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Identifying the underpinnings of δ²H discrepancies between plant stem and soil water: extraction-induced methodological artifacts *versus* biological fractionation effects

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Abstract. Recent studies have reported widespread presence of hydrogen isotope offset (HIO) between cryogenically-extracted plant stem and soil water, challenging the long-standing assumption that the isotopic composition of stem xylem water reliably represents that of its source water. Despite intensive researches on this topic over the past decade, it remains debated as to whether and/or to what extent HIO originates from extraction-related artifacts or from *in situ* isotope mixing/fractionation during water transport from soil to plants. Here, we used cryogenic vacuum distillation (CVD) to extract stem and soil water from eight species (trees, shrubs, and grasses) grown under two humidity regimes. We quantified species-specific HIO, tested its associations with ecophysiological and environmental variables, and conducted immersion-based rehydration experiments to assess CVD-induced biases. Across species, HIO ranged from −7.2‰ to 3.2‰: trees were consistently negative, whereas shrubs and grasses were near-zero to slightly positive. Rehydration experiments revealed CVD-induced δ²H biases in stem (−4.5‰) and soil water (−2.5‰). When these extraction-related biases in both stem and soil water were simultaneously corrected, species-level HIO (mean = 0.2‰) was no longer different from zero, and showed no significant correlations with ecophysiological or environmental variables. These results suggest that apparent HIO is largely driven by CVD-induced artifacts rather than ecophysiological/environmental processes that cause isotopic fractionation during water transport along the soil-xylem continuum. We conclude that simultaneously correcting CVD-induced biases in both stem and soil water is critical to avoid spurious HIO signals and to improve isotope-based estimation of plant water sources.

1 Introduction

Plant water uptake from soil is a fundamental process within the soil-plant-atmosphere continuum (Bachofen et al., 2024; Philip, 1966). Analyses of the stable hydrogen and oxygen isotope compositions (δ^2H and δ^{18}O) of plant source water provide a powerful means to infer about plant water uptake dynamics, and hence are widely used to elucidate plant water use strategies (Dai et al., 2023; Dawson and Ehleringer, 1991; Rothfuss and Javaux, 2017), partition ecosystem water fluxes (Good et al., 2015; Moreira et al., 1997; Xiao et al., 2018), and assess plant ecophysiological responses to climatic change (Gessler et al., 2022; Ren et al., 2025; Roden et al., 2000). A central assumption underlying these applications is that the isotopic composition of plant xylem water, most commonly inferred from stem water extracted via cryogenic vacuum distillation (CVD), faithfully reflects that of the plant source water, i.e. soil water (Ehleringer and Dawson, 1992; Zhao et al., 2024a).

However, this assumption has been increasingly challenged by observations of apparent hydrogen isotope offset (HIO) between cryogenically-extracted stem and soil water (de la Casa et al., 2022; Wang et al., 2025). Early reports of HIO were largely restricted to halophytic or xerophytic species (Ellsworth and Williams, 2007; Lin and Sternberg, 1993; Poca et al., 2019). More recent studies, however, have demonstrated under both field and controlled experimental settings that HIO occurs across a wide range of ecosystems, plant functional types, soil textures, and moisture conditions (Barbeta et al., 2020, 2022; Chen et al., 2020; Jiang et al., 2022; Newberry et al., 2017a; Poca et al., 2019; Vargas et al., 2017; Wang et al., 2025; Zhao et al., 2024a). For example, under controlled pot conditions, Barbeta et al. (2020) observed HIO values ranging from −11.4‰ to 15.8‰ in *Fagus sylvatica*. Similarly, in a field study of 12 species (trees, shrubs, and herbs) in the Heihe River Basin, Zhao et al. (2024a) documented a broad spectrum HIO that also spanned negative, near-zero, and positive values. More recently, a global meta-analysis by de la Casa et al. (2022) further highlighted the ubiquity of HIO, reporting values ranging from −44‰ to 36‰ across diverse plant traits and environmental conditions.

To date, multiple hypotheses have been proposed to explain the observed HIO. Early interpretations suggested that HIO might arise from *in situ*, ecophysiology-mediated isotope fractionation during soil-to-plant water transport. Proposed mechanisms include δ^2H kinetic fractionation during root water uptake, particularly under salinity or drought stress (Ellsworth and Williams, 2007; Lin and Sternberg, 1993), as well as potential influences of mycorrhizal associations (Poca et al., 2019). Nonetheless, recent advances in isotopic measurement techniques have enabled targeted analysis of xylem water, providing compelling evidence that root water uptake generally proceeds without measurable isotopic fractionation, thereby challenging, – or effectively invalidating, – these early explanations (Barbeta et al., 2022; Chen et al., 2020; Zhao et al., 2016). Consequently, several alternative mechanisms involving ecophysiologically-driven isotope mixing/fractionation processes have been put forward. One hypothesis suggests that plants may directly take up condensed vapor from soil pores, which is typically isotopically depleted and could therefore contribute to HIO (Vargas et al., 2017). Another posits that HIO arises from cellular metabolism-induced redistribution of deuterium signatures between xylem and non-xylem tissues within plant stems, resulting

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in alteration of the original source water signature preserved in the xylem water (Barbeta et al., 2020, 2022; Nehemy et al., 2022; Zhao et al., 2016). Beyond ecophysiological mechanisms, several recent studies have also examined the role of environmental drivers in modulating HIO. In this regard, hydroclimatic factors – including temperature, relative humidity, mean annual precipitation, soil water content, and net radiation – have been shown to provide plausible explanations for the magnitude and variability of HIO observed across species and ecosystems (Barbeta et al., 2020; de la Casa et al., 2022; Vargas et al., 2017; Wang et al., 2025, 2026; Zhang et al., 2026; Zhao et al., 2022, 2024a). Importantly, it has been suggested that these environmental effects are likely indirect, reflecting their roles in regulating plant ecophysiological processes, – such as transpiration and/or internal water mixing, – that may ultimately give rise to HIO (Barbeta et al., 2020; Vargas et al., 2017; Zhang et al., 2026; Zhao et al., 2024a).

The past decade has also witnessed intensive research efforts to understand methodological artifacts associated with CVD, which represent another potential mechanism for explaining HIO, as HIO, – after all –, is most often defined as δ^2H difference between CVD-extracted stem and soil water, as previously noted. On one hand, evidence has accumulated that CVD can introduce substantial biases in soil water isotope analyses, with reported effects linked to soil texture, water content, surface effects, extraction efficiency and extraction protocols (Chen et al., 2016; Gaj et al., 2017; Millar et al., 2018; Orlowski et al., 2016; Wen et al., 2021; Zhao et al., 2024b). In a global inter-laboratory comparison involving 16 laboratories, identical soil samples subjected to CVD yielded δ^2H biases ranging from −108.4‰ to 18.1‰, highlighting the urgent need to account for such methodological artifacts in soil water isotope analyses (Orlowski et al., 2018). On the other hand, comparable concerns have been raised for stem water extraction. Using a novel steady-state transpiration method as the benchmark, Chen et al. (2020) demonstrated significant CVD-caused δ^2H biases in stem water across nine plant species. Subsequent studies have confirmed the presence of such artifacts under rehydration experiments (Diao et al., 2022; Li et al., 2024; Wen et al., 2026), pot experiments (Jiang et al., 2022), and field trials (Duvert et al., 2024; He et al., 2023). The magnitude of these stem water biases has been shown to depend on stem relative water content (RWC), stem absolute water volume (AWA), and even the isotopic composition of the stem water itself (Chen et al., 2020; Diao et al., 2022; Wen et al., 2022). Collectively, these findings indicate that both soil and stem water measurements are vulnerable to CVD-induced artifacts, which may substantially contribute to the widespread occurrence of HIO.

Thus far, several studies have attempted to assessed the relative contributions of CVD-related artifacts and ecophysiology/environmental factors to the common presence of stem-soil water HIO (Barbeta et al., 2022; Chen et al., 2020; Jiang et al., 2022; Wen et al., 2022). On the one hand, researchers have actively explored alternative methods, such as centrifugation and direct vapor equilibration , etc, to more robustly determine the isotopic composition of stem xylem and soil water and thereby circumvent CVD-related artifacts, such that ecophysiological and/or environmental controls (if any) on HIO can be isolated (Barbeta et al., 2022; Gessler et al., 2022; Kühnhammer et al., 2023; Song & Barbour, 2016; Volkmann et al., 2016; Zhao et al., 2016). However, these alternative approaches have inherent limitations and, at present, are unable to fully

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replace CVD as a broadly applicable extraction technique (de la Casa et al., 2022; Wen et al., 2026). On the other hand, an increasing number of studies have relied on rehydration experiments to explicitly investigate CVD-related issues in stem and soil water isotope analyses (Diao et al., 2022; Millar et al., 2018; Newberry et al., 2017a; Wen et al., 2021, 2026). In this context, it has become increasingly clear that a comprehensive understanding of CVD-induced biases represents a necessary prerequisite for determining whether observed HIO reflects true ecophysiological and/or environmental signals or methodological artifacts (Allen and Kirchner, 2022; Jiang et al., 2022). Nevertheless, existing studies of CVD bias have relied almost exclusively on spiking-type rehydration, in which only limited amounts of water are added to dried stem and/or soil samples; but this approach that has recently been questioned because it may induce artifactual isotopic fractionation during rehydration itself (Diao et al., 2022; Newberry et al., 2017b; Wen et al., 2026). Moreover, to date, only a limited number of studies (e.g. Jiang et al., 2022) have attempted to simultaneously account for CVD-induced artifacts in both stem and soil water. As a result, the dominant source of stem-soil water HIO – whether arising from CVD-related artifacts or from ecophysiological and/or environmental processes – remains unresolved.

Here, we conducted a controlled, multi-species experiment designed to specially address the question of whether stem-soil HIO is driven primarily by CVD-related artifacts or by true ecophysiological/environmental processes that cause isotopic fractionation during water transport from the soil to the plant. For that purpose, eight plant species representing trees, shrubs, and grasses were grown under two humidity regimes. We first quantified species-specific HIO under each humidity treatment. We then used an immersion-based rehydration protocol to empirically estimate CVD-induced isotopic biases in both stem and soil samples, and evaluated three correction strategies – stem water-only, soil water-only, and dual correction – to assess how each approaches affects HIO estimates. Finally, we tested whether HIO values before and after correction were associated with measured ecophysiological and environmental factors. Together, these analyses aim to identify the dominant sources of HIO between cryogenically-extracted stem and soil water and to improve isotope-based inference of plant water sources, thereby strengthening ecohydrological and plant water use interpretations.

2 Materials and methods

2.1 Plant material and growing conditions

A total of eight plant species, including four tree species, two shrub species, and two grass species (Table 1), were included in this study. The tree species consisted of 2- to 3-year-old individuals, whereas the shrub and grass species were grown from seed. All plants were cultivated in 10 L plastic pots filled with a homogeneous mixture of soil and organic matter under controlled conditions. Plants were grown under a transparent Plexiglas shelter that prevented rainfall while allowing transmission of ambient light. Each pot was irrigated daily with *c.* 500 mL of local tap water, and excess water was allowed to freely drain from the bottom to maintain soil moisture close to field capacity. To minimize soil evaporation, the surface of each pot was covered with aluminum foil. In addition, a Hoagland nutrient solution, prepared with the same tap water, was applied weekly to ensure adequate nutrient supply (Pan et al., 2024). After two months of growth and acclimation, eight pots per species were selected, with four pots randomly assigned to each of two climate-controlled growth chambers set to different relative humidity (RH) levels (40% and 70%). Both chambers were maintained at a constant air temperature of 25°C under a 12 h light / 12 h dark photoperiod. During the light period (6:00 to 18:00), a photosynthetic photon flux density (PPFD) of 600 μmol m^{-2} s^{-1} was maintained at the leaf level. Plants remained in the chambers for seven days and continued to receive daily irrigation to full saturation using local tap water.

2.2 Gas exchange measurements

During the growth chamber treatments, leaf-level gas exchange was measured using a LI-6800 Portable Photosynthesis System (Li-Cor Inc., USA). Measurements were performed on the most recently matured, fully expanded leaves in the upper-middle canopy that were directly exposed to light. Gas exchange was recorded between 09:00 and 12:00, i.e. at least 3 h after the onset of chamber illumination, to ensure that leaves had reached a physiologically stable state. The LI-6800 was equipped with a 6 cm^2 fluorescence leaf chamber, and measurement conditions were standardized as follows: reference CO_2 concentration at 400 ppm, PPFD maintained at 600 μmol m^{-2} s^{-1}, flow rate of 500 μmol s^{-1}, and chamber air temperature of 25 °C. Chamber RH was adjusted to match the corresponding growth treatment (40% or 70%). Measurements were logged once gas exchange variables had stabilized, typically within 5-10 minutes after leaf clamping.

2.3 Sample collection

Destructive sampling was conducted on the final day of the humidity treatment to ensure that all physiological and isotopic measurements reflected steady-state conditions under the controlled environments. For trees and shrubs, stem segments (*c.* 3–4 cm long) were excised approximately 10 cm above the soil surface. After removing the bark, segments were immediately placed into 12 mL borosilicate glass vials. For grasses, basal non-photosynthetic tissues near the root-shoot junction were collected. In addition, recently fully expanded leaves were sampled from all species. Leaf blades were excised while avoiding major veins and immediately sealed in 12 mL vials. Soil samples were collected concurrently from the center portion of each corresponding pot, at a depth of approximately 15 cm. All glass vials containing samples were immediately sealed with Parafilm and stored at −20 °C until cryogenic vacuum extraction.

To determine stem tissue density (hereafter referred to as wood density), additional segments were collected from each individual (an 8 cm stem segment for trees and shrubs; sufficient basal non-photosynthetic shoot tissue for grasses). For trees and shrubs, bark was removed prior to processing. All segments were oven-dried at 65 °C to constant mass, and dry mass was recorded. The dried samples were then fully rehydrated by immersion in distilled water for 48 hours to ensure complete saturation. Volume was determined using the water displacement method, and wood density was calculated as the ratio of oven-dry mass to rehydrated volume (g cm^{-3}).

2.4 Rehydration experiment

To quantify isotopic artifacts potentially introduced during the CVD process, we performed immersion-based rehydration experiments for both plant stem and soil samples following the general approach described by Wen et al. 2026. In total, 28 stem samples and 25 soil samples were included. All samples were oven-dried at 65 °C to constant mass to ensure complete removal of pre-existing water. For plant stems, the dried tissues were fully immersed in > 60 L of reference water (local tap water) for 48 h to achieve rehydration. This duration allows the relative water content to recover to near-saturation levels, thereby facilitating isotopic equilibration between the plant tissues and the reference water. After immersion, residual surface water was carefully removed using lint-free tissue, and each sample was immediately sealed in 12 mL glass vials. For soils, the dried material was placed in filter bags prior to immersion to permit water exchange while preventing particle loss (Wen et al., 2026). The bagged soils were submerged in another batch of reference water for 48 h. After removal, the filter bags were gently squeezed to expel excess free water. This procedure yielded soil RWC comparable to that of the pot experiment. Soil samples were then transferred immediately into 12 mL glass vials. All vials were wrapped with Parafilm and stored at −20 °C until CVD extraction. Reference water was collected at the end of each 48-h rehydration period for isotope analysis, providing the baseline against which CVD-induced biases were evaluated.

2.5 Water extraction and isotope analysis

Water was extracted from plant tissue and soil samples using an automated cryogenic vacuum extraction system (LI-2100, LICA, Beijing, China). Samples were heated to 195 °C under near-vacuum for 2 h, allowing water vapor to be cryogenically trapped. Extracted water was then sealed in glass vials and stored at 4 °C until isotopic analysis. Each sample was weighed before and after extraction to determine (i) absolute water amount (AWA), defined as the mass of extracted water per sample, and (ii) relative water content (RWC), defined as the ratio of extracted water mass to fresh sample mass.

Hydrogen and oxygen isotope compositions of the extracted water were measured using a high-temperature conversion elemental analyzer (TC/EA) coupled to an isotope ratio mass spectrometer (Delta V Advantage; Thermo Fisher Scientific, Waltham, MA, USA). Isotope ratios are reported in per mil (‰) relative to the Vienna Standard Mean Ocean Water (VSMOW) scale. Analytical precision was better than ±1.0‰ for δ^2H and ±0.2‰ for δ^{18}O.

To quantify isotopic differences between CVD-execrated plant stem and soil water, we calculated hydrogen isotope offset (HIO) and oxygen isotope offset (OIO) as follows:

$$\mathrm{HIO} = \delta^2\mathrm{H}_{stem\ water} - \delta^2\mathrm{H}_{soil\ water} \tag{1}$$

$$\mathrm{OIO} = \delta^{18}\mathrm{O}_{stem\ water} - \delta^{18}\mathrm{O}_{soil\ water} \tag{2}$$

In addition, CVD-induced isotopic biases were quantified by comparing the isotopic composition of water extracted from rehydrated samples via CVD with that of the reference water:

$$\delta\ \mathrm{bias} = \delta_{rehydrated\ sample\ water} - \delta_{reference\ water} \tag{3}$$

2.6 Statistical analysis

All statistical analyses were performed in SPSS version 20.0 (IBM Corp., Armonk, NY, USA). One-sample *t*-tests were used to assess whether HIO and OIO differed significantly from zero. The effects of species identity and RH on HIO, OIO, and associated ecophysiological and environmental variables were evaluated using general linear models (GLMs), with species and RH treated as fixed factors. Because RH and the species × RH interaction were not significant for either HIO or OIO, data from the two RH treatments were pooled, and a one-way ANOVA was subsequently used to test for species-specific differences. For the rehydration experiments, one-sample t-tests were used to determine whether CVD-induced isotopic biases differed significantly from zero. To develop correction functions, linear regression models were fitted to relate CVD-induced biases and sample properties (RWC and AWA) for both stem and soil samples. Pearson correlation analyses were further used to examine relationships between HIO (before and after correction) and potential explanatory variables, including soil RWC, soil AWA, E, g_s, leaf water ^{18}O enrichment ($\Delta^{18}O_{lw}$), stem RWC, stem AWA, and wood density. Statistical significance was evaluated at three levels: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). Figures were produced using OriginPro 2025 (OriginLab Corp., Northampton, MA, USA).

3 Results

3.1 Variation in HIO across species and humidity treatments

Across all eight species and both humidity treatments, HIO displayed widely variability, spanning negative to positive values (Table 1; Fig. 1). Under 40% RH, tree species generally showed negative HIO, with *Quercus virginiana* exhibiting the strongest depletion (−6.4‰). In contrast, non-woody species showed near-zero to positive HIO, with the highest positive value observed in *Z. mays* (2.8‰). Under 70% RH, HIO also exhibited clear species-specific patterns, ranging from −7.9‰ to 3.6‰. Mean HIO were comparable between the two humidity levels (−1.8 ± 3.6‰ at 40% RH and −1.2 ± 4.4‰ at 70% RH), indicating limited influence of atmospheric humidity. GLMs confirmed that species identity had a highly significant effect on HIO ($P < 0.001$; Table 2), whereas RH showed no detectable influence ($P = 0.328$). Moreover, the species × RH interaction was not significant ($P = 0.233$), demonstrating that species-specific HIO patterns were consistent across humidity treatments. Thus, data from both RH treatments were combined for subsequent analysis. Functional group comparisons further revealed systematic differences: trees showed the most negative mean HIO (−4.4‰), whereas shrubs (0.6‰) and grasses (2.1‰) maintained near-zero to slightly positive values (Fig. 1), highlighting consistent variation across growth forms.

Table 1: HIO and OIO across species under two relative humidity (RH) treatments. The table lists plant species, plant functional types, and RH condition. HIO and OIO were calculated as the difference between the isotopic composition of stem water and that of soil water collected from the same pot (Eqns 1 and 2). No asterisk indicates no significant difference; *, **, and * denote significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.**

Acronym	Plant species	Type	40% RH				70% RH			
			HIO	SD	OIO	SD	HIO	SD	OIO	SD
QV	*Quercus virginiana* Mill.	Tree	**−6.4****	1.1	−0.8	0.7	**−7.9***	3.9	−0.8	0.6
PT	*Populus tomentosa* Carr.	Tree	**−5.3****	1.3	**−0.9***	0.3	−4.0	5.0	−0.5	0.4
ER	*Eucalyptus robusta* Smith	Tree	**−4.3***	1.7	−0.6	0.6	−1.6	2.5	−0.3	0.3
PE	*Pinus elliottii* Engelm.	Tree	−2.6	2.8	0.1	0.4	−2.6	2.3	−0.5	0.4
GH	*Gossypium hirsutum* L.	Shrub	−1.3	0.9	−0.04	0.6	0.8	3.2	−0.3	0.5
HA	*Helianthus annuus* L.	Shrub	0.1	2.0	−0.3	0.4	2.6	2.1	0.1	0.4
LP	*Lolium perenne* L.	Grass	**2.5***	1.1	0.03	0.3	−0.4	1.7	**−0.7 ***	0.3
ZM	*Zea mays* L.	Grass	2.8	2.0	0.04	0.8	**3.6****	0.5	**−0.5 ***	0.2

In contrast, OIO values were generally small and showed limited interspecific variation, with most measurements clustering around zero. The overall mean OIO (−0.4 ± 0.5‰) indicated only a slight isotopic difference between stem and source soil water. Although species identity had a modest effect, it was not statistically significant ($P = 0.064$, Table 2), and RH showed no detectable influence ($P = 0.466$). Similarly, the species × RH interaction was not significant for OIO ($P = 0.232$), indicating that variation in OIO was stable across humidity treatments. Furthermore, unlike HIO, OIO did not exhibit consistent patterns across plant functional types (Fig. 1b), suggesting that OIO is comparatively insensitive to species-specific traits or environmental conditions.

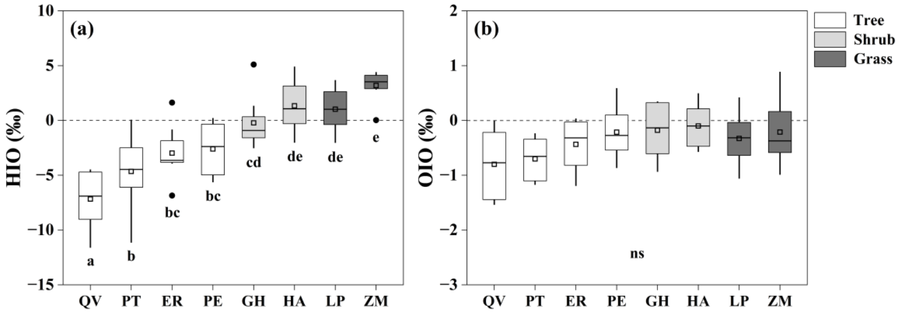

Figure 1: Boxplots of species-specific hydrogen isotope offset (HIO, a) and oxygen isotope offset (OIO, b) across different plant functional types. The acronyms QV, PT, ER, and PE represent tree species *Quercus virginiana*, *Populus tomentosa*, *Eucalyptus robusta*, *Pinus elliottii*, respectively. GH and HA denote the shrub species *Gossypium hirsutum* and *Helianthus annuus*, respectively. LP, and ZM indicate grass species *Lolium perenne* and *Zea mays*, respectively. The horizontal line within each box represents the median, and the square symbol denotes the mean. The vertical size of each box is the interquartile range (IQR) with the bottom and top edges marking the 25th and 75th percentile, respectively, and the whiskers extend to within 1.5 × IQR from each edge. Groups sharing the same letter below the box are not significantly different, while those with different letters are significantly different.

3.2 Variation in ecophysiological and environmental variables

General linear models (GLMs) revealed that the effects of species identity, RH, and their interaction varied across ecophysiological and environmental variables (Table 2). Species effects were significant for most traits, whereas RH effects and species × RH interactions were primarily observed for leaf-level variables. Soil water variables showed limited sensitivity to RH. Soil RWC ranged from 40.7% to 59.6%, with comparable mean values of 49.7±7.1% at 40% RH and 51.3±8.9% at 70% RH. Soil AWA varied within a narrow range (1.9 to 2.8 mL) and was not significantly affected by species or RH (Tables 2 and S1). In contrast, leaf ecophysiological traits exhibited pronounced variability and strong responses to both species and RH. E and g_s varied widely across species (E: 0.8–8.7 mmol m^{-2} s^{-1}, g_s: 0.08–1.20 mol m^{-2} s^{-1}) and were both significantly influenced by RH and species × RH interactions (all $P < 0.001$; Table 2). $\Delta^{18}O_{lw}$ was also strongly affected by RH, with higher values under 40% RH (16.8 ± 3.7‰) than under 70% RH (9.0 ± 3.1‰). Stem-related traits were dominated by species differences rather than RH effects. Stem RWC ranging from 41.3% to 95.9%, stem AWA from 0.9 to 2.2 mL, and wood density from 0.06 to 0.57 g cm^{-3}across species (Table S1). GLMs confirmed significant species effects for these stem-related traits (all $P < 0.001$), whereas RH effects were generally weak or non-significant, with the exception of a modest species × RH interaction detected for wood density ($P = 0.034$).

Table 2: *P*-values from general linear models (GLMs) testing the effects of species, relative humidity, and their interaction on HIO, OIO, and measured ecophysiological and environmental variables.

Variable	Species	Relative humidity	Species × Relative humidity
HIO	**<0.001**	0.328	0.233
OIO	0.064	0.351	0.115
Soil RWC	**<0.001**	0.315	0.571
Soil AWA	0.113	0.485	0.480
E	0.051	**<0.001**	**<0.001**
g_s	**0.038**	**<0.001**	**<0.001**
$\Delta^{18}O_{lw}$	**<0.001**	**<0.001**	**<0.001**
Stem RWC	**<0.001**	0.898	0.099
Stem AWA	**<0.001**	0.620	0.058
Wood density	**<0.001**	0.082	**0.034**

3.3 Cryogenic vacuum distillation introduces δ^2H biases in both stem and soil water

To evaluate isotopic biases introduced during CVD extraction, we compared δ^2H and δ^{18}O of water extracted from rehydrated stem and soil samples via CVD with those of the reference water. A clear pattern was detected for δ^2H: rehydrated stem water showed a substantial negative bias (−4.5 ± 1.4‰), significantly different from zero ($P < 0.001$; Fig. S1a). Soil water δ^2H also exhibited a significant but smaller bias (−2.5 ± 0.9‰, $P < 0.001$). In contrast, δ^{18}O biases were minimal (Fig. S1b); stem water (0.1 ± 0.2‰) did not differ significantly from zero, whereas soil water showed a slight but significant enrichment (0.2 ± 0.2‰).

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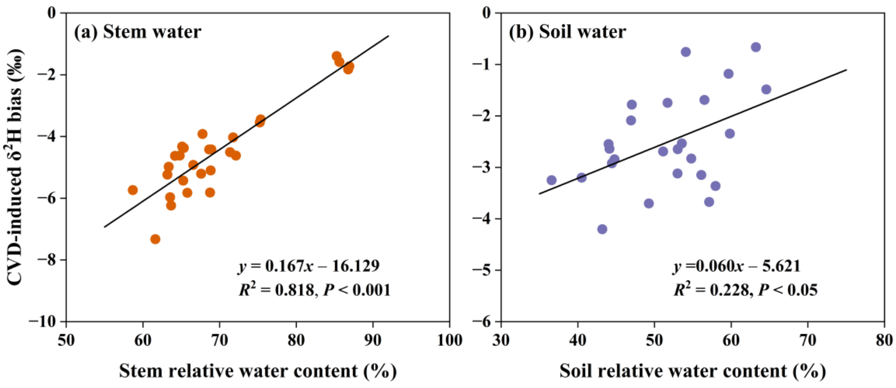

Figure 2: Relationship between cryogenic vacuum distillation-induced δ^2H bias and relative water content observed in the rehydration experiment for stem (a) and soil (b) samples. Solid line represents the best-fit linear regression.

CVD-induced δ^2H biases in stem water were strongly correlated with stem RWC ($R^2 = 0.818$, $P < 0.001$; Fig. 2a) but showed no significant relationship with stem AWA ($R^2 = 0.116$, $P = 0.076$; Fig. S2a). In soil water, a weaker yet statistically significant relationship was observed between CVD-induced δ^2H biases and soil RWC ($R^2 = 0.228$, $P < 0.05$; Fig. 2b), whereas no correlation was found with soil AWA ($R^2 = 0.028$, $P = 0.423$; Fig. S2b). Reflecting their much smaller overall magnitudes, CVD-induced δ^{18}O biases did not exhibit any detectable dependence on soil RWC or AWA (Fig. S3).

3.4 Effects of CVD-induced bias corrections on stem-soil water HIO

To evaluate how CVD-induced biases influence stem-soil water HIO, three correction strategies were applied: (1) CVD-bias correction for stem water δ^2H using the stem RWC-based regression (derived from Fig. 2a); (2) CVD-bias correction for soil water δ^2H using soil RWC-based regression (derived from Fig. 2b); and (3) dual bias correction, in which both stem and soil water δ^2H values were corrected simultaneously. Without correction, HIO values for five of the eight species differed significantly from zero (Fig. 3), indicating a measurable offset between stem and soil water δ^2H. Appling the stem-water bias correction alone increased HIO across all species, thereby reducing the magnitude of negative HIO in trees (i.e. shifting values toward zero) but causing HIO in non-woody species to deviate further from zero (Fig. 3, orange boxes). In contrast, applying the soil-water bias correction alone decreased HIO overall, leading to larger negative deviations from zero in trees while shifting HIO in non-woody species closer to zero (Fig. 3, purple boxes). Overall, single-pool bias corrections produced species-specific responses and did not consistently eliminate significant HIO deviations from zero across species. By comparison, under dual bias correction, HIO values for all species converged toward zero and were no longer significantly different from zero (Fig. 3, green boxes).

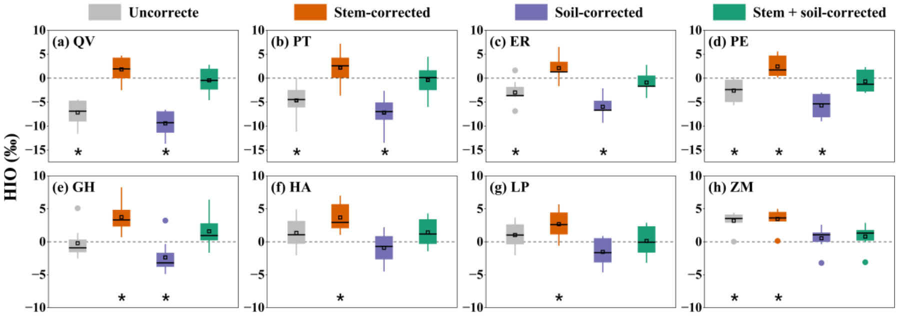

Figure 3: Effects of cryogenic vacuum distillation-induced bias corrections on stem-soil water hydrogen isotope offsets (HIO) across eight plant species. HIO values are shown for four scenarios: uncorrected values (grey), stem water δ^2H-corrected values based on stem RWC (orange), soil water δ^2H-corrected values based on soil RWC (purple), and values corrected simultaneously for both stem and soil water δ^2H (green). The acronyms QV, PT, ER, and PE represent tree species *Quercus virginiana*, *Populus tomentosa*, *Eucalyptus robusta*, *Pinus elliottii*, respectively. GH and HA denote the shrub species *Gossypium hirsutum* and *Helianthus annuus*, respectively. LP, and ZM indicate grass species *Lolium perenne* and *Zea mays*, respectively. The horizontal line within each box represents the median, and the square symbol denotes the mean. The vertical size of each box is the interquartile range (IQR) with the bottom and top edges marking the 25th and 75th percentile, respectively, and the whiskers extend to within 1.5 × IQR from each edge. Asterisks (*) denote HIO values that differ significantly from zero ($P < 0.05$).

3.5 Effects of CVD-bias correction on the relationship between HIO and environmental and physiological variables

Prior to correction, HIO showed no significant correlations with soil water status variables, including soil RWC and soil AWA, with correlation coefficients close to zero (r = 0.01 and −0.16, respectively; Fig. 4). In contrast, HIO showed significant correlations with several ecophysiological variables. Specifically, HIO was positively correlated with E ($r = 0.32$, $P < 0.01$) and g_s ($r = 0.28$, $P < 0.05$), and negatively correlated with $\Delta^{18}O_{lw}$ ($r = -0.34$, $P < 0.01$). In addition, HIO showed a strong positive correlation with stem RWC ($r = 0.81$, $P < 0.001$) and moderately positive correlation with stem AWA ($r = 0.42$, $P < 0.01$), while exhibiting a strong negative correlation with wood density ($r = -0.72$, $P < 0.001$).

After applying the dual correction for CVD-induced δ^2H bias in both stem water and soil water, HIO similarly showed no significant relationships with soil RWC or soil AWA (r = 0.23 and 0.00, respectively). Overall, CVD-bias correction substantially weakened the apparent relationships between HIO and ecophysiological variables (Fig. 4). Accordingly, corrected HIO showed no significant correlations with E (r =0.10), g_s (r =0.14), or $\Delta^{18}O_{lw}$ ($r = -0.17$), and associations with stem RWC ($r = 0.19$), stem AWA ($r = 0.10$), and wood density ($r = -0.20$) were markedly reduced. All correlations were non-significant ($P > 0.05$).

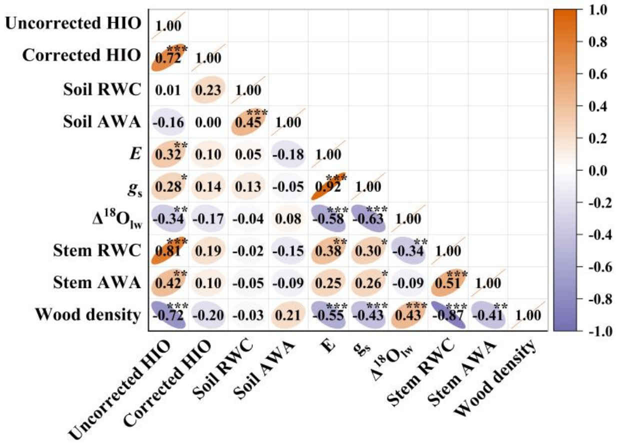

Figure 4: Correlation matrices summarizing relationships among uncorrected and dual-corrected hydrogen isotope offset (HIO) and measured ecophysiological and environmental variables. The variables include soil relative water content (Soil RWC), soil absolute water amount (Soil AWA), leaf transpiration rate (E), leaf stomatal conductance (g_s), leaf water oxygen isotope enrichment ($\Delta^{18}O_{lw}$), stem relative water content (Stem RWC), stem absolute water amount (Stem AWA), and wood density. Colors indicate the direction and magnitude of Pearson correlation coefficients (red = positive; blue = negative), while the shape and orientation of ellipses represent correlation strength. Numerical values show the Pearson correlation coefficients, and asterisks denote statistical significance (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

4 Discussion

4.1 Stem-soil water HIO and the influence of CVD-induced bias

Our controlled experiment showed that HIO between cryogenically-extracted stem and soil water did not differ across the two humidity treatments, yet varied markedly among species (Tables 1 and 2; Fig. 1). Notably, even under well-watered soil conditions (mean soil RWC > 40%), HIO values ranged from negative through near-zero to positive. This is consistent with results from controlled drought experiments on *Fagus sylvatica* (Barbeta et al., 2020) and with field observations spanning diverse species (Zhao et al., 2024a). Together, these lines of evidence suggest that HIO is neither confined to halophytic or xerophytic species (Ellsworth and Williams, 2007; Lin and Sternberg, 1993) nor limited to negative values, but instead represents a broadly observed phenomenon across functional types and environmental contexts (Barbeta et al., 2019, 2022; Chen et al., 2020; Jiang et al., 2022; Newberry et al., 2017a; Oerter and Bowen, 2017; Poca et al., 2019; Vargas et al., 2017; Wang et al., 2025; Zhao et al., 2016, 2024a).

In many cases, however, it remains unclear to what extent these widespread HIO patterns reflect true ecophysiological and/or environmental processes *versus* methodological artifacts associated with CVD itself (Barbeta et al., 2020, 2022; Chen et al., 2020; de la Casa et al., 2022; Jiang et al., 2022; Zhao et al., 2024a). To clarify this issue, we quantified the magnitude of CVD-induced isotopic biases using immersion-based rehydration experiments applied to both stem and soil samples, following established protocols from previous studies (Chen et al., 2020; Wen et al., 2026). Based on these rehydration experiments, we observed that CVD introduces substantial δ^{2}H biases in both rehydrated stem and soil samples (Figs. 2 and S1), consistent with a large body of earlier work (Chen et al., 2016, 2020; Duvert et al., 2024; Gaj et al., 2017; He et al., 2023; Li et al., 2024; Millar et al., 2018; Orlowski et al., 2016; Wen et al., 2021, 2022; Zhao et al., 2024b). Such evidence indicates that the observed HIO is, at least in part, affected by CVD-induced δ^{2}H biases. Therefore, it is essential to correct for such CVD-biases before interpreting HIO as a function of true ecophysiological and/or environmental processes (Allen and Kirchner, 2022; Jiang et al., 2022; Wen et al., 2021).

Importantly, we observed the presence of significant positive relationships between CVD-induced δ^{2}H biases and RWC for both stem and soil samples (Fig. 2). This is consistent with previous studies that have reported similar RWC-related patterns in stem water (Chen et al., 2020; He et al., 2023; Li et al., 2024; Wen et al., 2026; Younger et al., 2024) as well as in soil water (Meißner et al., 2014; Walker et al., 1994; Wen et al., 2021; Yang et al., 2023). These RWC-dependent relationships (i.e. the fitted regression equations showed in Fig. 2) therefore provide an empirical basis for correcting CVD-induced δ^{2}H biases in both stem and soil water, with the potential to reduce uncertainty in isotope-based inference of source soil water. However, correcting only a single water pool – either stem or soil – substantially altered both the magnitude and, in some cases, even the direction of HIO, with responses differing among species (Fig. 3). This sensitivity provides insight into why previous studies that corrected only one compartment have reached conflicting conclusions about plant-soil water interactions. For example, stem-water bias corrections have been shown to reduce HIO in apple trees (He et al., 2023) and willow (Li et al., 2024), whereas Gai et al. (2023) found that such corrections explained only *c.* 2% of the uncertainty in source-water partitioning. Soil-water bias corrections likewise appear study dependent: CVD-induced soil artifacts have been reported to affect interpretations of groundwater recharge and plant-water interactions (Orlowski et al., 2018; Wen et al., 2021), yet other work has documented negligible HIO despite measurable CVD-induced soil artifacts (Newberry et al., 2017a). These findings indicate that single-pool correction strategies can yield variable – and potentially misleading – estimates of plant water sources, with outcomes contingent on species traits, soil properties, and experimental conditions.

By contrast, in our experiment HIO values across all species approached zero only when CVD-induced δ^{2}H biases in both stem and soil water were corrected simultaneously (Fig. 3). This result indicates that reliable inference of plant water sources requires joint correction of CVD-related artifacts in both pools, rather than adjustment of either pool in isolation (Wen et al., 2021). This interpretation is supported by Allen and Kirchner (2022), who reanalyzed multiple published datasets and showed that correcting both stem and soil water can materially alter inferred source-water identities and relative contributions. An apparently different outcome was reported by Jiang et al. (2022), who found that soil-stem HIO in potted wheat and maize remained significantly different from zero even after correcting both pools. However, it is worth noting that their spiking-based protocol can induce additional isotopic artifacts via kinetic fractionation during rewetting (Diao et al., 2022; Newberry et al., 2017b; Wen et al., 2026), making the magnitude of their estimated CVD-related biases – and thus their corrected HIO – difficult to verify. In contrast, our immersion-based rehydration protocol avoids such spiking-related artifacts and allows more robust quantification of CVD-induced biases in both stem and soil water (Wen et al., 2026). When these empirically constrained corrections were applied simultaneously, HIO consistently collapsed toward zero across species (Fig. 3), providing strong evidence that, under controlled conditions, most apparent HIO primarily reflects methodological artifacts.

4.2 Ecophysiological and environmental influences on HIO

Recent studies have reported associations between HIO and a range of environmental factors, including temperature, relative humidity, mean annual precipitation, soil water content, and net radiation (Barbeta et al., 2020, 2022; de la Casa et al., 2022; Vargas et al., 2017; Wang et al., 2025, 2026; Zhang et al., 2026; Zhao et al., 2022, 2024a). However, the environmental correlates of HIO vary across studies, suggesting that the reported HIO-environment relationships may not be universal and may depend on differences among study systems. In our experiment, we did not detect such relationships: HIO showed no significant association with either relative humidity or soil water status (Table 2; Fig. 4). Mechanistically, previous studies have often attributed HIO-environment relationships to indirect effects mediated through g_s and/or E. For example, Vargas et al. (2017) linked HIO in avocado to transpiration-driven water loss, while Zhao et al. (2024a) inferred such relationships based on correlations with $\Delta^{18}O_{lw}$, a commonly used proxy for stomatal behavior. In our experiment, although HIO showed significantly correlations with g_s, E, and $\Delta^{18}O_{lw}$ prior to correction, these relationships disappeared after CVD-induced biases were removed (Fig. 4). This pattern indicates that the apparent HIO-ecophysiological associations observed prior to correction were influenced by extraction-related bias rather than genuine ecophysiological controls. We therefore interpret the initial correlations as indirect effects arising from the covariance between g_s, E, and $\Delta^{18}O_{lw}$ and stem RWC, rather than reflecting a direct ecophysiological control on HIO (Fig. 4).

Furthermore, several studies have proposed that within-stem isotopic heterogeneity, arising from metabolism-induced deuterium depletion in water within outside-xylem tissues relative to water in the xylem conduits, – may contribute to HIO (Barbeta et al., 2022, 2020; Bowers and Williams, 2022; Duvert et al., 2024; Zhao et al., 2016). It is important to note that in those studies, HIO patterns were often associated with interspecific wood anatomical traits. For example, Bowers and Williams (2022) reported that species with higher hydraulic vulnerability (ψ_{50}) showed smaller negative HIO, while Barbeta et al. (2022) observed that angiosperms with low wood density tended to have less negative HIO than gymnosperms with denser wood. In our dataset, however, the relationships between wood density and HIO disappeared entirely after dual correction (Fig. 4), suggesting that previously reported correlations may, at least in part, reflect unaccounted CVD-induced biases. We interpret this pattern as an indirect effect arising from the strong correlation between stem RWC and wood density (Fig. 4). We suggest that the dynamic nature of plant water transport processes should offer a physiological explanation for why within-stem isotopic heterogeneity should be of limited extent. As can be imagined, in transpiring plants, continuous flux of source water through the xylem essentially acts to effectively bath surrounding non-xylem tissues in a large, isotopically uniform water reservoir, – much like an immersion-based rehydration process. Under such conditions, even if non-xylem water were to develop localized deuterium signatures due to metabolic processes, these deviations would likely be rapidly attenuated via isotopic exchange with the dominant xylem water. Thus, in this sense it seems difficult to envision how a pronounced deuterium heterogeneity within the stem, – especially between xylem and non-xylem water, – could be maintained under sustained transpiration.

4.3 Limitations and perspectives of this study

This study highlights the importance of rigorously evaluating CVD-related artifacts to improve interpretation of plant-soil water isotope relationships, but several limitations should be acknowledged. Our experiments were conducted under controlled greenhouse conditions using potted plants and a single soil type. Accordingly, although our results suggest that apparent HIO under the conditions examined here is largely driven by CVD-related δ^2H biases in both stem and soil water, this outcome should not be interpreted as a universal solution to the wide range of stem-soil water HIO reported in the literature (de la Casa et al., 2022; Zhao et al., 2024a). Instead, broader validation across diverse plant functional types, soil properties, and field conditions spanning wider environmental stress gradients is required.

Nevertheless, a key contribution of this study is a dual-correction framework that jointly adjusts both stem- and soil- water δ^2H based on empirically constrained C VD-induced biases. The corrections are derived from an immersion-based rehydration approach, which has been highlighted as a reliable strategy for characterizing extraction bias (Wen et al., 2026). Applying such experimentally constrained corrections can improve the robustness of isotope-based assessments of plant water sources and reduce the risk of inference artifacts arising from uncorrected data. Looking forward, continued refinement and standardization of extraction and correction protocols will be essential for distinguishing methodological effects from genuine ecophysiological and/or environmental signals and for improving our understanding of plant-soil-atmosphere water interactions.

5 Conclusions

Our results suggest that well-documented occurrence of stem-soil water HIO is largely attributable to CVD-related artifacts, rather than unequivocally reflecting ecophysiological and/or environmental drivers. Prior to CVD-bias correction, HIO values in multiple species differed significantly from zero and appeared to correlate with several ecophysiological variables. However, after simultaneously correcting CVD-induced δ^2H biases in both stem and soil water, HIO values across all species were no longer significantly different from zero, and the previously observed associations with ecophysiological variables disappeared. Thus, these findings help to clarify that a considerable fraction of the HIO variability previously attributed to environmental factors (e.g. RH, soil water status) or ecophysiological variables (e.g. E, g_s, and wood density), – as reported in a number of studies (Barbeta et al., 2020; Vargas et al., 2017; Wang et al., 2025, 2026; Zhang et al., 2026; Zhao et al., 2022, 2024a), – may instead reflect extraction-related bias. Nevertheless, we cannot exclude the possibility that genuine biological and environmental processes may manifest to influence HIO under more extreme conditions; testing this hypothesis will require targeted experiments spanning broader stress gradients and field settings in future studies.

Data availability

All relevant data generated and analyzed to support the findings of this study are within this paper and the Supplement.

Author contributions

WW and XS conceived the study. WW and BC performed experiments and collected the data. WW performed the analysis and prepared the first draft of the manuscript. CY, XT, YW, WAK and XS contributed to writing the final manuscript.

Declaration of Competing Interest

The contact author has declared that none of the authors has any competing interests.

Disclaimer

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