

Hydrogen isotope fractionation in plants with C₃, C₄, and CAM CO₂ fixation

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Summary

- Measurements of stable isotope ratios in organic compounds are widely used tools for plant ecophysiological studies. However, the complexity of the processes involved in shaping hydrogen isotope values ($\delta^2\text{H}$) in plant carbohydrates has limited its broader application.
- To investigate the underlying biochemical processes responsible for ^2H fractionation among water, sugars, and cellulose in leaves, we studied the three main CO₂ fixation pathways (C₃, C₄, and CAM) and their response to changes in temperature and vapor pressure deficit (VPD).
- We show significant differences in autotrophic ^2H fractionation (ϵ_{A}) from water to sugar among the pathways and their response to changes in air temperature and VPD. The strong ^2H depleting ϵ_{A} in C₃ plants is likely driven by the photosynthetic H⁺ production within the thylakoids, a reaction that is spatially separated in C₄ and strongly reduced in CAM plants, leading to the absence of ^2H depletion in the latter two types. By contrast, we found that the heterotrophic ^2H -fractionation (ϵ_{H}) from sugar to cellulose was very similar among the three pathways and is likely driven by the plant's metabolism, rather than by isotopic exchange with leaf water.
- Our study offers new insights into the biochemical drivers of ^2H fractionation in plant carbohydrates.

Introduction

The hydrogen isotopic composition ($\delta^2\text{H}$) of plant carbohydrates (e.g. sugar, cellulose) is a proxy for hydrological (Yakir, 1992; Roden *et al.*, 2000), biochemical (Zhou *et al.*, 2018) and physiological processes (Augusti *et al.*, 2006; Lehmann *et al.*, 2022; Wieloch *et al.*, 2022b). For an accurate interpretation of the observed $\delta^2\text{H}$ in the various carbohydrates of plants, such as in ecohydrological studies, it is crucial to understand the underlying biochemical processes behind the ^2H fractionation in plant compounds – from the CO₂ fixation to the formation of cellulose.

The current knowledge on ^2H fractionation processes in the carbohydrate metabolism of plants with C₃ CO₂ fixation indicates an initial ^2H depletion in leaf sugars compared with the leaf water (ϵ_{A}) during the light-dependent reactions of CO₂ fixation (Estep & Hoering, 1981; Luo *et al.*, 1991; Schuler *et al.*, 2023). Later, cellulose becomes ^2H enriched compared with leaf sugars (ϵ_{H}) (Yakir & DeNiro, 1990; Roden *et al.*, 2000; Cormier *et al.*, 2018; Holloway-Phillips *et al.*, 2022; Lehmann *et al.*, 2022). While the exact mechanism behind the autotrophic

^2H fractionation (ϵ_{A}) is yet to be identified, its amplitude is related to the phylogeny of a plant (Schuler *et al.*, 2023), and might explain a large part of the observed variability of the $\delta^2\text{H}$ in leaf cellulose across different species (Arosio *et al.*, 2020; Holloway-Phillips *et al.*, 2022).

Carbohydrates of plants with different CO₂ fixation pathways differ in their $\delta^2\text{H}$ (Ziegler *et al.*, 1976; Sternberg & DeNiro, 1983; Luo & Sternberg, 1991; Schmidt *et al.*, 2003). Leaf sugars and cellulose of plants fixing CO₂ with the C₃ CO₂ fixation pathway are more strongly ^2H depleted compared with leaf water than those with C₄, and even more compared to those with CAM CO₂ fixation (Sternberg *et al.*, 1984; Luo & Sternberg, 1991; Schmidt *et al.*, 2003; Schuler *et al.*, 2022). Investigating these differences in ^2H fractionation within and between CO₂ fixation types and its response to environmental conditions might enable us to identify, and thus understand, the most important biochemical ^2H fractionation processes that cause $\delta^2\text{H}$ variation in plant carbohydrates.

In C₃ plants, water molecules are split by the hydrogen splitting complex of photosystem II (PS II) during the

light-dependent reactions of CO₂ fixation in the chloroplasts' thylakoid. This reaction produces protons (H⁺), which are used to generate energy and reducing equivalents for the Calvin–Benson–Bassham (CBB) cycle that occurs in the stroma of the same chloroplasts. The water-splitting reaction establishes a high H⁺ concentration inside the thylakoid, with a simultaneous low H⁺ concentration inside the chloroplasts stroma (Heldt *et al.*, 1973; Falkner *et al.*, 1976; Heldt, 1980). The water-splitting reaction discriminates against ²H, leading to a strongly ²H-depleted pool of reducing equivalents such as NADPH (Luo *et al.*, 1991). New findings show a strong phylogenetic pattern behind the δ²H of leaf sugars (Schuler *et al.*, 2023), indicating a relatively simple enzymatic process underlying the ²H fractionation during photosynthesis.

In C₄ CO₂ fixation, the water-splitting reaction in the thylakoids is spatially separated from the CBB cycle in mesophyll (M) and bundle-sheath (BS) cells, respectively (Oswald *et al.*, 1990; Höfer *et al.*, 1992; Meierhoff & Westhoff, 1993). The protons for NADH or NADPH synthesis are mostly derived from malate in the chloroplast stroma of BS cells (Drincovich *et al.*, 2001; Rao & Dixon, 2016), which is synthesized in, and imported from, mesophyll cells. Thus, during the transport among the two cell types, the signal of the strong ²H depletion of PS II is likely not or much less carried into the fresh assimilates.

In CAM CO₂ fixation, initial CO₂ fixation into organic acids takes place over night (Winter & Smith, 2022), and the contribution of PSII to CO₂ fixation is strongly reduced (Niewiadomska *et al.*, 2011). Additionally, in CAM plants, NADH is generated from malic acid as the proton source, a reaction which happens in the cytosol (Drincovich *et al.*, 2001; Chen *et al.*, 2019), and thus, an isotopic exchange with the cytosol water may occur. However, several species of facultative CAM plants are operating along a wide spectrum of C₃ or C₄ and CAM CO₂ fixation (Winter *et al.*, 2008; Winter, 2019), making it challenging to disentangle the contribution of a certain type of CO₂ fixation to a plant's carbon budget.

Moreover, climatic factors such as air temperature and relative humidity are known to influence isotopic fractionation (Augusti *et al.*, 2006; Farquhar *et al.*, 2007; Cernusak *et al.*, 2016). The three types of CO₂ fixation show different physiological responses to changes in temperature (Yamori *et al.*, 2014; Kumar *et al.*, 2017; dos Santos *et al.*, 2022). For instance, C₃ plants have a lower temperature optimum than C₄ plants (Orsenigo *et al.*, 1997). This might be reflected in the temperature response of their autotrophic ²H fractionation, as ²H fractionation reflects plant performance (Sanchez-Bragado *et al.*, 2019), for example ε_A is likely strongest at the physiological climatic optimum of a given plant species, where their growth rate is highest. In addition, CO₂ availability, which can be influenced by the stomatal response to changes in vapor pressure deficit (VPD) (Grossiord *et al.*, 2020), can alter hydrogen isotope fractionation in C₃ plants (Wieloch *et al.*, 2022a). Hence, these biochemical and physiological differences in C₃, C₄, and CAM plants may contribute to the differences in their δ²H.

Since the type of CO₂ fixation is independent of a plant's secondary metabolism, the biochemical pathways responsible for ε_H

should not be directly affected by the type of CO₂ fixation. Current views on the heterotrophic ²H fractionation assume an isotopic exchange of carbon-bound hydrogen with hydrogen atoms from the surrounding water. This process, which is derived from the analogy with the exchange of carbon-bound oxygen during cellulose synthesis, is assumed to be responsible for the observed ²H enrichment during cellulose formation (Yakir & DeNiro, 1990; Augusti *et al.*, 2008; Holloway-Phillips *et al.*, 2022). However, this assumption has not been systematically investigated.

Therefore, the main aim of this study was to determine ε_A and ε_H in various C₃, C₄, and CAM plants at the leaf level, growing under different climatic conditions (20 and 30°C air temperature, VPD of 1.2, 1.3. and 2.6 kPa), in order to unravel the processes leading to δ²H variations of plant carbohydrates. Since the transition from an overall heterotrophic to an overall autotrophic state in dicot leaves occurs when the leaf reaches 30–60% of its final size (Turgeon, 1989), and much earlier in grasses, the major proportion of leaf cellulose (including the secondary cell wall) is synthesized after this transition, when leaf sugars and leaf water have the same δ²H as measured in the mature leaf.

We expected strong but opposite responses in the ²H fractionation of C₃ and C₄ plants to changes in temperature under a constant VPD. In C₃ plants, we expected the strongest ε_A, leading to leaf bulk sugar and cellulose being the most ²H depleted at 20°C, which is closer to their optimum temperature, where they perform best (Sanchez-Bragado *et al.*, 2019), that is where they have the highest carbon gain (Vitali *et al.*, 2024). By contrast, in C₄ plants, we expected leaf sugar and cellulose to be most ²H depleted at 30°C, as C₄ plants are adapted to higher temperatures. We expected CAM plants to respond marginally to temperature, with slightly lower δ²H at 30°C than at 20°C, as they are adapted to higher temperatures but do not show a strong ²H depletion in general. A higher VPD increases the evaporative ²H enrichment of leaf water, which is expected to increase the δ²H of the leaf sugar and cellulose in C₃ and C₄ plants, while CAM plants should not be affected, as they perform their gas exchange only during the night, when VPD was constant between the treatments.

To conclude, we hypothesized that:

- (1) The autotrophic ²H fractionation (ε_A) is driven by biochemical reactions during a plant's CO₂ fixation, which differs in plants with different biochemical CO₂ fixation pathways. Furthermore, based on the idea that the optimum temperature for carbon gain regulated δ²H values in plant organic matter and carbohydrates, ε_A is presumably strongest at 20°C in C₃ and at 30°C in C₄ and CAM CO₂ fixation.
- (2) The heterotrophic ²H fractionation (ε_H) is independent of a plant's CO₂ fixation type and not impacted by exchange reactions with leaf water isotopes.
- (3) Temperature and VPD impact the autotrophic and heterotrophic ²H fractionation depending on the physiological response of the three types of CO₂ fixation, as well as between species within each type of CO₂ fixation.

Materials and Methods

Growing conditions, and sampling of leaf material

Twenty-six plant species with three different CO₂ fixation pathways were grown between May 2020 and March 2021 in two walk-in climate chambers (Bouygues E&S InTec Schweiz AG, Zurich, Switzerland) under three different controlled climates. The climatic conditions included 20°C with a VPD of 1.2 kPa, 30°C with a VPD of 1.3 kPa, and 30°C with a VPD of 2.6 kPa. The climate chambers were set to 14 h of daylight with the target conditions, a photosynthetic active irradiance of 110 μmol m⁻² s⁻¹ was maintained using uniform fluorescent tubes (OSRAM L 36 W 777 Fluora; Osram Licht AG, Germany), and 10 h of nighttime with uniform conditions across all treatments (15°C with a VPD of 0.7 kPa).

The selected species covered 11 C₃ (*Abelmoschus esculentus* (L.) Moench, *Anthurium* Linden ex André, *Begonia maculata* C. DC. ex Huber, *Begonia semperflorens* C. L. Willdenow, *Cyperus alternifolius* Rottb., *Euphorbia pulcherrima* Willd. ex Klotzsch, *Hordeum vulgare* L., *Oryza sativa* L., *Quercus pubescens* Willd., *Salvia hispanica* L., *Zantedeschia aethiopica* (L.) Spreng.), 7 C₄ (*Amaranthus caudatus* L., *Amaranthus tricolor* L. (both are dicot forbs and belong to the C₄ NAD-Me subtype), *Pennisetum glaucum* L., *Salsola soda* L., *Setaria italica* (L.) P. Beauv., *Sorghum bicolor* (L.) Moench, *Zea mays* L. (the latter 5 monocot grass species belong to the C₄ NADP-Me subtype)), and 7 CAM plant species (*Curio repens* P. V. Heath, *Delosperma cooperi* (Hook. f.) L. Bolus, *Hyllocereus* (A. Berger) Britton & Rose, *Mesembryanthemum cordifolium* L. f., *Phalaenopsis* Blume, *Rhipsalis* (J. S. Muell.) Stearn, *Sedum* L.). The species were selected based on (1) photosynthetic pathways, (2) different phylogenetic (including mono- and dicots) and functional groups (grasses, forbs, and trees), and (3) propagule availability. Triplicates of seeds or plantlets of all but three species (*H. vulgare*, *O. sativa*, and *Phalaenopsis*) were sown in 3-l pots containing potting soil (Kübelpflanzenerde; RICO-TER Erdaufbereitung AG, CH-3270 Aarberg, Switzerland). Due to the small plant size, three times 30 seeds of *H. vulgare* and *O. sativa* were sown in individual 20-l pots containing the same potting soil, and triplicates of each of the 7 CAM species were bought in a plant shop. Because all plants of a species were grown in only one climate chamber per climatic condition, the chamber experiments are pseudoreplicated. All CAM plants but *Phalaenopsis* were replanted in the common potting soil in 3-l pots. However, *Phalaenopsis* was continuously grown in the original 0.5-l pots containing orchid potting mix. The C₃ and C₄ plants were watered twice a week to soil capacity, while the CAM plants were watered only once per week to induce drought stress and a high percentage of CAM CO₂ fixation in all species. The δ²H of the irrigation water was −79.9‰, with a maximum SD of 2‰ throughout the experimental period.

To ensure that only plant material was sampled growing under the selected climatic conditions, the growing tip was marked on the first day of each condition either with a string, a permanent marker, or, in the case of the cacti (*Hyllocereus* and *Rhipsalis*), by cutting off the tip, forcing the plants to grow distinguishable side

shoots. After 1–3 months of treatment, depending on the specific growth rate, fully developed leaves, or photosynthetic active stems in the case of the two cacti, of the three replicates per species were sampled. In the case of *H. vulgare* and *O. sativa* (both C₃ grasses), three pools of leaves each consisting of four plants were sampled. The sampling was conducted after 7 h of light to allow the plants to synthesize a sufficient amount of fresh assimilates on the day of harvest and to ensure steady-state leaf water enrichment (Cernusak *et al.*, 2016). Depending on their size, whole leaf samples were transferred to gas-tight 12-ml or 30-ml glass vials (prod. no.: 738 W; Exetainer glass vials; Labco, Lampeter, Wales, UK; culture vials with screw cap DURAN® glass, 30 ml, height: 180 mm, Labware from Carl Roth GmbH + Co. KG, Karlsruhe, Germany, prod. no.: K250.1, respectively), and were kept on ice until the harvest was complete (i.e. within 2 h). Subsequently, the samples were then stored at −20°C until water extraction.

Extraction of leaf water, sugars, and cellulose

Leaf water was cryogenically extracted (Diao *et al.*, 2022) and stored in glass vials at −20°C until δ²H measurement. Later, the dry leaf material of each sample was cut and crushed into small pieces, homogeneously mixed, and separated into two subsamples. The first subsample was milled (MM400, Retsch, Germany), and the bulk leaf sugar fraction (i.e. 'leaf sugar') was extracted following established protocols for carbon, oxygen, and hydrogen isotope analysis (Rinne *et al.*, 2012; Lehmann *et al.*, 2020; Schuler *et al.*, 2023). In brief, the water-soluble content (including sugars and amino acids) was extracted by mixing the ground leaf material with deionized water at 85°C for 30 min. The extracted leaf sugar was then separated from the other water-soluble compounds using ion exchange cartridges (OnGuard II A, H and P, Dionex; Thermo Fisher Scientific, Bremen, Germany). The sugar solutions were frozen, freeze-dried, and the total weight of dried sugar per sample measured. Then, deionized water was added to reach a concentration of 1 mg per 20 μl, and the samples were stored at −20°C until further use.

The second subsample was used for the extraction of leaf holocellulose. One hundred milligrams of leaf material was crushed and packed in F57 fiber filter bags (ANKOM Technology, Macedon, NY, USA). The samples were washed two times for 2 h with 5% NaOH at 60°C. After that, the samples were rinsed three times with boiling deionized water and incubated three times in a mixture of 7% NaClO₂ and 96% acetic acid with a pH of 4–5 at 60°C for 8 h. After that, the samples were rinsed three times with boiling deionized water, squeezed using a spatula, and dried for at least 4 h in the drying oven at 60°C. In a final step, the purified holocellulose was mixed with deionized water, homogenized with an ultrasonic transducer (UP200St; Hielscher, Berlin, Germany), and freeze-dried overnight.

δ²H analysis of leaf water

The δ²H values of leaf water samples were measured using a high-temperature conversion elemental analyzer coupled to a

Delta^{Plus} XP isotope ratio mass spectrometer (TC/EA-IRMS; Finnigan MAT, Bremen, Germany). Calibration was performed with a range of certified waters of different isotope $\delta^2\text{H}$ ratios, resulting in a precision of analyses of 2‰.

$\delta^2\text{H}$ analysis of sugar and cellulose using a hot water vapor equilibration method

The $\delta^2\text{H}$ of non-exchangeable hydrogen ($\delta^2\text{H}$) analyses of sugar and cellulose were performed according to the hot water vapor equilibration method (Schuler *et al.*, 2022). Two sets of each sugar sample were prepared by pipetting 20 μl into pre-weighed 5 $\times$ 9 mm silver foil capsules (Prod. no.: SA76981106; Santis, Switzerland). Both sets were frozen at -20°C , freeze-dried, and packed into a second silver foil capsule. Similarly, two sets of all cellulose samples were prepared by transferring two replicates of 1 mg into 3.3 $\times$ 5 mm silver foil capsules (Prod. no.: SA76980506; Santis, Switzerland). All samples were stored in a desiccator at low relative humidity (2–5%) until $\delta^2\text{H}$ measurement.

The two sets of the samples were then equilibrated with hot water vapor at 130°C for 2 h, using one of two isotopically distinct waters for each set ($\delta^2\text{H}$ (Water 1) = -160‰ and $\delta^2\text{H}$ (Water 2) = -428‰). The equilibration apparatus consisted of an electrical heating oven (ED23; Binder, Tuttlingen, Germany) where an equilibration chamber was inserted (Schuler *et al.*, 2022). After the 2 h, excess water was pumped back and discarded, and the feeding capillary was switched to a dry nitrogen gas delivering capillary (N25.0, Prod. no.: 2220 912; PanGas AG, Dagmersellen, Switzerland) for 2 h at 130°C . The samples were then immediately transferred into a Zero Blank Autosampler (N.C. Technologies S.R.L., Milano, Italy), which was installed on a sample port of the TC/EA-IRMS system. The autosampler was evacuated to 0.001 bar and filled with dry helium gas to avoid re-exchange of exchangeable hydrogen of the samples with the hydrogen of ambient water vapor. Pyrolyzation took place in a reactor according to Gehre *et al.* (2004), and the products were carried in a flow of dry helium (150 ml min^{-1}) to the IRMS. Offset corrections of the raw $\delta^2\text{H}$ values were performed using polyethylene foil standards (PEF, IAEA-CH-7 polyethylene foil, International Atomic Energy Agency, Vienna, Austria; SD < 0.7‰ within one run).

Calculation of the isotope ratio ($\delta^2\text{H}$)

All isotope ratios (δ) were calculated as given in Eqn 1 (Coplen, 2011):

$$\delta = \frac{R_{\text{Sample}} - R_{\text{Standard}}}{R_{\text{Standard}}} \quad \text{Eqn 1}$$

where $R = {}^2\text{H}/{}^1\text{H}$ is the hydrogen isotope ratio of the sample (R_{Sample}) and the Vienna Standard Mean Ocean Water (VSMOW2; R_{Standard}). To express the resulting δ values in permil (‰), results have been multiplied by 1000.

The percentage of hydrogen exchanged during the equilibrations (x_e , Eqn 2, from Filot *et al.* (2006)) can be calculated as:

$$x_e = \frac{\delta^2\text{H}_{e1} - \delta^2\text{H}_{e2}}{\alpha_{e-w} (\delta^2\text{H}_{w1} - \delta^2\text{H}_{w2})} \quad \text{Eqn 2}$$

Then, $\delta^2\text{H}_{ne}$ can then be calculated using x_e , the measured $\delta^2\text{H}$ of one of the two equilibrations, and α_{e-w} , which is the fractionation factor of 1.082 for carbohydrates (Filot *et al.*, 2006; Schuler *et al.*, 2022):

$$\delta^2\text{H}_{ne} = \frac{\delta^2\text{H}_{e1} - x_e \cdot \alpha_{e-w} \cdot \delta^2\text{H}_{w1} - 1000 \cdot x_e \cdot (\alpha_{e-w} - 1)}{1 - x_e} \quad \text{Eqn 3}$$

Three internal sucrose standards were used for the equilibrations of leaf sugars, three internal cellulose standards for the equilibrations of the leaf cellulose, and results normalized to the international scale with PEF standards, each measured as triplicates. Throughout the manuscript, $\delta^2\text{H}$ has been used instead of $\delta^2\text{H}_{ne}$.

Eqn 4 was used to calculate the autotrophic fractionation between leaf water and leaf sugar ϵ_A , and Eqn 5 to calculate the heterotrophic fractionation between leaf sugar and leaf cellulose ϵ_H as in Schuler *et al.* (2023):

$$\epsilon_A = \delta^2\text{H}_{\text{leaf sugar}} - \delta^2\text{H}_{\text{leaf water}} \quad \text{Eqn 4}$$

$$\epsilon_H = \delta^2\text{H}_{\text{leaf cellulose}} - \delta^2\text{H}_{\text{leaf sugar}} \quad \text{Eqn 5}$$

Since ϵ_A and ϵ_H are calculated as the difference between the $\delta^2\text{H}$ of the precursor and the product, their unit is $\Delta^2\text{H}$. More detailed information on the background of the calculations of the $\delta^2\text{H}$ can be found in Schuler *et al.* (2022).

Statistical analyses

Statistical analyses were performed using R v.4.1.2 (R Core Team, 2023). Equal variance among the three types of CO_2 fixation was tested with Bartlett's test. Bartlett's tests of leaf sugar and leaf cellulose showed a different variance for the three types of CO_2 fixation. Thus, for the analysis within and between the three types of CO_2 fixation and their response to changes in temperature and VPD, Kruskal–Wallis tests were performed followed by pairwise Wilcoxon rank sum tests as the *post hoc* analysis, with a P -value adjustment using the Bonferroni method. Subsequently, results were displayed by applying compact letter displays (CLD). The within and between CO_2 fixation types and the species-specific response to temperature and VPD were tested by one-way ANOVA followed by Tukey *post hoc* analysis, and results were displayed by applying CLD. Regression analyses were used to determine the general drivers behind the ${}^2\text{H}$ fractionation processes with linear models within the package GGPlot2, including the measurements of individual plant samples belonging to the corresponding groups. Due to sample loss, sugar and cellulose of *Amaranthus caudatus* (C_4), and *Phalaenopsis* (CAM) could not

be analyzed for all climatic conditions. For the same reason, *Setaria italica* (C₄) could only be included in the general analysis of the CO₂ fixation pathways. Due to the unbalanced sample design for the NADP-Me (5 monocot grass species) and NAD-Me C₄ (2 dicot forb species) subtypes, no statistical analysis was performed comparing these two types. As the $\delta^2\text{H}$ of carbohydrates and the ϵ_A of the two facultative CAM species (e.g. performing C₃ CO₂ fixation if the drought stress is not severe enough) indicated a strong contribution of C₃ CO₂ fixation, the comparison between the regression analyses has been conducted with and without these two species.

Results

General patterns of $\delta^2\text{H}$ fractionation within and between plants with C₃, C₄, and CAM CO₂ fixation

We observed distinct patterns of $\delta^2\text{H}$ in leaf water, leaf sugar, and leaf cellulose, as well as in the autotrophic and heterotrophic $\delta^2\text{H}$ fractionation factors ϵ_A and ϵ_H , within and between the three CO₂ fixation pathways across all tested species and climatic conditions (Figs 1, 2, Supporting Information Figs S1–S3; Tables 1, 2).

Interquartile Range: The box itself spans from the first quartile (25th percentile) to the third quartile (75th percentile). This range contains the middle 50% of the data.

Whiskers: The lines extending from the box (whiskers) indicate variability outside the upper and lower quartiles. They typically extend to 1.5 times the IQR from the quartiles. Data points outside this range are considered outliers.

While the $\delta^2\text{H}$ in leaf water across all treatments (Figs 1, 2; Tables 1, 2) was similar in plants with C₃ and C₄ CO₂ fixation (−29.4‰, SD = 14.1‰, and −29.8‰, SD = 14.6‰; NAD-Me = −16.1‰, SD = 7.2‰; NADP-Me = −36.3‰, SD = 13.4‰, respectively), leaf water of plants with CAM CO₂ fixation was significantly more ^2H enriched (−6.1‰,

SD = 15.2‰). With an average $\delta^2\text{H}$ of −89.6‰ (SD = 40.5‰), leaf sugar was significantly more ^2H depleted in plants with C₃ compared with leaf sugar of plants with C₄ (−24.7‰, SD = 19.8‰; NAD-Me = −39.9‰, SD = 7.6‰; NADP-Me = −16.8‰, SD = 20.1‰) or CAM (−5.9‰, SD = 60.9‰) CO₂ fixation. This autotrophic $\delta^2\text{H}$ fractionation (ϵ_A) was most pronounced in C₃ (−60.2‰, SD = 41.6‰), close to zero in CAM (0.2‰, SD = 54.4‰), and slightly positive in C₄ plants (5.0‰, SD = 23.2‰; NAD-Me = −23.8‰, SD = 9.1‰; NADP-Me = 19.5‰, SD = 13.3‰). The $\delta^2\text{H}$ of leaf cellulose roughly reflected the pattern observed in leaf sugar, with −44.1‰ in C₃ (SD = 21.8‰), 17.8‰ in C₄ (SD = 28.2‰; NAD-Me = 14.8‰, SD = 11.2‰; NADP-Me = 20.5‰, SD = 33.8‰), and 27.1‰ in CAM (SD = 44.3‰) CO₂ fixation pathways. There was no significant difference in ϵ_H observed among the three types of CO₂ fixation (C₃ = 45.5‰, SD = 30.0‰; C₄ = 42.6‰, SD = 20.5‰; NAD-Me = 54.7‰, SD = 14.1‰; NADP-Me = 37.3‰, SD = 21.4‰; and CAM = 33.0‰ (SD = 41.4‰) (Table 1).

If the two facultative CAM species *D. cooperi* and *M. cordifolium* were excluded (Table 1), the $\delta^2\text{H}$ of the leaf water increased to −2.1‰ (SD 15.7‰), $\delta^2\text{H}$ of leaf sugar to 28‰ (SD = 34.4‰), ϵ_A to 30.2‰ (SD = 31.6‰), the $\delta^2\text{H}$ of leaf cellulose increased to 48.5‰ (SD = 32.1‰), and ϵ_H decreased to 20.5‰ (SD = 41.1‰). In this case, average $\delta^2\text{H}$ of leaf sugar and $\delta^2\text{H}$ of leaf cellulose (Fig. S1) of plants with CAM CO₂ fixation were significantly ^2H enriched compared with the compounds in C₄ CO₂ fixation and ϵ_A was significantly higher. By contrast, ϵ_H of this CAM subset was significantly lower than the ones observed in plants with C₃ and C₄ CO₂ fixation.

Across all tested species, temperature increase from 20°C to 30°C alone did not lead to significant overall changes in $\delta^2\text{H}$ of leaf water, leaf sugar, and leaf cellulose, and $\delta^2\text{H}$ fractionation among the three types of CO₂ fixation (Fig. 3 for the main pattern, Table 2 for the values and SD). However, the increase in VPD from 1.3 to 2.6 kPa significantly increased the $\delta^2\text{H}$ of leaf

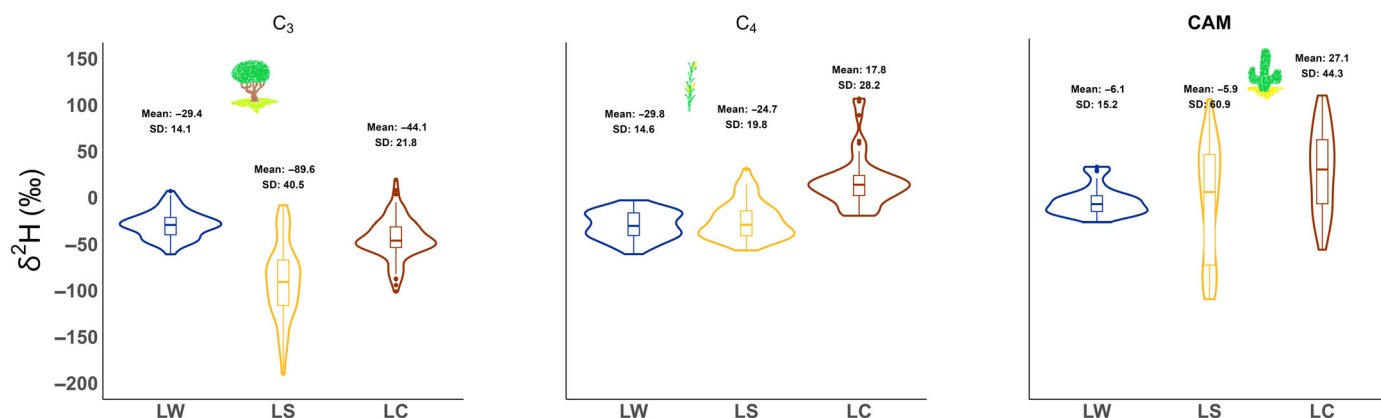


Fig. 1 $\delta^2\text{H}$ of leaf water (LW), leaf sugar (LS), and leaf cellulose (LC) of plants with C₃, C₄, and CAM CO₂ fixation, including all measurements of this study. SD, standard deviation from the mean. The horizontal line inside the box represents the median, the box itself spans from the first quartile to the third quartile and contains the middle 50% of the data, and the lines extending from the box (whiskers) indicate variability outside the upper and lower quartiles. They typically extend to 1.5 times the interquartile range (IQR) from the quartiles. Data points outside this range are considered outliers.

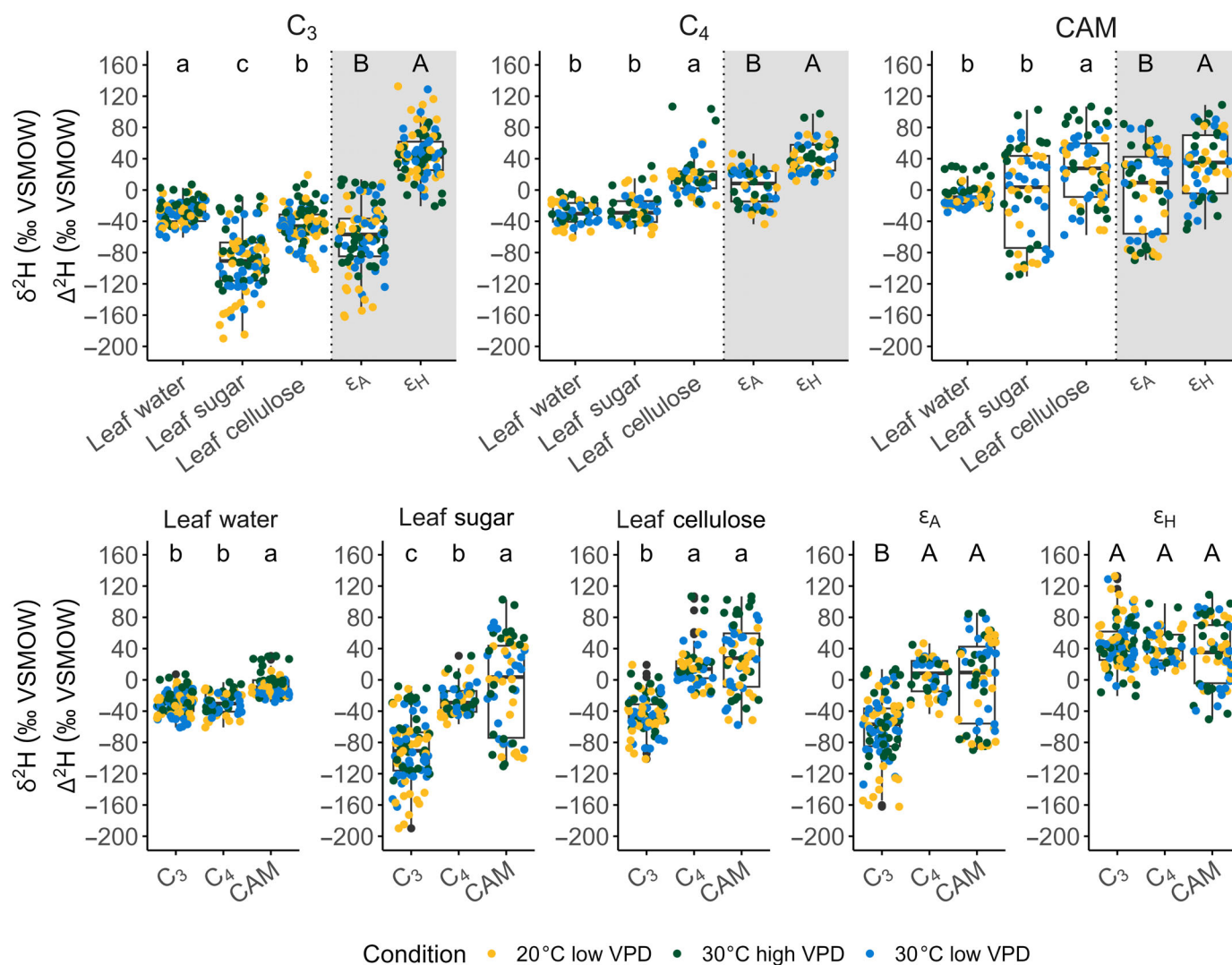


Fig. 2 Comparison of the $\delta^2\text{H}$ (compounds) and $\Delta^2\text{H}$ (fractionation factors) within (top row) and between (bottom row) the three types of CO₂ fixation pathways and the three climate treatments (yellow points 20°C, vapor pressure deficit (VPD) = 1.2 kPa; green points 30°C, VPD = 1.3 kPa; blue points 30°C, VPD = 2.6 kPa). ϵ_A , autotrophic ^2H fractionation factor, ϵ_H , heterotrophic ^2H fractionation factor. Letters indicate the grouping of results from pairwise Wilcoxon rank sum tests by CLD, analyzed separately for the compounds (lower case) and fractionation factors (upper case), with significant differences indicated by different letters. The fractionation factors have been separated from the compounds by a grey coloring and a dotted line in the upper three panels. Circles represent individual data points, with their position reflecting their actual $\delta^2\text{H}$ value, the horizontal line represents the median value of $\delta^2\text{H}$ for each category, and whiskers indicate the extend from the edges of the box (representing the first and third quartiles) to the smallest and largest values within 1.5 times the interquartile range (IQR).

water of plants with C₃ and CAM CO₂ fixation, but not in those with C₄ CO₂ fixation. Increasing VPD also lead to higher $\delta^2\text{H}$ values of leaf sugar and leaf cellulose in C₃ plants; however, no change was observed in both of their ^2H fractionation factors. Neither the $\delta^2\text{H}$ of leaf sugar and leaf cellulose nor the ^2H fractionation factors of plants with C₄ and CAM CO₂ fixation showed a general response to changes in temperature and VPD. In summary, the treatments did not significantly change the overall pattern of the $\delta^2\text{H}$ values and ^2H fractionation factors within the three types of CO₂ fixation across all tested species (Figs S2, S3).

Species-specific $\delta^2\text{H}$ response to changes in temperature and VPD

Analyzing the response to changes in temperature and VPD on a species level revealed a diverse pattern for plants with C₃, C₄, and CAM CO₂ fixation pathways (Tables 3–5).

Plants with C₃ CO₂ fixation (Table 3) showed a distinct response in their $\delta^2\text{H}$ of leaf water, leaf sugar, and leaf cellulose as well as fractionation factors to changes in temperature and VPD. The temperature response of the autotrophic ^2H fractionation ϵ_A varied among the species. ϵ_A was more negative at 20°C for two

Table 1 Average $\delta^2\text{H}$ and $\Delta^2\text{H}$, the SD, and the grouping of the results from the pairwise Wilcoxon rank sum tests by compact letter display (CLD) of leaf water, leaf sugar, and leaf cellulose (lower case letters), and of the autotrophic ^2H fractionation factor ϵ_A , and the heterotrophic ^2H fractionation factor ϵ_H (upper case letters).

	Leaf water			Leaf sugar			Leaf cellulose			ϵ_A			ϵ_H		
	$\delta^2\text{H}$	SD	CLD	$\delta^2\text{H}$	SD	CLD	$\delta^2\text{H}$	SD	CLD	$\Delta^2\text{H}$	SD	CLD	$\Delta^2\text{H}$	SD	CLD
C ₃	−29.4	14.1	a b	−89.6	40.5	c c	−44.1	21.8	b b	−60.2	41.6	B B	45.5	30.0	A A
C ₄	−29.8	14.6	b b	−24.7	19.8	b b	17.8	28.2	a a	5.0	23.2	B A	42.6	20.5	A A
CAM	−6.1	15.2	b a	−5.9	60.9	b a	27.1	44.3	a a	0.2	54.4	B A	33.0	41.4	A A
C ₃	−29.4	14.1	a b	−89.6	40.5	c c	−44.1	21.8	b c	−60.2	41.6	B C	45.5	30.0	A A
C ₄	−29.8	14.6	b b	−24.7	19.8	b b	17.8	28.2	a b	5.0	23.2	B B	42.6	20.5	A A
CAM*	−2.1	15.7	c a	28.0	34.4	b a	48.5	32.1	a a	30.2	31.6	A A	20.5	41.1	A B

The CLD are separated into within CO₂ fixation type of the different compounds and fractionation factors (first letter; horizontal comparison), and between CO₂ fixation type within one compound and isotope fractionation factor (second letter; vertical comparison). In the second set of data including CAM*, the facultative CAM species *Delosperma cooperi* and *Mesembryanthemum cordifolium* were excluded.

Table 2 Average $\delta^2\text{H}$ (‰), the SD, and the grouping of the results from the pairwise Wilcoxon rank sum tests by compact letter display (compact letter display (CLD); vertical comparison between treatments) of leaf water, leaf sugar, leaf cellulose, the autotrophic fractionation factor ϵ_A , and the heterotrophic fractionation factor ϵ_H (the latter two as $\Delta^2\text{H}$) among the three CO₂ fixation pathways, in response to the respective growing condition.

		$\delta^2\text{H}$ Leaf water			$\delta^2\text{H}$ Leaf sugar			$\delta^2\text{H}$ Leaf cellulose			ϵ_A			ϵ_H		
		‰	SD	CLD	‰	SD	CLD	‰	SD	CLD	‰	SD	CLD	‰	SD	CLD
C ₃	20°C, VPD = 1.2 kPa	−31.0	12.6	b	−99.1	47	ab	−47	23.8	a	−68.1	50.9	a	52.1	34.4	a
	30°C, VPD = 1.3 kPa	−34.9	13.8	b	−95.5	33.6	b	−49.2	19.6	a	−60.5	33.8	a	46.2	27.3	a
	30°C, VPD = 2.6 kPa	−22.3	13.2	a	−74.5	36.8	a	−36.1	20.4	a	−52.1	38.4	a	38.4	27.1	a
C ₄	20°C, VPD = 1.2 kPa	−31.5	19.1	a	−26.8	23.2	a	14.2	19.5	a	4.7	28.3	a	41	19.3	a
	30°C, VPD = 1.3 kPa	−33.3	9.3	a	−26	14.3	a	11.5	22.2	a	7.2	19.7	a	37.5	19.4	a
	30°C, VPD = 2.6 kPa	−24.6	13.2	a	−21.4	21.7	a	27.8	38.2	a	3.2	22.1	a	49.2	22.3	a
CAM	20°C, VPD = 1.2 kPa	−9	9.1	b	−22.2	58.3	a	12.7	35	a	−13.2	56	a	34.9	29.8	a
	30°C, VPD = 1.3 kPa	−15.5	7.3	b	−2.1	51.6	a	26	41.9	a	13.4	46.6	a	28	42.3	a
	30°C, VPD = 2.6 kPa	5.9	17.8	a	4.9	71	a	41.1	51.4	a	0.9	59.5	a	36.2	50.3	a
CAM*	20°C, VPD = 1.2 kPa	−5.3	8.8	CLD	14	31.1	b	32.4	20.9	a	19.2	37.1	a	18.5	18.8	a
	30°C, VPD = 1.3 kPa	−13.5	7.2	b	22.8	30.2	ab	43	30	a	36.4	25.6	a	20.1	42.9	a
	30°C, VPD = 2.6 kPa	12.7	15.4	a	45.7	36	a	68.3	33.6	a	32.9	32.4	a	22.7	54	a

In the subset CAM*, the facultative CAM species *Delosperma cooperi* and *Mesembryanthemum cordifolium* were excluded.

species (*B. maculata* and *H. vulgare*), and more negative at 30°C for three species (*A. esculentus*, *O. sativa*, and *Q. pubescens*). The heterotrophic ^2H fractionation ϵ_H was significantly more positive at 20°C in two species (*B. maculata* and *B. semperflorens*), and more positive at 30°C in one species (*Q. pubescens*). The autotrophic ^2H fractionation ϵ_A was more negative at a VPD of 1.3 kPa for one species (*Z. aethiopica*), and a VPD of 2.6 kPa for two species (*A. esculentus*, *O. sativa*). The heterotrophic ^2H fractionation ϵ_H was more positive at a VPD of 1.3 kPa in two species (*H. vulgare* and *Z. aethiopica*), and a VPD of 2.6 kPa in two species (*O. sativa* and *Q. pubescens*). When ϵ_A decreases (e.g. becoming more negative), ϵ_H usually simultaneously increases (e.g. becoming less positive), as seen for instance in *Quercus pubescens*, the two species of *Begonia*, *Oryza sativa*, and *Hordeum vulgare*.

Plant species with C₄ CO₂ fixation showed the least response to changes in temperature and VPD, independent of their C₄ subtype (Table 4). *Z. mays* and *A. caudatus*, the latter only analyzed for the temperature effect due to sample loss of the high VPD treatment, did not show any significant differences in any of the analyzed compounds or ^2H fractionation factors between

the treatments. ϵ_A was more negative in the high VPD treatment in *P. glaucum*, whereas ϵ_H was more positive in *P. glaucum* and *S. bicolor* under high VPD.

Plant species with CAM CO₂ fixation showed a distinct treatment response in their isotopic composition (Table 5). ϵ_A was more negative at 20°C than at 30°C in three species (*Hylerocereus*, *M. cordifolium*, and *Sedum*), and at 30°C than at 20°C in one species (*Rhipsalis*). ϵ_H was more positive at 20°C than at 30°C in one species (*Hylerocereus*), and higher at 30°C than at 20°C in one species (*C. repens*). The autotrophic fractionation ϵ_A was more negative at a VPD of 2.6 kPa in one species (*D. cooperi*). The heterotrophic fractionation factor ϵ_H did not significantly respond to changes in VPD.

Drivers of the observed $\delta^2\text{H}$ values and ^2H fractionation factors

The linear regression analysis revealed relationships of various strength between the $\delta^2\text{H}$ of the different compounds and ^2H fractionation factors in the three types of CO₂ fixation (Figs 4,

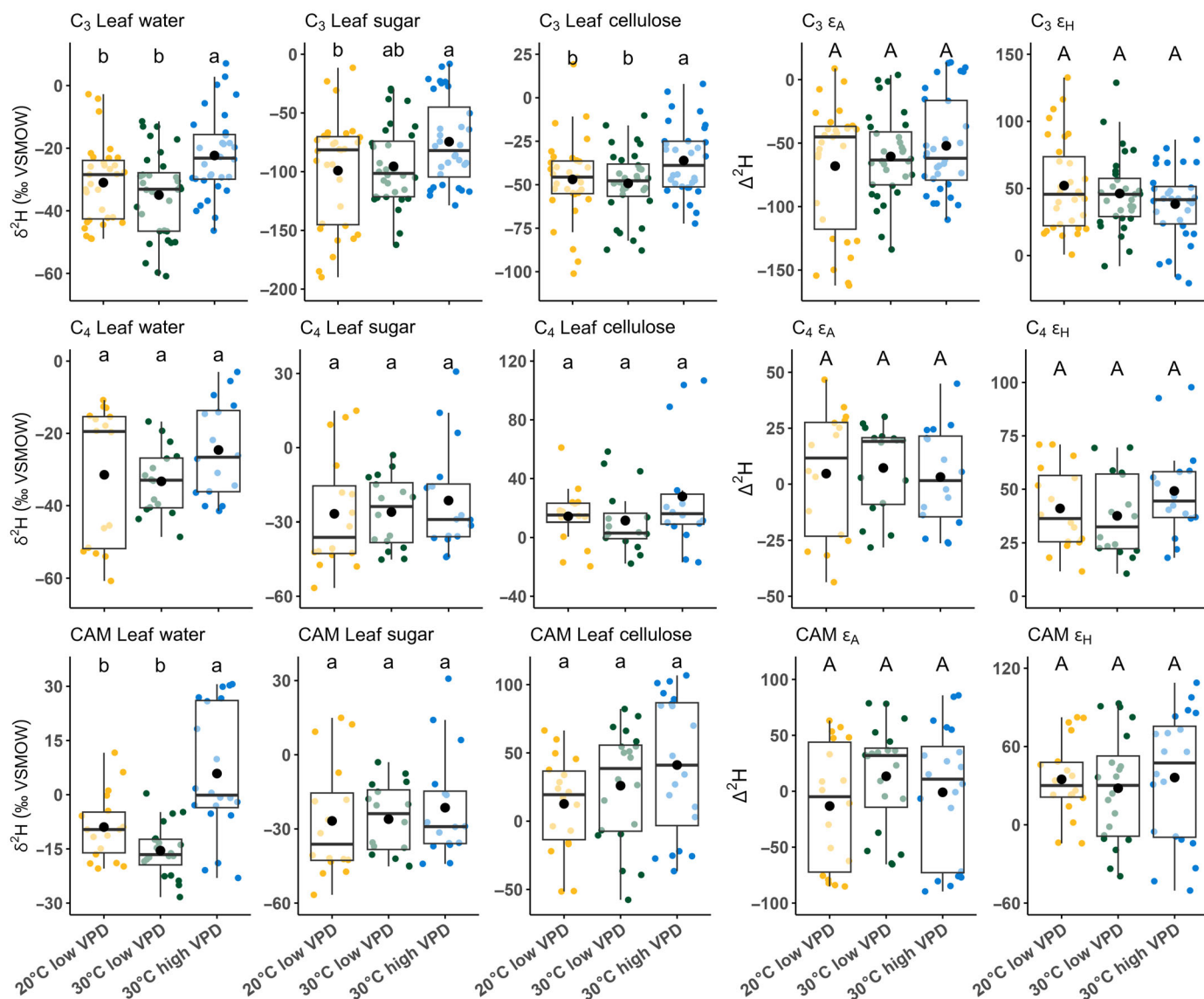


Fig. 3 $\delta^2\text{H}$ of leaf water, leaf sugar, leaf cellulose, and ϵ_A (autotrophic ^2H fractionation) and ϵ_H (heterotrophic ^2H fractionation) as $\Delta^2\text{H}$ in response to changes in temperature and VPD among the three types of CO_2 fixation. Letters display the grouping of the results from the pairwise Wilcoxon rank sum tests by CLD (compounds in lower case, fractionation factors in upper case). Circles represent individual data points, with their position reflecting their actual $\delta^2\text{H}$ value, the horizontal line represents the median value of $\delta^2\text{H}$ for each category, and whiskers indicate the extend from the edges of the box (representing the first and third quartiles) to the smallest and largest values within 1.5 times the interquartile range (IQR). The three climate treatments are indicated by different colors (yellow points 20°C, vapor pressure deficit (VPD) = 1.2 kPa; green points 30°C, VPD = 1.3 kPa; blue points 30°C, VPD = 2.6 kPa).

S4–S9). $\delta^2\text{H}$ of leaf water did not significantly correlate with $\delta^2\text{H}$ of leaf sugar in C_3 and C_4 CO_2 fixation, but explained 28% of the variation in CAM plants. ϵ_A was highly related to $\delta^2\text{H}$ of leaf sugar, explaining 94% of the variation in plants with CAM, 89% in plants with C_3 , and 61% in plants with C_4 CO_2 fixation. About half of the variation in $\delta^2\text{H}$ of leaf cellulose was explained by the $\delta^2\text{H}$ of leaf sugar (i.e. 48% for C_3 , 47% for C_4 , and 54% for CAM plants). ϵ_H only related to $\delta^2\text{H}$ of leaf cellulose of the plants with C_4 CO_2 fixation, explaining 51% of the observed variation. In all three types of CO_2 fixation, ϵ_H was negatively related to ϵ_A , with the latter explaining 16% of the observed variation of ϵ_H in C_4 , 44% of the observed variation of ϵ_H in CAM, and 73% of the observed variation of ϵ_H in C_3 CO_2 fixation,

indicating a common driver behind ϵ_A and ϵ_H . These patterns largely remained after separating the three types of CO_2 fixation by the three treatments (Figs S4–S6).

Discussion

The biochemical drivers of autotrophic ^2H fractionation ϵ_A among C_3 , C_4 , and CAM CO_2 fixation

We were able to determine and investigate ϵ_A for plants of different CO_2 fixation pathways (Figs 1, S1; Tables 1–5) for the first time. ϵ_A caused a strong photosynthetic ^2H depletion from leaf water to leaf sugar in C_3 plants, an autotrophic ^2H enrichment in C_4 plants and no autotrophic ^2H fractionation in CAM plants

Table 3 Treatment response of the $\delta^2\text{H}$ of leaf water ($\delta^2\text{H W}$), leaf sugar ($\delta^2\text{H S}$), and leaf cellulose ($\delta^2\text{H C}$), the autotrophic ^2H fractionation factor ϵ_{A} and the heterotrophic ^2H fractionation factor ϵ_{H} among the species with C_3 CO_2 fixation pathway.

		20°C, VPD = 1.2 kPa			30°C, VPD = 1.3 kPa			30°C, VPD = 2.6 kPa		
		‰	SD	CLD	‰	SD	CLD	‰	SD	CLD
<i>Quercus pubescens</i>	$\delta^2\text{H W}$	−30	2.8	b	−42.1	4	c	−20.3	1.9	a
	$\delta^2\text{H S}$	−69.3	1.7	a	−118.4	7.5	b	−86.9	10.9	a
	ϵ_{A}	−39.2	3	a	−76.2	5.9	b	−66.6	12.7	b
	$\delta^2\text{H C}$	−48.4	4.2	a	−85.8	3.1	b	−43.1	9	a
	ϵ_{H}	20.9	2.6	c	32.6	4.5	b	43.8	3.9	a
<i>Oryza sativa</i>	$\delta^2\text{H W}$	−43.2	0.9	ab	−48.8	2.1	b	−42.9	3.2	a
	$\delta^2\text{H S}$	−76.6	8.6	a	−102.8	2.5	b	−116.9	3.1	c
	ϵ_{A}	−33.4	7.7	a	−54.0	3.9	b	−74	5.4	c
	$\delta^2\text{H C}$	−37.4	3.2	a	−54.3	4.3	b	−47.7	3.3	b
	ϵ_{H}	39.2	8.8	b	48.5	2.8	b	69.1	0.5	a
<i>Hordeum vulgare</i>	$\delta^2\text{H W}$	−28.8	4.8	a	−33.1	0.7	a	−30.3	1.9	a
	$\delta^2\text{H S}$	−173	25	b	−112.0	13	a	−74.8	5.1	a
	ϵ_{A}	−144.2	29.5	b	−78.9	13	a	−44.6	6.9	a
	$\delta^2\text{H C}$	−94.2	7	c	−41.1	2.4	a	−59	4.9	b
	ϵ_{H}	78.7	18.9	a	70.9	12.6	a	15.8	8.5	b
<i>Salvia hispanica</i>	$\delta^2\text{H W}$	−48.4	11.5	b	−27.2	3.3	a	−29.3	0.4	a
	$\delta^2\text{H S}$	−126.4	28.6	a	−96.9	19.1	a	−91.8	4.1	a
	ϵ_{A}	−77.9	37.6	a	−69.6	18.2	a	−62.5	3.8	a
	$\delta^2\text{H C}$	−71.1	22.6	b	−36.3	10	a	−49.8	2.3	a
	ϵ_{H}	55.2	44.6	a	60.6	9.2	a	42	1.8	a
<i>Begonia semperflorens</i>	$\delta^2\text{H W}$	−26.6	4.4	a	−29.6	1.3	a	−18.7	6.3	a
	$\delta^2\text{H S}$	−161.7	9.9	b	−136.1	15.1	ab	−119	8.8	a
	ϵ_{A}	−135.1	12.8	b	−106.5	16.2	ab	−100.3	9.3	a
	$\delta^2\text{H C}$	−42.3	6.3	a	−54.1	5.5	a	−40.2	13.3	a
	ϵ_{H}	119.4	12	a	82.0	18.4	b	78.8	8.3	b
<i>Begonia maculata</i>	$\delta^2\text{H W}$	−3.5	2.9	b	−18.2	2.7	c	3.4	3.4	a
	$\delta^2\text{H S}$	−143.4	14.1	b	−106.8	8.9	a	−79.3	15.2	a
	ϵ_{A}	−139.9	14.5	b	−88.6	11.6	a	−82.7	12.3	a
	$\delta^2\text{H C}$	−53.6	3	b	−48.9	2.1	b	−21.6	8.8	a
	ϵ_{H}	89.8	12.6	a	58.0	10.4	b	57.7	13.6	b
<i>Anthurium</i>	$\delta^2\text{H W}$	−32.8	9.7	a	−35.0	2.5	a	−21.8	15.6	a
	$\delta^2\text{H S}$	−71.4	2.5	c	−38.9	1	b	−25.9	4.3	a
	ϵ_{A}	−27.8	19.3	b	−3.9	3.5	ab	−4.1	12.7	a
	$\delta^2\text{H C}$	−53.8	16.3	b	−21.5	21.8	ab	−14.6	10.4	a
	ϵ_{H}	16.4	3.9	a	3.1	15.6	a	11.3	14	a
<i>Abelmoschus esculentus</i>	$\delta^2\text{H W}$	−43.2	1.2	c	−12.5	1	a	−18.0	2	b
	$\delta^2\text{H S}$	−83.8	9	a	−73.7	7.7	a	−110.5	7.3	b
	ϵ_{A}	−40.6	8.1	a	−61.1	6.7	b	−92.5	8.8	c
	$\delta^2\text{H C}$	−29.5	5.3	a	−35.0	10.5	a	−63.4	10.6	b
	ϵ_{H}	54.3	13.7	a	38.7	4.3	a	47.1	5.2	a
<i>Euphorbia pulcherrima</i>	$\delta^2\text{H W}$	−21.7	1.4	a	−29.8	3.7	ab	−33.7	4.6	b
	$\delta^2\text{H S}$	−21.8	9.7	a	−40.6	18.1	a	−32.6	15.3	a
	ϵ_{A}	−0.1	8.3	a	−10.8	21.7	a	1.1	15.8	a
	$\delta^2\text{H C}$	−2.1	18.6	a	−17.6	8.3	a	1.2	8.1	a
	ϵ_{H}	19.7	21	a	23.1	21.5	a	33.8	8.1	a
<i>Zantedeschia aethiopica</i>	$\delta^2\text{H W}$	25.1	1.9	a	−48.9	2.2	b	−22.7	7.3	a
	$\delta^2\text{H S}$	−74.1	8	b	−90.4	13.7	b	−14.1	8.2	a
	ϵ_{A}	−49	7.9	b	−41.6	15.5	b	8.6	3.8	a
	$\delta^2\text{H C}$	−53.3	6.9	b	−51.7	4.9	b	−28.4	1.8	a
	ϵ_{H}	20.8	4.8	a	38.7	10.3	a	−14.4	7.2	b
<i>Cyperus alternifolius</i>	$\delta^2\text{H W}$	−42.6	8.3	b	−59.1	2.1	c	−6.1	4.7	a
	$\delta^2\text{H S}$	−86.6	10.2	ab	−114.6	14.2	b	−63.8	0.5	a
	ϵ_{A}	−44.1	17.4	a	−55.5	15.9	a	−57.6	4.2	a
	$\delta^2\text{H C}$	−39.6	3.4	b	−76.4	1.3	c	−27.4	3.3	a
	ϵ_{H}	47	10.4	a	38.2	14.1	a	36.4	3.8	a

Letters display the grouping of the results from the pairwise Wilcoxon rank sum tests by compact letter display (CLD), comparing the treatment effect within each species (horizontally).

Table 4 Treatment response of the $\delta^2\text{H}$ of leaf water ($\delta^2\text{H}_\text{W}$), leaf sugar ($\delta^2\text{H}_\text{S}$), and leaf cellulose ($\delta^2\text{H}_\text{C}$), the autotrophic ^2H fractionation factor ϵ_A and the heterotrophic ^2H fractionation factor ϵ_H among the species with C_4 CO_2 fixation pathway. Missing material for analysis and not possible statistical comparisons (CLD) are indicated by na.

		20°C, VPD = 1.2 kPa			30°C, VPD = 1.3 kPa			30°C, VPD = 2.6 kPa		
		‰	SD	CLD	‰	SD	CLD	‰	SD	CLD
<i>Zea mays</i>	$\delta^2\text{H}_\text{W}$	−45.4	na	a	−42.1	2.3	a	−38.9	3.6	a
	$\delta^2\text{H}_\text{S}$	−42.2	na	a	−39.8	3.1	a	−30	19.5	a
	ϵ_A	3.2	na	a	2.3	0.9	a	8.9	15.9	a
	$\delta^2\text{H}_\text{C}$	−16.9	na	a	−9	12.4	a	−7.5	13.2	a
<i>Pennisetum glaucum</i>	ϵ_H	25.3	na	a	30.8	9.3	a	22.5	6.3	a
	$\delta^2\text{H}_\text{W}$	−50.6	4	b	−40	1.9	a	−38.8	2.3	a
	$\delta^2\text{H}_\text{S}$	−17.5	9.6	a	−17.6	2.6	a	−18.9	9.1	a
	ϵ_A	33.1	11.8	a	22.4	4.1	a	19.9	7.7	a
<i>Sorghum bicolor</i>	$\delta^2\text{H}_\text{C}$	11.5	9.6	a	6.2	5.6	a	18.9	10.5	a
	ϵ_H	29	4.8	ab	23.8	3.3	b	37.9	2.2	a
	$\delta^2\text{H}_\text{W}$	−55.5	4.6	b	−40.9	7.7	ab	−30.8	5.2	a
	$\delta^2\text{H}_\text{S}$	−36.3	15.8	a	−16.9	12.5	a	−36.5	10.7	a
<i>Amaranthus tricolor</i>	ϵ_A	19.2	14.4	a	24.0	5.3	a	−5.8	16	a
	$\delta^2\text{H}_\text{C}$	−4.9	17.2	a	−0.4	7.2	a	10.5	1.3	a
	ϵ_H	31.3	7.2	ab	16.5	5.3	b	47	12	a
	$\delta^2\text{H}_\text{W}$	−18.1	2.3	b	−25.2	2.5	ab	−6	3.2	a
<i>Salsola soda</i>	$\delta^2\text{H}_\text{S}$	−38.1	5.5	a	−39.2	5.2	a	−31.5	4.2	a
	ϵ_A	−19.9	6.9	a	−14.0	7.7	a	−25.5	1.1	a
	$\delta^2\text{H}_\text{C}$	17.4	5.6	a	1.3	12.9	a	23.3	8.5	a
	ϵ_H	55.4	10.2	ab	40.5	17.7	b	54.8	11	a
<i>Amaranthus caudatus</i>	$\delta^2\text{H}_\text{W}$	−14.6	1.8	a	−31.3	1.6	b	−13.7	1.2	a
	$\delta^2\text{H}_\text{S}$	12.2	2.8	a	−10.2	2.3	b	17	12.6	a
	ϵ_A	26.8	4.3	a	21.1	3.5	a	30.6	12.7	a
	$\delta^2\text{H}_\text{C}$	39.3	19.4	b	51.1	6.8	b	99.8	9.6	a
<i>Setaria italica</i>	ϵ_H	27.2	21.6	b	61.3	6.9	ab	82.8	21.6	a
	$\delta^2\text{H}_\text{W}$	−13.9	3.6	a	−18.0	1.8	a	na	na	na
	$\delta^2\text{H}_\text{S}$	−49.0	7.1	a	−42.6	3.2	a	na	na	na
	ϵ_A	−35.2	7.4	a	−24.6	5	a	na	na	na
<i>Setaria italica</i>	$\delta^2\text{H}_\text{C}$	18.2	4.4	a	13.5	15.7	a	na	na	na
	ϵ_H	67.3	6.3	a	56.2	18.9	a	na	na	na
	$\delta^2\text{H}_\text{W}$	na	na	na	na	na	na	−26.3	4.6	na
	$\delta^2\text{H}_\text{S}$	na	na	na	na	na	na	−36.3	0.7	na
<i>Setaria italica</i>	ϵ_A	na	na	na	na	na	na	−10.0	3.9	na
	$\delta^2\text{H}_\text{C}$	na	na	na	na	na	na	4.1	16.9	na
	ϵ_H	na	na	na	na	na	na	40.4	16.2	na

Letters display the grouping of the results from the pairwise Wilcoxon rank sum tests by compact letter display (CLD), comparing the treatment effect (horizontally).

(Table 1). However, after excluding the two facultative CAM species (*D. cooperi* and *M. cordifolium*) from the analysis, ϵ_A in CAM plants resulted in a ^2H enrichment from leaf water to leaf sugar (Table 1). It is not clear whether the higher $\delta^2\text{H}$ values of CAM plant leaf water is due to more ^2H enriched soil water caused by reduced irrigation, possibly enriching the soil water, or due to differences in leaf water enrichment. The moderate relationship between leaf water $\delta^2\text{H}$ and leaf sugar $\delta^2\text{H}$ in CAM plants (Fig. 4) is likely due to a partial contribution of C_3 CO_2 fixation in some species, as leaf water $\delta^2\text{H}$ did not affect ϵ_A (Fig. S8). The relationship between $\delta^2\text{H}$ of the different compounds and the two ^2H fractionation factors of C_3 , C_4 , and CAM plants (Fig. 4) point to distinct physiological and anatomical aspects that influence the hydrogen isotope composition of plant carbohydrates. $\delta^2\text{H}$ of leaf sugars and ϵ_A are highly related in C_3 and CAM plants, whereas this relationship is reduced in C_4 plants

(Fig. 4). A strong relationship between $\delta^2\text{H}$ of leaf sugars and ϵ_A indicates an isotopic fractionation in equilibrium (Schuler *et al.*, 2023). Assuming that C_4 plants were in an isotopic steady state under all tested climatic conditions, this pattern was likely caused by the $\delta^2\text{H}$ of the water in BS cells, reflecting the ^2H -depleted source water. By contrast, the water in the mesophyll M cells is more likely to experience transpirative enrichment (Smith *et al.*, 1991; Zhou *et al.*, 2016). Thus, ϵ_A might not be correctly calculated in C_4 plants when using bulk leaf water $\delta^2\text{H}$. Comparing the biochemical reactions among the three types of CO_2 fixation (Figs 5–7) illustrates the biochemical and anatomical background that might be responsible for these observed patterns. In C_3 CO_2 fixation (Fig. 5), H^+ is generated via the splitting of H_2O in the chloroplast's thylakoids and results in a strongly ^2H depleted NADPH pool (Luo *et al.*, 1991). This

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Table 5 Treatment response of the $\delta^2\text{H}$ of leaf water ($\delta^2\text{H}_\text{W}$), leaf sugar ($\delta^2\text{H}_\text{S}$), and leaf cellulose ($\delta^2\text{H}_\text{C}$), the autotrophic ^2H fractionation factor ϵ_A and the heterotrophic ^2H fractionation factor ϵ_H among the species with CAM CO_2 fixation. Missing material for analysis and not possible statistical comparisons (CLD) are indicated by na.

		20°C, VPD = 1.2 kPa			30°C, VPD = 1.3 kPa			30°C, VPD = 2.6 kPa		
		‰	SD	CLD	‰	SD	CLD	‰	SD	CLD
<i>Delosperma cooperi</i>	$\delta^2\text{H}_\text{W}$	−19.3	0.5	a	−23.7	1.3	b	−21	2	ab
	$\delta^2\text{H}_\text{S}$	−91.6	8.4	ab	−82.2	6.7	a	−104.7	7.7	b
	ϵ_A	−72.4	8.8	ab	−58.5	6.2	a	−83.8	6.3	b
	$\delta^2\text{H}_\text{C}$	−13.8	9.3	a	−10.3	27.4	a	−25	2.7	a
<i>Mesembryanthemum cordifolium</i>	ϵ_H	77.8	5	a	71.9	25.9	a	79.7	8.3	a
	$\delta^2\text{H}_\text{W}$	−13.4	4	b	−17.7	1	b	0.5	1.2	a
	$\delta^2\text{H}_\text{S}$	−97.2	3.8	b	−68.6	18.4	a	−75.6	5.6	ab
	ϵ_A	−83.7	1.4	b	−50.8	19.5	a	−76.1	4.4	ab
<i>Rhipsalis</i>	$\delta^2\text{H}_\text{C}$	−39.8	20	a	−47.1	14.9	a	−19.6	20.4	a
	ϵ_H	57.4	21.6	a	21.5	3.5	a	55.9	17.1	a
	$\delta^2\text{H}_\text{W}$	−8.9	2.8	b	−8.7	4.2	b	27.6	2.3	a
	$\delta^2\text{H}_\text{S}$	46	5.1	a	23.4	11.8	b	57.2	7.6	a
<i>Hylerocereus</i>	ϵ_A	54.9	7.6	a	32.1	7.7	b	29.6	7	b
	$\delta^2\text{H}_\text{C}$	37.3	8.5	a	−7.4	4.4	b	21.2	12	a
	ϵ_H	−9.7	9	a	−30.8	10.4	a	−35.9	19.2	a
	$\delta^2\text{H}_\text{W}$	−11.5	8.1	b	−5.5	6.3	ab	14	5.9	a
<i>Sedum</i>	$\delta^2\text{H}_\text{S}$	34.4	10.1	c	68.5	4.3	b	99.2	5	a
	ϵ_A	46	12.2	b	74.0	7.7	a	85.1	0.9	a
	$\delta^2\text{H}_\text{C}$	58.7	8.4	a	65.1	14.9	a	89	6.6	a
	ϵ_H	24.2	1.8	a	−3.3	10.5	b	−10.1	1.6	b
<i>Curio repens</i>	$\delta^2\text{H}_\text{W}$	−3	12.7	b	−16	2	b	29.1	1.9	a
	$\delta^2\text{H}_\text{S}$	−0.4	1.5	c	16.5	2.6	b	45.3	8.3	a
	ϵ_A	2.5	11.5	b	32.5	1.1	a	16.2	10.2	ab
	$\delta^2\text{H}_\text{C}$	23.2	10.9	c	56.9	8.3	b	97.5	11.5	a
<i>Phalaenopsis</i>	ϵ_H	23.7	9.9	a	40.4	6.3	a	52.2	19.6	a
	$\delta^2\text{H}_\text{W}$	2.4	3.4	a	−19.0	3.1	b	−4.6	1.5	a
	$\delta^2\text{H}_\text{S}$	−24.2	23	a	−19.6	5.5	a	−12	5.7	a
	ϵ_A	−26.5	26.1	a	−0.8	8.6	a	−7.4	7.1	a
<i>Phalaenopsis</i>	$\delta^2\text{H}_\text{C}$	10.6	15.3	b	64.1	15.7	a	86.1	16.2	a
	ϵ_H	34.8	13	b	83.8	13.7	a	98.1	10.5	a
	$\delta^2\text{H}_\text{W}$	na	na	na	−18.5	8.6	b	−2.0	1.1	a
	$\delta^2\text{H}_\text{S}$	na	na	na	25.4	16.8	b	56.5	3.5	a
<i>Phalaenopsis</i>	ϵ_A	na	na	na	43.9	9	a	58.5	4.2	a
	$\delta^2\text{H}_\text{C}$	na	na	na	36	13.2	a	54.7	31.7	a
	ϵ_H	na	na	na	10.6	30	a	−1.8	33.2	a

Letters display the grouping of the results from the pairwise Wilcoxon rank sum tests by compact letter display (CLD), comparing the treatment effect (horizontally).

reaction might even decrease the $\delta^2\text{H}$ of the water inside the chloroplast stroma during photosynthetically active periods (Schuler *et al.*, 2023). This ^2H -depleted hydrogen pool is imprinted to the fresh assimilates during the CBB cycle, leading to the observed negative ϵ_A (Table 1). In addition, the strength of the autotrophic ^2H fractionation in C_3 CO_2 fixation is species-specific (Table 3) and likely driven by one or few proteins (Schuler *et al.*, 2023).

By contrast, water-splitting during the light-dependent reactions of C_4 CO_2 fixation (Fig. 6) takes place in the chloroplasts of M cells (Sage & Monson, 1998) and is not functional in BS cells (Meierhoff & Westhoff, 1993), where carbohydrate synthesis takes place. Thus, H^+ is imported to the BS cell chloroplast from M cells via the malate-pyruvate shuttle in the NADP-Me subtype (Fig. 6a), and via the import of aspartic acid in the NAD-Me subtype (Fig. 6b). Therefore, the strong ^2H depletion during PS II is not or only partially imprinted in the fresh

assimilates (Figs 1, 2; Table 1; Zhou *et al.*, 2016). Photorespiration can be excluded as a major driver of the observed relative ^2H enrichment compared with C_3 plants (Zhou *et al.*, 2018), as it is limited to a minimum in plants with C_4 CO_2 fixation (Bauwe, 2011). We therefore speculate that the ϵ_A observed here in C_4 plants (Table 4) may be caused by NADPH generation in the NADP-Me subtype at the end of the malate-pyruvate shuttle in the BS chloroplast (Fig. 6a step 3), and respectively by NADH generation in the BS mitochondria during the decarboxylation of oxaloacetic acid (Fig. 6b step 3) in the NAD-Me subtype. However, due to an unbalanced sampling design for the two C_4 subtypes (2 NAD-Me forbs vs 5 NADP-Me grasses), the data could not analyze for differences between the two C_4 subtypes. However, since significant differences in $\delta^2\text{H}$ in leaf organic matter of NADP-Me and NAD-Me grasses have been observed in earlier studies (Smith & Ziegler, 1990), further studies comparing the photosynthetic isotope fractionations of these two subtypes are

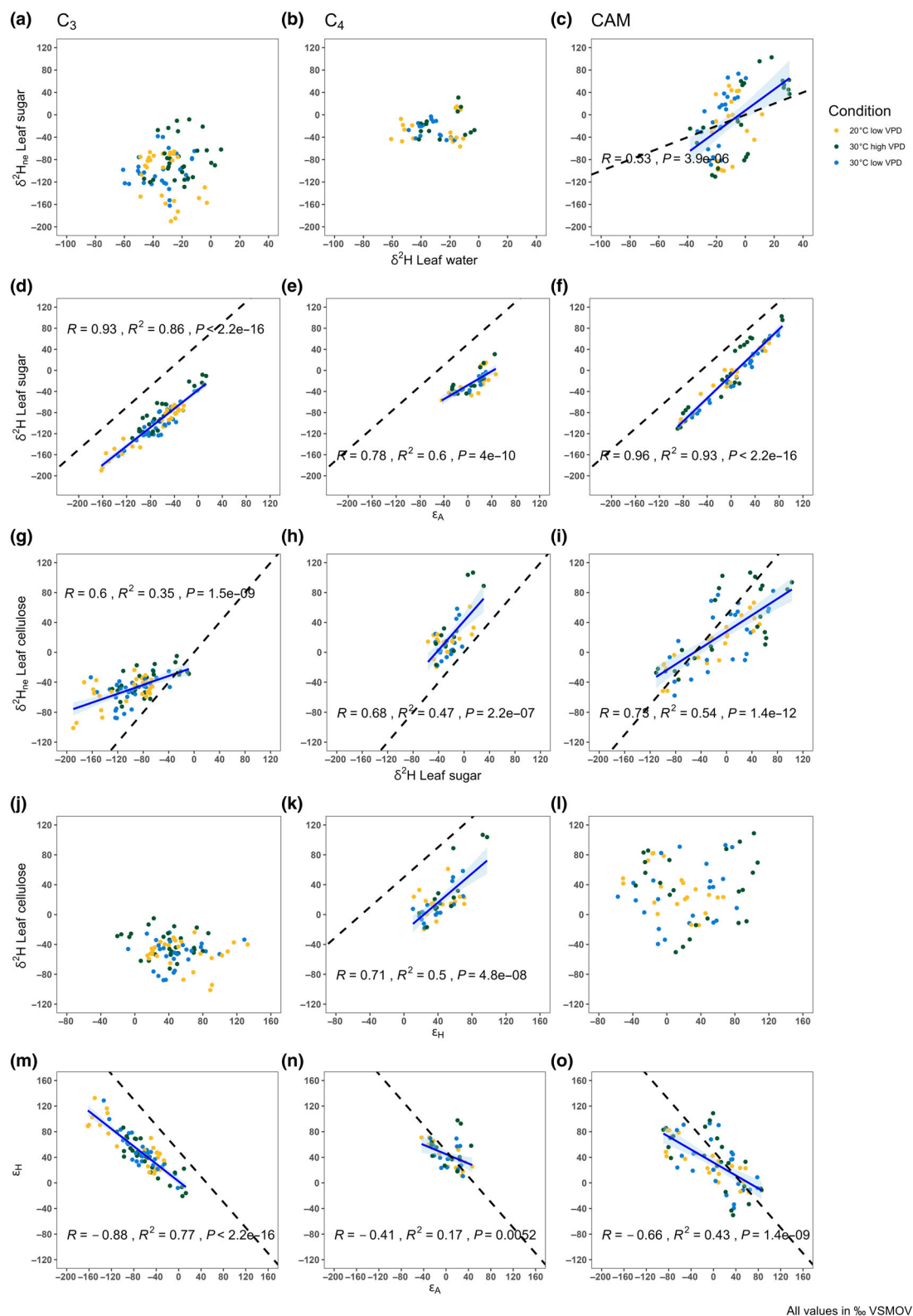


Fig. 4 Regression analysis within and between the three types of CO₂ fixation showing R , the relationships; R^2 , the proportion of variance that can be explained, and P values indicating significant differences: First column C₃ CO₂ fixation, second column C₄ CO₂ fixation, third column CAM CO₂ fixation. (a–c) $\delta^2\text{H}$ leaf sugar/ $\delta^2\text{H}$ leaf water; (d–f) $\delta^2\text{H}$ leaf sugar/ ϵ_A ; (g–i) $\delta^2\text{H}$ leaf cellulose/ $\delta^2\text{H}$ leaf sugar; (j–l) $\delta^2\text{H}$ leaf cellulose/ ϵ_H , (m–o) ϵ_A/ϵ_H . The blue line indicates the linear model, the light blue shading represents the 95% confidence interval of the linear model, and the dashed grey line the 1 : 1 line. VSMOW, Vienna Standard Mean Ocean Water.

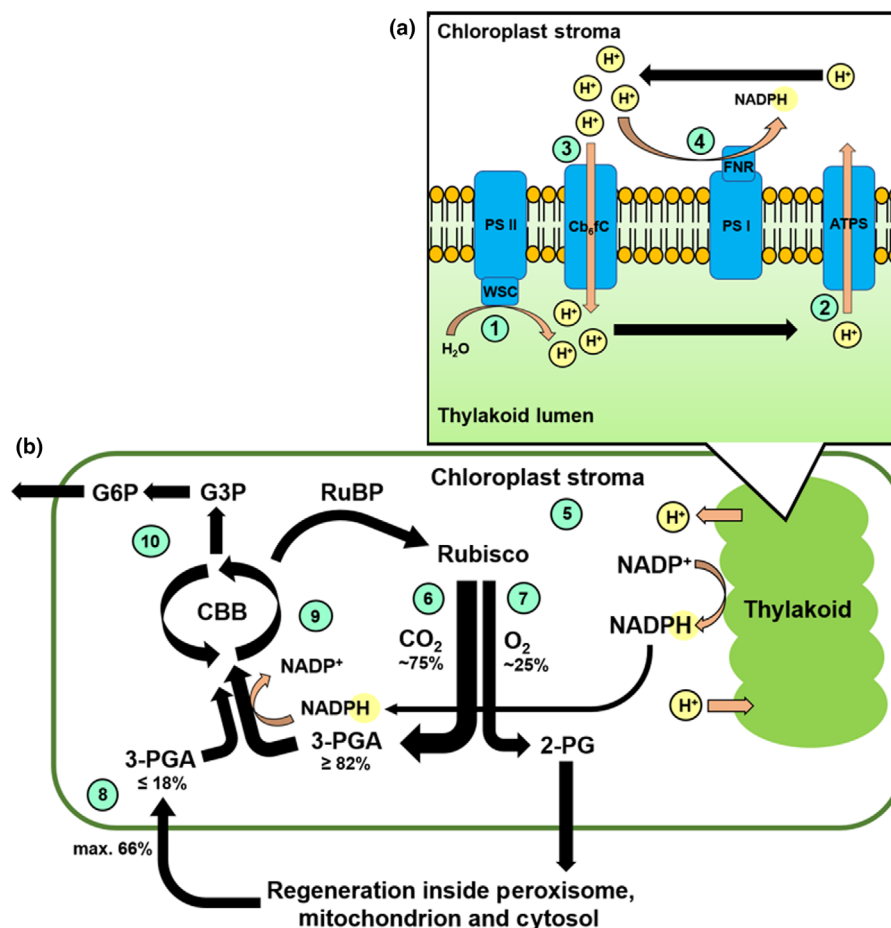


Fig. 5 Simplified scheme of C₃ CO₂ fixation (modified from Schuler *et al.* (2023)): Light-dependent reactions (a) according to Allen *et al.* (2011), light-independent reactions (b) according to Busch (2020). Proton fluxes are indicated by arrows, and orange arrows indicate reactions directly involving hydrogen. Light-dependent reactions (a, 1–4): (1) Initial proton production by the splitting of the water molecules by the water-splitting complex (WSC) of PS II (Ferreira *et al.*, 2004). (2) The proton pump of the ATP synthase (ATPS, Seelert *et al.* (2000)), which pumps protons from the thylakoid lumen into the chloroplast stroma. The $\delta^2\text{H}$ value of the proton pool in the chloroplast stroma can be potentially influenced by a selective H⁺ transport by the ATPS, or (3) the proton transfer back into the thylakoid lumen by the cytochrome b₆f complex (Cb₆fC, Cramer *et al.* (2011)). (4) The NADPH synthesis by the ferredoxin-NADP⁺ reductase (FNR, Nelson & Ben-Shem (2005)), which is connected to photosystem I (PS I), uses protons from the pool of the chloroplast stroma, which derives from the light-dependent reactions of the thylakoid. During the light-independent reactions (b, 5–10), the $\delta^2\text{H}$ value of the (5) proton pool in the chloroplast stroma is incorporated during the CO₂ assimilation process and probably further altered by other ²H fractionation processes. (6) c. 75% of RuBisCO binds CO₂ to 3-PGA, and (7) c. 25% of RuBisCO binds oxygen in a process called photorespiration (Busch, 2020) and needs to be regenerated as 2-phosphoglycolate (2-PG) to (8) 3-PGA (Bauwe, 2018). At least 82% of the 3-PGA pool comes from direct CO₂ fixation and maximum 18% from photorespiration (Busch, 2020). Further exchanges in biochemical reactions involving hydrogen occur during (9) the Calvin–Benson–Bassham (CBB) cycle, and (10) the synthesis of glucose-6-phosphate (G6P) out of glyceraldehyde-3-phosphate (G3P).

needed, which should also consider to measure the $\delta^2\text{H}$ of leaf water in BS and M cells.

In CAM CO₂ fixation (Fig. 7), water splitting during the light-dependent reactions is strongly reduced (Niewiadomska *et al.*, 2011). As in the C₄ NAD-Me subtype, the organic acid sequence of oxaloacetate, malate, and malic acid acts as the main proton source for the light-independent reactions (Winter & Smith, 1996b). Unlike in C₄ CO₂ fixation, the organic acids are synthesized separated by time during the night in the same cells as the CBB cycle (Winter & Smith, 1996a). During the day, NADH is produced by the decarboxylation of malate into CO₂ and pyruvate, pyruvate is transferred to the pool of storage

carbohydrates, and NADH and CO₂ are transferred to the chloroplasts for CO₂ assimilation (Winter & Smith, 1996b). Thus, it can be speculated that the reactions of the organic acid cycle are responsible for the observed ²H enrichment (Fig. 1; Table 5). In facultative CAM plants, PS II activity remains high in the absence of sufficient environmental stress, allowing C₃-type CO₂ fixation. Consequently, a high percentage of H⁺ comes from NADPH produced during PS II, resulting in C₃-type ϵ_A and hence $\delta^2\text{H}$ of sugar and cellulose, as observed in *D. cooperi* and *M. cordifolium* (Table 5). This obscured the ²H enriching ϵ_A in CAM plants, which was only seen after excluding these two species from the analysis (Tables 1, 2).

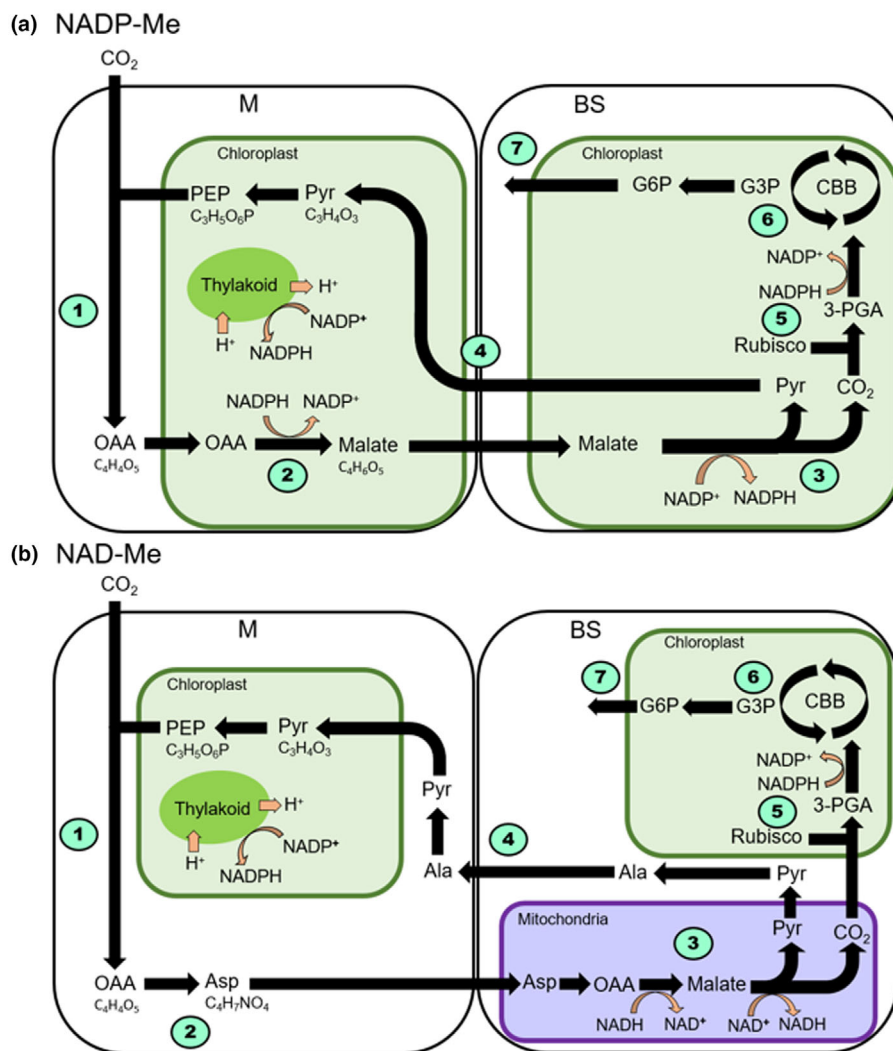


Fig. 6 Simplified scheme of C_4 CO_2 fixation according to Ludwig (2016) and Rao & Dixon (2016). (a) The reactions involving hydrogen within the NADP-Me subtype, while (b) the reactions within the NAD-Me subtype. In the NADP-Me subtype (a), (1) CO_2 is initially bound to phosphoenolpyruvate (PEP) by the enzyme phosphoenolpyruvate carboxylase (PEPC) in the mesophyll (M) cells cytosol, forming oxaloacetate (OAA). OAA is transferred into the M cells chloroplasts, (2) where it receives two more hydrogen atoms from NADPH, which was formed during the light-dependent reactions around the M cells thylakoids, forming malate. The malate is transported into the chloroplasts of the bundle-sheath (BS) cells and acts as a proton carrier. (3) There, malate is decarboxylated by NADP-Me, producing CO_2 and pyruvate, as well as two NADPH from $NADP^+$ using the carried protons. The pyruvate is transported back into the M cell chloroplasts (4), where it is used to regenerate the PEP pool. The CO_2 release from malate in combination with the absence of the O_2 production by the light-dependent reactions is creating a high CO_2 and low O_2 environment in the chloroplasts of the BS cells. Thus, (5) photorespiration is minimized given that O_2 uptake is strongly reduced by RUBISCO, which increases CO_2 uptake efficiency. As during the C_3 CO_2 fixation, the (6) final carbohydrate synthesis takes place in the CBB cycle, and (7) carbohydrates are further allocated within the cell and eventually the leaf tissue. The (b) NAD-Me subtype differs from the former as (2) aspartic acid (Asp) is formed out of the OAA in the M cells cytosol, which is eventually transported into the BS cells mitochondria, where (3) it is decarboxylated to produce the CO_2 which is transferred to the BS cells chloroplasts.

Patterns and drivers of the heterotrophic 2H fractionation ϵ_H

The δ^2H of leaf sugar explains roughly 70% of the observed variation in δ^2H in leaf cellulose (Fig. 4), similar to the observations by Holloway-Phillips *et al.* (2022), indicating a common mechanism for the heterotrophic 2H fractionation. However, δ^2H of leaf cellulose does only significantly correlate with ϵ_H in C_4 plants. This might be explained by a different timing of the cellulose synthesis, since leaves of grasses, which represent the

majority of the C_4 plants in this study, grow mainly during the day (Poire *et al.*, 2010), while the leaves of dicotyledons continue to grow during the night (Kronenberg *et al.*, 2021). This assumption is also supported by the steeper slope of the linear regression between δ^2H of the leaf cellulose and δ^2H of the leaf sugar in C_4 species (Fig. 4), which is close to the 1 : 1 line. Thus, the observed pattern might be caused by an imbalanced composition regarding monocotyledon and dicotyledon plant species among the three types of CO_2 fixation, rather than by the type of CO_2 fixation

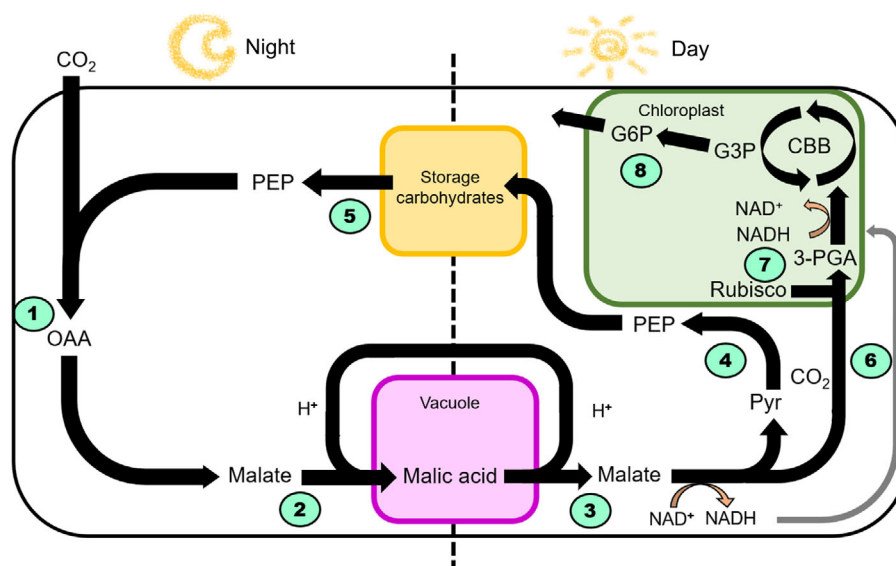


Fig. 7 Simplified scheme of CAM CO₂ fixation, including only the steps crucial for hydrogen isotopes, according to Schiller & Bräutigam (2021) and Winter & Smith (2022). The chemical reactions in CAM CO₂ fixation are temporally separated, with the primary CO₂ fixation (Steps 1, 2, and 5) occurring during the night, and the carbohydrate synthesis (Steps 3, 4, 6, 7, and 8) during the day (Winter & Smith, 1996a,b). During the night, (1) CO₂ is taken up via the stomata and used to carboxylate phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxylase (PEPC), forming oxaloacetate (OAA) as a H⁺ carrier. Subsequently, (2) malate is formed out of OAA, which is eventually being stored as malic acid in the cell's vacuole. During the day, (3) malic acid is exported out of the vacuole, transformed into malate and decarboxylated in the cytoplasm, releasing CO₂ and generating H⁺ for regenerating NADH, which is (6) transferred into the chloroplasts, where (7) the high CO₂ concentration inhibits photorespiration, and the NADH from the decarboxylation of the organic acid carrier is consumed for the CBB cycle. Eventually, (8) the fresh assimilates are exported out of the chloroplast and redistributed within the leaf.

itself. However, potential differences between monocots and dicots will need to be investigated in further studies.

Significant negative relationships between ϵ_A and ϵ_H were observed in all three types of CO₂ fixation (Fig. 4). We speculate that, in C₃ plants, both the autotrophic ²H depletion and the heterotrophic ²H enrichment of the sugar pool are affected by the metabolic activity of a plant. These negative relationships could therefore correspond to a general reflection of the source–sink relationship in ϵ_A vs ϵ_H . Hence, the more new ²H depleted sugars are produced through a strong source activity, the stronger the signal of ϵ_A will become. At the same time, a stronger sink activity might enforce the ²H enriching ϵ_H , leading to more ²H enriched leaf cellulose. However, this hypothesis needs to be tested in further studies. The absence of a relationship between ϵ_A and ϵ_H in C₄ at 30°C (both at low and high VPD) can be explained by the earlier discussed relative ²H depletion of the water inside BS compared with M cells (Zhou *et al.*, 2018), which can be expected to be stronger at higher temperatures (Cernusak *et al.*, 2022). The different timing of cellulose synthesis in monocotyledonous compared with dicotyledonous plant species (Poire *et al.*, 2010) could contribute to the observed pattern. The reduced relationship between ϵ_A and ϵ_H in CAM plants compared with C₃ plants might be caused by the overall slower metabolic rate of these plants, causing a reduced ϵ_H , which was observed in species with strong CAM CO₂ fixation (Fig. S1).

There were significant but inconsistent relationships between $\delta^2\text{H}$ of leaf cellulose and leaf water (Fig. S7), with distinct relationships between ϵ_H and $\delta^2\text{H}$ of leaf water in different types of CO₂ fixation (Fig. S8). In C₃ plants, ϵ_H did not correlate with

$\delta^2\text{H}$ of leaf water. In C₄ plants, there was a positive relationship, while in CAM plants under high temperatures, there was a negative relationship between ϵ_H and leaf water $\delta^2\text{H}$. These findings are surprising because the biochemical reactions responsible for ²H fractionation during cellulose synthesis should not fundamentally differ among the three types of CO₂ fixation. However, the structure and composition of the grass cell walls, representing the majority of the C₄ plants in this study, are fundamentally different from those of dicotyledons (Vogel, 2008; Zhong *et al.*, 2018). These structural and biochemical differences between grasses and dicotyledons may explain the positive relationship observed here between ϵ_H and $\delta^2\text{H}$ of leaf water in C₄ plants. These results suggest that dicotyledons should be separated from monocotyledons such as grasses in further studies of ²H fractionation during cellulose synthesis.

One possible driver of the variability of ϵ_A and ϵ_H could be based on variations in the plant's carbohydrate metabolism. For instance, the timing and reactions involving leaf transitory starch dynamics varies in plants with C₃, C₄, and CAM CO₂ fixation (Weise *et al.*, 2011). Another explanation for the negative relationship between ϵ_H and $\delta^2\text{H}$ of leaf water in CAM plants is a gradual contribution of C₃ and CAM CO₂ fixation among different species, since C₃ CO₂ fixation shows more negative leaf water $\delta^2\text{H}$ and more ²H enriching ϵ_H than CAM CO₂ fixation. Notably, excluding the two facultative CAM species (Fig. S1; Table 1) resulted in lower ϵ_H values, supporting the idea that ϵ_H could be influenced by a plant's metabolic rate, which is reduced in CAM compared with C₃ and C₄ plants (Lüttge, 2004).

Component-specific ^2H response to changes in temperature and VPD is highly species-specific

Across all species (Table 2), we found no general temperature and minor VPD effects on $\delta^2\text{H}$ of leaf water, leaf sugar, and leaf cellulose in C_3 and CAM plants (Fig. 3). However, we observed various significant species-specific responses of $\delta^2\text{H}$ of leaf water, leaf sugar and leaf cellulose, as well as their ϵ_A and ϵ_H , to changes in temperature and VPD (Tables 3–5). We suggest that the observed lack of general responses is caused by the generally high variability of $\delta^2\text{H}$, which masks general temperature and VPD effects that can be seen only at the species level.

In C_3 plants (Fig. 3), species-specific changes might result from various processes, such as an up- or downregulation of the photosynthetic activity (i.e. the light-dependent reactions), transpiration, photorespiration, respiration or metabolic activity (Sanchez-Bragado *et al.*, 2019; Holloway-Phillips *et al.*, 2022). This can depend on a plant's physiological climate response (Bolstad *et al.*, 2003; Middleby *et al.*, 2024) due to their species-specific climate adaption (Cavieres *et al.*, 2000; Loveys *et al.*, 2002), such as changes in the ratio of photosynthesis (P) to respiration (R). These climate responses might be further modified by the unsaturated photosynthetic active radiation of $110 \mu\text{mol m}^{-2} \text{s}^{-1}$ during this experiment, which likely directly impacted the P : R ratio. As ϵ_A in this study is calculated as the difference between $\delta^2\text{H}$ of leaf sugar and $\delta^2\text{H}$ of leaf water, it is an apparent fractionation factor, combining autotrophic and heterotrophic processes (Holloway-Phillips *et al.*, 2022; Vitali *et al.*, 2024). Thus, follow-up studies are needed to disentangle the response of ϵ_A and ϵ_H , as well as $\delta^2\text{H}$ of sugar and cellulose to changes in temperature and PAR, including measurements of gas exchange and Chl fluorescence. C_4 plants showed a much more limited response to changes in both temperature and VPD (Fig. 4). This might be due to the lower overall autotrophic ^2H fractionation in C_4 compared with C_3 and CAM plants (Fig. 1). An interesting outlier among the tested C_4 plants was *Salsola soda*, a species already known to switch from C_3 to C_4 CO_2 fixation after its seedling stage (Lauterbach *et al.*, 2017). The increase in ϵ_H observed here with increasing temperature and VPD is comparable to what was observed in some CAM species (Fig. 5). *S. soda* is a succulent halophyte native to the Mediterranean Basin. Thus, the species is adapted to limited water availability, and one can speculate that it might perform some level of CAM CO_2 fixation in response to harsher climatic conditions. Similarly, we assume that the observed variation in some of the CAM plants was triggered by an upregulation of CAM with a simultaneous downregulation of C_3 CO_2 fixation in response to higher temperature and VPD.

Conclusions

We demonstrated that the different biochemical reactions involved in C_3 , C_4 , and CAM CO_2 fixation are the principal drivers of their autotrophic ^2H fractionation and their responses to changes in climatic conditions. While ϵ_H from sugar to cellulose is probably based on the same biochemical reactions among

all plants, it is likely shaped by processes such as a plant's metabolic activity, the diel timing of its growth, and the structure and biochemical composition of the cell wall, especially when comparing grasses with dicotyledons. The diversity of the processes involved in ^2H fractionation in plant carbohydrates might make it less straightforward to implement $\delta^2\text{H}$ analysis in a broad range of studies. Further experiments combining $\delta^2\text{H}$ measurements of leaf water, non-structural carbohydrates, and cellulose with measurements of gas exchange and Chl fluorescence are needed to identify the drivers of ^2H fractionation during the carbohydrate metabolism of plants. Such studies might further reveal the driving forces behind the here and elsewhere observed $\delta^2\text{H}$ variability in carbohydrates of C_3 plants, and in species with a more variable biochemical background, for example which can switch between C_3 , C_4 , and CAM CO_2 fixation. However, it could become a useful tool for various plant eco-physiological investigations, such as studies on facultative CAM or plant internal carbon dynamics.

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Competing interests

None declared.

Author contributions

PS designed the experiment and took the lead in writing the manuscript. PS, OR and MO performed the experiment and processed the samples. VV, OR, MS, MO, LAC, AG, NB and MML contributed to the interpretation of the results and the writing of the manuscript. MML supervised the project.

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Data availability

All data are included in the tables of the manuscript.

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Supporting Information

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

Fig. S1 Comparison of the $\delta^2\text{H}$ (compounds) and $\Delta^2\text{H}$ (fractionation factors) values within and between the three types of CO₂ fixation pathways and the three climate treatments without the two facultative CAM species.

Fig. S2 $\delta^2\text{H}$ (compounds) and $\Delta^2\text{H}$ (fractionation factors) values across all tested plant species within the three CO₂ fixation pathways (i.e. C₃, C₄, CAM) of leaf water, leaf sugar, leaf cellulose, ϵ_A and ϵ_H in response to changes in temperature and VPD.

Fig. S3 $\delta^2\text{H}$ (compounds) and $\Delta^2\text{H}$ (fractionation factors) of all tested plant species with strong CAM CO₂ fixation pathway of leaf water, leaf sugar, leaf cellulose, ϵ_A and ϵ_H in response to changes in temperature and VPD without the two facultative CAM species.

Fig. S4 Regression analysis showing the effect of changing temperature and VPD on C_3 CO_2 fixation: δ^2H leaf sugar/ δ^2H leaf water; δ^2H leaf sugar/ ϵ_A ; δ^2H leaf cellulose/ δ^2H leaf sugar; δ^2H leaf cellulose/ ϵ_H ; ϵ_A/ϵ_H .

Fig. S5 Regression analysis showing the effect of changing temperature and VPD on C_4 CO_2 fixation: δ^2H leaf sugar/ δ^2H leaf water; δ^2H leaf sugar/ ϵ_A ; δ^2H leaf cellulose/ δ^2H leaf sugar; δ^2H leaf cellulose/ ϵ_H ; ϵ_A/ϵ_H .

Fig. S6 Regression analysis showing the effect of changing temperature and VPD on CAM CO_2 fixation: δ^2H leaf sugar/ δ^2H leaf water; δ^2H leaf sugar/ ϵ_A ; δ^2H leaf cellulose/ δ^2H leaf sugar; δ^2H leaf cellulose/ ϵ_H ; ϵ_A/ϵ_H .

Fig. S7 Relation between δ^2H of leaf cellulose and leaf water between the three treatments and the three types of CO_2 fixation. The blue line represents the linear regression.

Fig. S8 Relationship between ϵ_A and δ^2H of leaf water of C_3 , C_4 , CAM plants, as well as CAM plants excluding the two facultative CAM species *Delosperma cooperi* and *Mesembryanthemum cordifolium*, separated by growing condition.

Fig. S9 Relationship between ϵ_H and δ^2H of leaf water of C_3 , C_4 , CAM plants, as well as CAM plants excluding the two facultative CAM species *Delosperma cooperi* and *Mesembryanthemum cordifolium*, separated by growing condition.

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