an updated Cavitron protocol with sampling time, comparison between with and without the bark and phloem, and supporting data is urgently needed. Further investigation is also needed to understand this across different species and wetness conditions.

4.3. Limitations and future research

We acknowledge that our results are limited to only one species and single sampling period, but the investigation of the use of distinct CVD protocols and comparison with different methods like Cavitron is crucial given the varieties of protocols present in the literature and the urgent need for standardization (Millar et al., 2022). Furthermore, xylem water from other plant species should be tested using CVD protocols and Cavitron systems, especially those growing in environments where the deuterium fractionation is known to occur (Ellsworth and Williams 2007; Lin et al., 1993; Poca et al., 2019), such as xerophytes and halophytes. Future research should also compare the same species under distinct soil texture, water content, and salt stress conditions.

5. Conclusion

Cryogenic vacuum distillation (CVD) was used to extract plant water under different extraction protocols (i.e., temperature and time) to determine these effects on stable isotope values of the extracted water. The isotopic compositions of CVD-xylem water were intercompared and compared with Cavitron-xylem water. Our results showed that Cavitron-xylem water was close to CVD-xylem water with total extraction efficiency below 98 % but was more depleted in heavy isotopes than CVD-xylem water with total extraction efficiency above 98 %. With total extraction efficiency above 98 %, isotopic compositions of CVD-xylem water extracted using different protocols are similar but higher extraction temperatures yielded more positive $\delta^{2}H$. Cross-comparisons of the Cavitron- and CVD-xylem water at different temperatures for more plant organs, tree species, and water-stress conditions are strongly suggested for future research to determine the robustness of CVD extractions for plant water in future studies.

CRediT authorship contribution statement

Hongxiu Wang: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Haiyang Yu:** Methodology, Investigation. **Dong He:** Methodology, Investigation. **Min Li:** Writing – review & editing, Funding acquisition. **Bingcheng Si:** Writing – review & editing. **Jeffrey J. McDonnell:** Writing – review & editing. **Magali F. Nehemy:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhydrol.2024.131853.

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