RESEARCH ARTICLE

The effect of processing medium on the $^2\text{H}/^1\text{H}$ of carbon-bound hydrogen in α -cellulose extracted from higher plants

Zhenyu Zhu^{1,4} | Xijie Yin² | Fengyan Lu³ | Bo Wang¹ | Ran Ma¹ | Yu Zhao¹ | Ying Wang¹ | Yi Ma¹ | Jing Su² | Qiulin Yan¹ | Charles H. Hocart^{1,5} | Youping Zhou^{1,4}

¹Isotopomics in Chemical Biology & Shaanxi Key Laboratory of Chemical Additives for Industry, School of Chemistry & Chemical Engineering, Shaanxi University of Science & Technology, Weiyang University Park, Xi'an 710021, China

²Laboratory of Marine & Coastal Geology, MNR Third Institute of Oceanology, Xiamen 361005, China

³Institute of Earth Environment, Chinese Academy of Sciences, Xi'an 710061, China

⁴Southern Marine Science and Engineering Guangdong Laboratory, Zhuhai 519000, China

⁵Research School of Biology, Australian National University, Canberra 2601, Australia

Correspondence

Y.-P. Zhou, Isotopomics in Chemical Biology, School of Chemistry & Chemical Engineering, Shaanxi University of Science & Technology, Weiyang University Park, Xi'an 710021, China. Email: youping.zhou@sust.edu.cn

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Rationale: Although the $^2\text{H}/^1\text{H}$ ratio of the carbon-bound hydrogens (C-Hs) in α -cellulose extracted from higher plants has long been used successfully for climate, environmental and metabolic studies, the assumption that bleaching with acidified NaClO_2 to remove lignin before pure α -cellulose can be obtained does not alter the $^2\text{H}/^1\text{H}$ ratio of α -cellulose C-Hs has nonetheless not been tested.

Methods: For reliable application of the $^2\text{H}/^1\text{H}$ ratio of α -cellulose C-H, we processed plant materials representing different phytochemistries and photosynthetic carbon assimilation modes in isotopically contrasting bleaching media (with an isotopic difference of 273 mUr). All the isotope ratios were measured by elemental analyzer/isotope ratio mass spectrometry (EA/IRMS).

Results: Our results show that H from the bleaching medium does appear in the final pure α -cellulose product, although the isotopic alteration to the C-H in α -cellulose due to the incorporation of processing H from the medium is small if isotopically "natural" water is used to prepare the processing medium. However, under prolonged bleaching such an isotope effect can be significant, implying that standardizing the bleaching process is necessary for reliable $^2\text{H}/^1\text{H}$ measurement.

Conclusions: The currently adopted method for removing lignin for α -cellulose extraction from higher plant materials with acidified NaClO_2 bleaching is considered acceptable in terms of preserving the isotopic fidelity if isotopically "natural" water is used to prepare the bleaching solution.

1 | INTRODUCTION

The natural abundance $^2\text{H}/^1\text{H}$ ratio of the carbon-bound hydrogens (C-Hs) of α -cellulose in higher plants has been shown to provide a useful record of the growth environment,¹ climate,^{2–8} and plant metabolic status.^{9,10} Accurate and precise isotope measurement, a must for reliable interpretation of the signal recorded in the isotopic compositions of α -cellulose, can be obtained only if (1) the α -cellulose

is of an acceptable purity, (2) the original isotopic signature is retained during sample processing to α -cellulose, and (3) isotopic analysis is conducted with robust instrumentation techniques. A chemically pure α -cellulose product does not necessarily guarantee isotopic fidelity.

To extract α -cellulose, solvent mixtures of various polarities are used to, first, remove the non-structural components of the cell wall preparation. It is not expected that this process will affect the H isotopic composition of α -cellulose as the solvent mixture works by pure dissolution and involves no chemical bond formation or fission on the α -cellulose. Likewise, the strong and concentrated alkaline

Zhenyu Zhu, Xijie Yin, and Fengyan Lu contributed equally to this work.

solution (NaOH or KOH) usually used to hydrolytically remove hemicellulose is predicted to have no hydrogen isotope effect on the α -cellulose as it is unable to hydrolyze α -cellulose. Oxidative removal of lignin, the other structural component, with (acidified) chlorite (ClO_2^-) or similar oxidants such as NO_3^- , $^{11}\text{Cl}_2$, $^{12}\text{HOCl}$, 13 and ClO_2 ,¹⁴ is necessary for obtaining acceptably pure α -cellulose and works by oxidative cleavage of the lignin (producing a mixture of CO_2 , small water-soluble molecules, or lignin fragments that can be removed subsequently by centrifugation) via a free radical mechanism. This process has been found to result in the incorporation of O from the processing medium into the structure of the purified α -cellulose product when the inferred O-containing chemical bond between α -cellulose and lignin is oxidatively cleaved.^{15,16}

Although breaking the O-containing bond between lignin and α -cellulose theoretically induces no H isotope effect to the α -cellulose, the free radical that oxidizes the lignin may weaken the C-H bonds in the glucose units of α -cellulose, and therefore cause a replacement of the C-Hs in the α -cellulose. Indeed, evidence was presented for the replacement of these hydrogens in plant cell wall polysaccharides by H from the processing medium when attacked by hydroxyl radicals ($\text{HO}^\bullet$)¹⁷ under aerobic conditions. If this occurs during the oxidative removal of lignin by bleaching, the H isotope fidelity of the extracted α -cellulose will be disturbed. It is therefore the purpose of the work reported in this paper to investigate if, and to what extent, the processing medium affects the isotopic compositions of the C-Hs in the final α -cellulose product. Structural components of plant materials from different organs (leaf and stem) representing all three photosynthetic tissue types were first hydrolyzed with NaOH and then bleached with NaClO_2 solutions of contrasting $^2\text{H}/^1\text{H}$ ratios. The isotopic ratios of the isolated α -cellulose products at different stages of the bleaching process were then compared to gain insights into the effect of the processing medium on the measured $^2\text{H}/^1\text{H}$ ratios in α -cellulose.

2 | MATERIALS AND METHODS

2.1 | Plant samples for the test

The effect of processing media was tested on the leaves of two C3 species: *Pinus palustris* (pine) and *Eucalyptus longifolia* (eucalypt) (grown on the campus of Curtin University, Perth, Australia); two C4 species, *Saccharum officinarum* (sugarcane) and *Poa* (an unknown grass); and two CAM species, *Bromelia serra variegata* (bromeliad) and *Ananas bracteatus* (pineapple) (grown in glasshouses at the Research School of Biology, Australian National University, Canberra, Australia). We also compared photosynthetic (leaf) and non-photosynthetic (stem) organs for the two investigated C3 species as the stems (tree ring) are the favored subject from which an isotopic signature is extracted for ecological and climatological studies. The phytochemistries of these two C3 species are known to be different.

2.2 | Experimental design and sample processing

Samples for isotopic analysis were processed using the method of Zhou et al.¹⁶ with slight modification (Figure 1). Briefly, samples were first washed in deionized water, oven-dried, powdered and sieved. The samples were then exhaustively extracted with methanol/dichloromethane, acetone, water and finally absolute ethanol to remove non-structural components of the cell wall preparation. The pelleted holocellulose-lignin complex (HCLC) was oven-dried after centrifuging, weighed and split into two halves. The two halves were then hydrolyzed under dry N_2 in sealed centrifuge tubes (to minimize ambient CO_2 and vapor entering into the alkaline solutions and loss of vapor to the air, thus changing the pH and $\delta^2\text{H}$ values of the hydrolytic solutions) with either unenriched ($\delta^2\text{H} = -24.2$ mUr, equivalent to ‰) or ^2H -enriched ($\delta^2\text{H} = 248.7$ mUr, prepared by diluting 99.9% $^2\text{H}_2\text{O}$ with deionized water) aqueous NaOH (17%, w/v, ~4 M) for 2 h at 70°C to remove hemicellulose.

On completion of the hydrolysis, the samples (α -cellulose-lignin complex, α -CLC) were centrifuged and washed repeatedly with deionized water ($\delta^2\text{H} = -24.2$ mUr) at 70°C for 2 h, and finally dried with absolute ethanol. An aliquot of the α -CLC was then taken for $\delta^2\text{H}$ measurement following the procedure described in section 2.4. The lignin in the remaining α -CLC (in the range of 20–100 mg) was then removed by bleaching in solutions made of 15 g NaClO_2 and 10 mL CH_3COOH (AcOH) dissolved in 1050 mL of waters of contrasting $\delta^2\text{H}$ values (unenriched: -24.2 mUr; ^2H -enriched: 248.7 mUr) at 70°C . The contribution of H to the processing media by the CH_3COOH (0.14%) is negligible. During the process of bleaching, the centrifuge tube headspace was filled with dry N_2 and sealed to minimize change in the $\delta^2\text{H}$ values of the bleaching media due to exposure to ambient water vapor and hydrogen-containing compounds. We have shown previously^{15,16} that, after 1.5 h of bleaching, acceptably pure α -cellulose products can be obtained. To ensure the completeness of bleaching and to examine the effect of prolonged bleaching on the isotopic composition of α -cellulose product, in this work we extended the bleaching time to 3 h.

On being recovered after different periods of bleaching, the α -cellulose products were immediately frozen in liquid N_2 and freeze-dried. The samples were then washed with copious amounts of deionized water ($\delta^2\text{H} = -12.9$ mUr) at 70°C for 2 h to allow complete replacement of any adsorbed bleaching solution. After being pelleted by centrifuging, the samples were washed with absolute ethanol and dried and stored in a desiccator filled with dry N_2 before being analyzed for their $\delta^2\text{H}$ values following the procedure described below.

In our previous work,^{15,16} we have shown that our method is capable of producing α -cellulose of high purity (lignin content was below gas chromatography/mass spectrometry (GC/MS) detection limits) with residual hemicellulose under 4% by weight (GC/MS assay of hydrolyzed monosaccharides derivatized as alditol acetates). However, we have not quantified (a) the effect of adsorption of water on the final (pure) α -cellulose recovered from different bleaching solutions or (b) the effect of possible exposure to laboratory air while

FIGURE 1 Flowchart for sample preparation and IRMS analysis. As shown in Figures 3, loss of lignin was minimal after 2 h bleaching, indicating that pure α -cellulose was obtained after 2 h of bleaching. The α -CLC and crude cellulose (obtained in the first 2 h bleaching) and pure α -cellulose (obtained after 2 h bleaching) were analyzed for their $\delta^2\text{H}$ values [Color figure can be viewed at wileyonlinelibrary.com]

the α -CLC and the α -cellulose samples (tightly wrapped but unsealed) waited on an autosampler tray to be analyzed for their $^2\text{H}/^1\text{H}$ ratios. As these effects may alter/skew the isotopic compositions of α -CLC and α -cellulose samples, we added two more tests to address these issues.

2.2.1 | Time needed to completely replace hydrolyzing and bleaching solutions with deionized water after the hydrolyzing and bleaching steps

To determine the time needed for complete replacement of the hydrolyzing and bleaching solutions adsorbed onto the α -CLC and α -cellulose samples, we randomly chose one pair (recovered from ^2H -enriched versus unenriched processing media) of the original α -CLC and α -cellulose samples (Table 1) on completion of hydrolysis and bleaching, and immediately froze them in liquid N_2 . Following freeze-drying, the samples were dispersed in deionized water ($\delta^2\text{H} = -12.9 \text{ mUr}$) at 70°C to allow replacement of adsorbed water. Aliquots taken at 0.5, 1, 1.5 and 2 h were frozen in liquid N_2 and then freeze-dried. The freeze-dried samples were kept sealed in a desiccator filled with dry N_2 before being analyzed for their $\delta^2\text{H}$ values following the procedure described below.

2.2.2 | Time needed to establish isotopic equilibrium between laboratory air and the exchangeable H in the recovered samples from hydrolysis and bleaching steps

To find the minimum time for re-equilibrium to be established between the laboratory air moisture and the exchangeable H in the α -CLC and α -cellulose samples (see Table 2), we tightly wrapped the samples in silver capsules (not an air-tight seal) and allowed them to equilibrate with the laboratory air (22°C and 50% relative humidity, $\delta^2\text{H}$ value of air moisture is -12.9 mUr) for 1, 2, 3, 4.5, 5.5 and 6 days and measured the isotopic compositions of the samples taken at each time point using the procedure described below.

Finally, as the isotopic composition of the processing media may change as a result of exchange with α -CLC during the bleaching process, we also monitored the variation of the isotopic composition of the bleaching media by taking aliquots of the bleaching liquids (Table 3) at six time points along the bleaching process. Cryogenic distillation was then used to extract the water from the aliquots (after they were filtered to remove solids) and the isotopic compositions of these water samples were measured following the procedures described below.

TABLE 1 Isotopic ($\delta^2\text{H}$ value, mUr, V-SMOW) change for the pairs (^2H -enriched and unenriched) of α -CLC and α -cellulose processed from *Pinus palustris* stem over a period of 2 h heating in pure water to establish the minimal time for complete replacement of residual (adsorbed) water. All averages reported here are the results of quadruplicated analysis

	$\delta^2\text{H}_{\text{enriched}}$ (mUr)		$\Delta_{\text{enriched-unenriched}}$	$\delta^2\text{H}_{\text{unenriched}}$ (mUr)	
Sample	Avg	Stdev		Avg	Stdev
α -CLC					
0 h	−22.9	0.3	4.2	−18.7	0.4
0.5 h	−23.3	0.4	4.8	−18.5	0.4
1 h	−23.5	0.2	4.4	−19.1	0.3
1.5 h	−23.4	0.3	5.2	−18.2	0.3
2 h	−23.8	0.1	5.1	−18.7	0.2
Average	−23.4			−18.6	
α -cellulose					
0 h	−22.3	0.4	1.9	−20.4	0.3
0.5 h	−22.0	0.2	1.5	−20.5	0.7
1 h	−22.2	0.3	2.0	−20.2	0.4
1.5 h	−21.9	0.2	2.2	−19.7	0.3
2 h	−22.7	0.3	2.8	−19.9	0.2
Average	−22.2			−20.1	

2.3 | Correction for the adsorption of laboratory air moisture on the isotopic compositions of α -cellulose

As described below, all the crude and pure α -cellulose samples (including the α -CLCs recovered from hydrolysis) and standards were tightly wrapped in silver capsules (not air-tight) and dried in an oven (temperature 70°C; relative humidity 50%) for a minimum of 6 days to allow complete equilibration of adsorbed moisture with laboratory air. To estimate the maximum effect of laboratory air moisture on the measured isotopic compositions of the samples, we cryogenically collected laboratory air moisture (-15.0 mUr) with a dry-ice/ethanol trap under the suction of a pump following the procedure of Zhou et al.¹⁸ The laboratory air temperature and relative humidity were kept constant and recorded, and were used to calculate the true $\delta^2\text{H}_{\text{C-H}}$ value of the pure α -cellulose product using Equation (1):

$$\delta^2\text{H}_{\alpha\text{-cellulose}} \approx \frac{\left[\left(\delta^2\text{H}_{\text{vapor}} + 1000 \right) \alpha_{\text{vapor_liquid}} - 1000 \right] \left(\frac{w}{18} * 2 \right) + \left[\frac{(100-w)}{162} * 10 \right] \left[(1-f) \left[\left(\delta^2\text{H}_{\text{vapor}} + 1000 \right) \alpha_{\text{vapor_O-H}} - 1000 \right] + f \delta^2\text{H}_{\text{C-H}} \right]}{\left(\frac{w}{18} * 2 \right) + \frac{(100-w)}{162} * 10} \quad (1)$$

where $\delta^2\text{H}_{\alpha\text{-cellulose}}$ and $\delta^2\text{H}_{\text{vapor}}$ are the measured values of the pure α -cellulose products and laboratory air, respectively, while w , $\alpha_{\text{vapor_O-H}}$ and $\alpha_{\text{vapor_liquid}}$ are the weight percentage of adsorbed air moisture in the samples, and the isotopic fractionation factors between vapor and the O-H, and between vapor and the adsorbed moisture (liquid), respectively. In this study, we use a value of 1.0562

(calculated following Horita and Wesolowski¹⁹) for $\alpha_{\text{vapor_liquid}}$ as an approximation for the EIF (equilibrium isotope fractionation) between water adsorbed onto pure α -cellulose and the surrounding vapor, and 3–5% for w according to Saurer and Siegwolf²⁰ and Stamm.²¹ Note that when $w \leq 5\%$, only part of the exchangeable H in the α -cellulose can be in equilibrium with liquid moisture while the rest must be in equilibrium with vapor. We tested the non-exchangeable fraction H $f = 72\%$ and $f = 73\%$ (instead of the theoretical maximum 70%, note that 7 of the 10 H atoms in the glucose unit of the α -cellulose are non-exchangeable and that not all the theoretically exchangeable H atoms in the α -cellulose are accessible for exchange due to physical protection by the microcrystallinity) in the range of $w = 3\text{--}5\%$ (see Table S1, supporting information, for detailed calculations). The molecular weights of water and the glucose unit in the α -cellulose are 18 and 162, respectively.

The correction can be applied only to the pure α -cellulose products and not to the crude α -cellulose products or the α -CLCs, as the lignin in the latter two does not have a fixed chemical composition and the isotopic fractionation between exchangeable H in the lignin and the processing media varies as bleaching proceeds. A similar approach was used by Feng et al.²² to calculate the $\delta^2\text{H}_{\text{C-H}}$ value in

TABLE 2 Isotopic change ($\delta^2\text{H}$ value, mUr, V-SMOW) of *Pinus palustris* α -cellulose (recovered from 2.5 h bleaching) over a period of 5 days heating in an oven at 70°C to establish the minimal time needed for the re-equilibration of adsorbed water with laboratory air moisture

Date	Time	$\delta^2\text{H}_{\alpha\text{-cellulose, enriched (mUr)}}$		$\delta^2\text{H}_{\alpha\text{-cellulose, unenriched (mUr)}}$	
		Avg	Stdev	Avg	Stdev
Day 1	10: 30	−28.2	0.3	−29.9	0.4
Day 2	12: 20	−27.3	0.6	−29.1	0.5
Day 3	11: 25	−26.3	0.4	−28.4	0.3
Day 4	18: 27	−25.9	0.4	−28.6	1.1
Day 5	10: 35	−26.0	0.5	−28.2	0.2
Day 5	18: 50	−26.2	0.8	−28.4	0.6
Day 6	11: 40	−25.9	0.5	−28.7	0.3
Day 6	16: 20	−25.9	0.5	−28.4	0.5

α -cellulose but these authors failed to recognize that there is always water adsorbed onto α -cellulose whatever methods are used to dry the samples.

2.4 | Isotopic analysis

All the solid (α -CLC, and crude and pure α -cellulose products and certified reference materials EMA P1: $\delta^2\text{H} = -87.80$ mUr, purchased from Elemental Analysis Ltd, Okehampton, UK; referenced to IAEA V-SMOW) and water samples (including in-house water isotope standards GBW-0440: $\delta^2\text{H} = -0.4$ mUr and GBW-0443: $\delta^2\text{H} = -189.1$ mUr, also referenced to IAEA V-SMOW) were analyzed for their H isotope composition following the method described in Zhou et al.¹⁶ Briefly, the H in the solid samples (0.2–0.3 mg), standards and reference materials wrapped in silver capsules (but not air-tight) was introduced by a AI 3000 autosampler and quantitatively pyrolyzed in a glassy-carbon-lined ceramic tube at 1380°C to H_2 in a Flash HT (1112 series) EA (elemental analyzer) coupled to a MAT 253 Plus isotope ratio mass spectrometer via a Conflo III interface (all instruments from Thermo Fisher Scientific GmbH, Bremen, Germany). The water samples (1 μL withdrawn from a vial sealed with rubber septum by the AI 3000 autosampler) were injected directly into the same Flash HT elemental analyzer, thermally converted into H_2 and analyzed under exactly the same conditions on the same isotope ratio

mass spectrometry (IRMS) system as was used for the solid samples. We kept the temperature and humidity around the mass spectrometry facility stable throughout the analytical course to ensure batch-to-batch comparability.

Because of the hygroscopic nature of the samples, isotopic change to the samples is still therefore possible due to progressive adsorption of air moisture while samples are sitting in the autosampler tray. To minimize the isotopic effect of such exposure, only six samples per batch were loaded into the autosampler at any one time; thus, no sample was exposed to the ambient laboratory atmosphere for more than 1 h. Under such conditions, no significant changes in isotopic composition were observed (Table S2, supporting information).

In all H isotopic analysis, the H_3^+ factor was monitored daily and the average value was always less than 6 ppm/nA. The isotopic compositions were reported in the standard δ -notation relative to V-SMOW (Vienna Standard Mean Ocean Water):

$$\delta = (\text{R}_{\text{sample}} - \text{R}_{\text{standard}}) / \text{R}_{\text{standard}} (\text{mUr})$$

where R_{sample} and $\text{R}_{\text{standard}}$ are the isotopic ratios ($^2\text{H}/^1\text{H}$) of sample and standard, respectively. All analyses were carried out in duplicate or triplicate unless indicated otherwise. The external precision of repeated analyses of the isotopic standards was better than 2.5 mUr for solids and 1.4 mUr for water.

TABLE 3 Variations in $\delta^2\text{H}$ values (mUr, V-SMOW) of bleaching media during the course of bleaching experiments with a randomly chosen α -CLC sample (processed from *Pinus palustris* leaf)

Time (h)	$\delta^2\text{H}_{\text{medium, enriched (mUr)}}$					$\Delta_{\text{enriched-unenriched}}$	$\delta^2\text{H}_{\text{medium, unenriched (mUr)}}$				
	rpt1	rpt2	rpt3	Avg	Stdev		rpt1	rpt2	rpt3	Avg	Stdev
0.5	248.2	247.7	248.2	248.0	0.3	272.7	−24.2	−24.6	−25.1	−24.6	0.5
1.0	250.7	248.7	248.8	249.4	1.1	274.4	−24.3	−24.9	−25.7	−25.0	0.7
1.5	252.1	250.3	250.1	250.8	1.1	274.8	−23.7	−24.4	−23.9	−24.0	0.4
2.0	254.1	251.2	250.9	252.1	1.8	276.2	−23.3	−24.1	−24.9	−24.1	0.2
2.5	253.1	252.1	255.5	253.6	1.7	277.4	−24.1	−23.7	−23.7	−23.8	0.2
3.0	254.1	251.2	252.9	252.7	1.5	275.6	−24.8	−22.7	−23.1	−22.9	0.2

2.5 | Statistical analysis

The inbuilt statistic functions of Microsoft Excel (2010)[®] was employed to *t* test if the means of the averages of recovered crude and pure α -celluloses from the ^2H -enriched and the ^2H -unenriched bleaching media are significantly different.

3 | RESULTS AND DISCUSSION

3.1 | Residual water and moisture adsorption effects on the isotopic compositions of recovered hydrolysis and bleaching products

3.1.1 | Minimum time to completely replace residual water

The $\delta^2\text{H}$ values of the α -CLC and pure α -cellulose extracted from a *P. palustris* stem, recovered at various time points over a period of 2 h heating in pure water of known $\delta^2\text{H}$ value to replace the residual waters, are listed in Table 1. No significant variation in $\delta^2\text{H}$ values was observed in either of the two α -CLC and pure α -cellulose products investigated, indicating that residual water was completely replaced, probably in the first half hour of heating.

3.1.2 | Minimum time to reach equilibrium between adsorbed and vapor

The changes in the $\delta^2\text{H}$ values of α -cellulose products (recovered from 2.5 h bleaching in both ^2H -enriched and unenriched media) of *P. palustris* over a period of 5 days are listed in Table 2. There were

slight increases of 2.3 mUr and 1.7 mUr in the first 4 days in the α -celluloses recovered from the ^2H -enriched and unenriched bleaching media, respectively, and no significant changes after 4 days, indicating that, by the 4th day, isotopic equilibria between adsorbed water and vapor were already established.

3.1.3 | Medium isotopic change over the bleaching course

The $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of the bleaching media sampled at six time points during the bleaching process are listed in Table 3. A slight increasing trend for both $\delta^2\text{H}$ (5.6 mUr) and $\delta^{18}\text{O}$ (1.6 mUr) values was observed, probably associated with evaporative loss of lighter isotopes during the bleaching process.

Thus, we have shown that any isotopic differences observed between the crude and pure α -cellulose products must be mainly due to the bleaching chemistries rather than measurement errors.

3.2 | Isotopic differences between α -CLC recovered from hydrolysis in solutions of contrasting isotopic compositions

The isotopic differences (uncorrected for the adsorption of moisture) of α -CLC recovered from ^2H -enriched and ^2H -unenriched hydrolytic media vary from a negligible 1.9 mUr for the eucalypt leaf to a significant 20.2 mUr for the grass leaf (Figure 2, see also Table S1, supporting information, for data). Since only two species for each photosynthetic mode were investigated, we are not sure if the lack of discernable difference between leaf and stem and the larger difference found in CAM and C4 plants than in C3 plants are

FIGURE 2 Raw and corrected (for the contribution of exchangeable H) $\delta^2\text{H}$ values of the recovered crude and pure α -cellulose ($\delta^2\text{H}_{\text{crude-cellulose}}$ in Equation 1) over the course of bleaching in both ^2H -enriched and unenriched media. Following Equation (1), the correction was based on: Measured vapor $\delta^2\text{H}_{\text{vapor}} = 15.0$ mUr; a relative humidity of 50%; room temperature $T = 22^\circ\text{C}$ (295 K); $\alpha_{\text{v-C-OH}} = 1.1000$; $\alpha_{\text{vapor-liquid}} = 1.0562$; percentage of adsorbed water $w = 5\%$ and the exchangeable H in pure α -cellulose of 28% [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 3 Hydrogen isotopic exchange between the α -H (C-H) of the ester bonds in lignin and the processing media during hemicellulose removal in strong alkali (NaOH) [Color figure can be viewed at wileyonlinelibrary.com]

statistically significant. The notably larger differences found in the grass (C4) and the CAM leaves are probably a result of the phytochemical differences between C3, C4 and CAM plants.

Hydrolysis in strong alkali breaks up the glycosidic (ether) bonds between the monomers of hemicelluloses, and the ester bonds among the lignin units and between lignin and cellulose (including α -cellulose) monomers (Figure 3). At the end of the hydrolysis, the isotopic differences between the two α -CLC products must be either due to (1) the introduction of H from the media to the C-H in the α -CLCs and/or (2) the adsorption of water (H) from the processing media. Since the adsorbed waters in the two recovered α -CLCs were fully replaced with water of the same isotopic composition, introduction of H from the hydrolysis media to the C-H positions in the α -CLCs must be responsible for the observed differences. This alteration of the C-H in the α -CLC must be due to isotopic exchange between the C-H in lignin α to the ester bond (between the lignin monomer and cellulose and/or among the lignin monomeric units) and the hydrolysis media. This is consistent with the prediction that in strongly alkaline solution, the H atom α to the carbonyl group is highly exchangeable due to its acidity. Other possibilities seem less likely; glycosidic bonds in the α -cellulose and the (non-glycosidic) ether bonds, for example, are normally stable under alkaline conditions.

The presence of ester bonds in milled wood lignin isolated from poplar wood was confirmed by Sun et al.²³ The fact that the grass-derived cellulose investigated here showed much larger isotopic difference implies that the lignin of grass leaves probably contains

more ester bonds than are found in trees. Such isotopic differences between the two α -CLCs were obviously passed on to the bleaching stage.

3.3 | Isotopic composition differences between α -celluloses recovered from bleaching in isotopically contrasting media

As bleaching in acidified NaClO_2 proceeded, the oxidative loss of lignin led to the observed continual decrease in the weight of recovered crude α -cellulose. The accumulated % loss of lignin (calculated as the percentage of weight loss at a particular bleaching stage relative to that of the final stage when all lignin is lost) is plotted against the bleaching time in Figure 4 (see also Table S2, supporting information). Leaf lignin oxidizes faster than stem lignin for the two investigated C3 species. In C4 and CAM species, lignin loss proceeded more slowly than for C3 species. On a stepwise basis, the inferred lignin loss between the ^2H -enriched and unenriched media is statistically indistinguishable, consistent with the prediction that isotopic composition at close to natural abundance should have minimal effect on the oxidation kinetics of the lignin under the conditions used here. It can also be seen that, for all the samples investigated, weight loss after 2 h bleaching was minimal, implying that pure α -cellulose was obtained after 2 h, consistent with the findings of Zhou et al.^{15,16}

FIGURE 4 Weight loss over the course of bleaching. Lignin loss % is calculated with the amount of weight loss to that of the total weight loss when pure α -cellulose was obtained after 2 h of bleaching. Those points where no values were available were due to partial sample loss during processing [Color figure can be viewed at wileyonlinelibrary.com]

The raw (uncorrected) $\delta^2\text{H}$ values of the recovered crude and pure α -cellulose products of all samples investigated (Figure 2, see also Table S1, supporting information, for the data) showed an overall concomitant increasing trend as bleaching proceeded, consistent with the prediction that more medium-derived H (relatively ^2H -enriched against the H in the samples) will be incorporated into the remaining α -CLC (crude α -cellulose). Nevertheless, there are notable exceptions to this overall trend: (1) the first step (0.5 h) of bleaching in pine and eucalypt stems and sugarcane and pineapple leaves saw a significant dip rather than an increase in the $\delta^2\text{H}$ value; (2) eucalypt stem exhibited significant fluctuations in its $\delta^2\text{H}$ values throughout the course of bleaching; and (3) the isotopic differences between the ^2H -enriched and unenriched treatments exhibited no consistent increasing or decreasing trend throughout the bleaching.

To provide a quantitative explanation for the trend and in particular the notable exceptions to it, we first express the $\delta^2\text{H}$ value of the crude α -cellulose ($\delta^2\text{H}_{\text{crude-cellulose}}$) at any stage of bleaching in terms of its non-exchangeable (C-H) and exchangeable H (-OH and -COOH) in both the lignin and the α -cellulose components using Equation (2):

$$\delta^2\text{H}_{\text{crude-cellulose}} \approx \frac{\left[\delta^2\text{H}_{\text{lig}}^{\text{ne}} f_{\text{lig}}^{\text{ne}} + \left(\delta^2\text{H}_{\text{air}} \alpha_{\text{lig}}^{\text{ex}} + \alpha_{\text{lig}}^{\text{ex}} - 1 \right) \left(1 - f_{\text{lig}}^{\text{ne}} \right) \right] (1 - p_{\text{cel}}) A_{\text{H-lig}} + \left[\left(\delta^2\text{H}_{\text{air}} \alpha_{\text{cel}}^{\text{ex}} + \alpha_{\text{cel}}^{\text{ex}} - 1 \right) \left(1 - f_{\text{cel}}^{\text{ne}} \right) + \delta^2\text{H}_{\text{cel}}^{\text{ne}} f_{\text{cel}}^{\text{ne}} \right] p_{\text{cel}} A_{\text{H-cel}}}{(1 - p_{\text{cel}}) A_{\text{H-lig}} + p_{\text{cel}} A_{\text{H-cel}}} \quad (2)$$

where $\delta^2\text{H}_{\text{crude-cellulose}}$ is the $\delta^2\text{H}$ value of the total H in the recovered α -cellulose; $\delta^2\text{H}_{\text{lig}}^{\text{ne}}$ and $\delta^2\text{H}_{\text{cel}}^{\text{ne}}$ are the lumped $\delta^2\text{H}$ values of the non-exchangeable H in lignin and α -cellulose, respectively, at any stage of bleaching; $f_{\text{lig}}^{\text{ne}}$ and $f_{\text{cel}}^{\text{ne}}$ are the fractions of non-exchangeable H of

lignin and α -cellulose, respectively; $\alpha_{\text{cel}}^{\text{ex}}$ and $\alpha_{\text{lig}}^{\text{ex}}$ are the lumped fractionation factors between exchangeable H and the moisture with which isotopic equilibration was reached before isotopic analysis of lignin and α -cellulose, respectively; $\delta^2\text{H}_{\text{air}}$ is the $\delta^2\text{H}$ value of air in equilibrium with the exchangeable H in the recovered α -cellulose; p_{cel} is the weight fraction of α -cellulose in the recovered crude α -cellulose; and $A_{\text{H-lig}}$ and $A_{\text{H-cel}}$ are the percentages of H in the lignin and α -cellulose components in the crude cellulose products, respectively.

We then tried relating the chemistry of lignin bleaching to that of the hydrogen isotope effects occurring to both lignin and cellulose components of the remaining α -CLC (crude α -cellulose). As lignin is a three-dimensional (3D) cross-linked heterogeneous polymer made up of three monomeric units (syringyl **S**, guaiacyl **G** and *p*-hydroxyphenyl **H**) with specific phytochemistry-dependent ratios, it is very difficult (if not impossible) to know with certainty the exact chemistry during the bleaching process in acidified NaClO_2 . Nevertheless, it is generally accepted from past work^{24,25} that the actual oxidant is ClO_2 and that species such as Cl_2 , ClO^- and $\text{O}_2^{\bullet -}$ are probably formed in the bleaching process and are actively involved in the bleaching (Figure 5).

The bleaching proceeds via the following confirmed pathways: (1) aromatic substitution of the C-Hs on the aromatic ring by Cl; (2) electrophilic displacement of the alkyl group connected to the C-1 of the aromatic ring by Cl; (3) oxidation of the hydroxyl group on the

FIGURE 5 Lignin bleaching chemistry and isotopic effects associated with replacement of the aromatic H, displacement of the propanoid side chain H and loss of the H in the methoxyl group. For clarity, only the most abundant β -O-4' linkage between monomers is shown. Acidic hydrolysis of the residual hemicellulose (xylose) is also possible, exposing H-1 and H-2 (the red Hs in the lower panel) for exchange with the processing medium [Color figure can be viewed at wileyonlinelibrary.com]

aromatic ring by Cl_2 to form a quinone; (4) hydrolysis of the RO- (radicoxyl) group by Cl_2 and H_2O to form aromatic OH; (5) hydrolysis of the (residual) ester bonds within the lignin network and between lignin and residual (hemi)cellulose; and (6) ring opening (between C-3 and C-4) to form 2,4-hexadienoic diacid. Chemically and gravimetrically, these processes lead to (a) gradual depolymerization and decomposition of the lignin network; and (b) loss of lignin as the decomposition products are solubilized and removed by successive washings. As the lignin is progressively lost, the elemental composition of the increasingly pure cellulose exhibits a decreasing $A_{\text{H-lig}}$ as lignin H is lost due to chlorination (and other reactions) and because of the increasing incorporation of O into the remaining lignin. This is also accompanied by an increase in the $A_{\text{H-cel}}$ value. In terms of the relative contributions of the non-exchangeable and exchangeable Hs in the lignin, the non-exchangeable fraction $f_{\text{lig}}^{\text{ne}}$ decreases due to continual loss of C-H as a result of the aromatic H replacement with Cl, demethoxylation and displacement of the propanoid side chain in the lignin monomeric unit.

Isotopically, replacement with H from relatively ^2H -enriched processing media and displacement (with Cl) of ^2H -depleted C-H in the lignin monomeric units should lead to an overall ^2H enrichment of lignin C-H (the $\delta^2\text{H}_{\text{lig}}^{\text{ne}}$ value). As the $\delta^2\text{H}$ value of, and the ratio amongst the three C-H components (the aromatic, methoxyl and the propanoid side chain) of a monomeric unit of lignin are phytochemistry-dependent and hard to predict, bleaching-caused change in the $\delta^2\text{H}$ value of each of the three components and/or the ratios amongst them may lead to a fluctuation (rather than a monotonic increase) in the lumped $\delta^2\text{H}_{\text{lig}}^{\text{ne}}$ value for a given monomeric unit along the bleaching process. Bleaching may also change the ratio of the three monomeric units **S**, **G** and **H** and, if so, will undoubtedly affect the $\delta^2\text{H}$ value of the C-H in the lignin as the replaceability, displaceability and exchangeability and the original (intact) $\delta^2\text{H}$ values of the three component C-Hs are different, making fluctuation of the lumped lignin $\delta^2\text{H}_{\text{lig}}^{\text{ne}}$ value along the bleaching process all the more likely.

Similarly, during the bleaching, the exchangeable H is replaced with the relatively ^2H -enriched media H, leading also to an elevation of the $\delta^2\text{H}_{\text{lig}}^{\text{ex}}$ value. Bleaching also leads to a change in the ratio

between the exchangeable H in the forms of $-\text{OH}$ and $-\text{COOH}$, and therefore a change in the (lumped) fractionation factor $\alpha_{\text{lig}}^{\text{ex}}$ between the exchangeable H and processing media, with the direction of change being hard to predict.

Therefore, even if the C-H in α -cellulose is isotopically stable, i.e., the $\delta^2\text{H}_{\text{cel}}^{\text{ne}}$ value is not affected by the bleaching, changes in p_{cel} alone, or together with the change in $\alpha_{\text{lig}}^{\text{ex}}$ (caused by the opening up the network of α -CLC therefore exposing the previously inaccessible H for exchange), will cause, albeit small, changes (and fluctuations) in the $\delta^2\text{H}$ value of crude α -cellulose as bleaching proceeds.

In summary, $A_{\text{H-lig}}$ and $f_{\text{lig}}^{\text{ne}}$ are predicted to decrease monotonically with bleaching, while $\delta^2\text{H}_{\text{air}}$ and $A_{\text{H-cel}}$ are known fixed values. The directions of change in the values of $\delta^2\text{H}_{\text{lig}}^{\text{ne}}$ and $\alpha_{\text{lig}}^{\text{ex}}$ are uncertain while $\alpha_{\text{cel}}^{\text{ex}}$, $\delta^2\text{H}_{\text{cel}}^{\text{ne}}$ and $\delta^2\text{H}_{\text{lig}}^{\text{ne}}$ can be treated as unchanged and the value of p_{cel} is predicted to increase monotonically with bleaching. Since the $\delta^2\text{H}_{\text{lig}}^{\text{ne}}$ value is predicted to be initially smaller but perhaps later greater (as bleaching proceeds) than the $\delta^2\text{H}_{\alpha\text{-cellulose}}$ value due to the continued incorporation of H from the medium, these asynchronous and opposing isotope effects at some stage of bleaching make fluctuation in the $\delta^2\text{H}_{\text{cel}}^{\text{ne}}$ value almost a certainty.

The dip in the recovered crude cellulose after 0.5 h of bleaching observed with the *P. palustris* and *E. longifolia* stem samples, and *S. officinarum* and *A. bracteatus* leaf samples (Figure 2), can also be explained by the preferential loss of the fragments carrying relatively ^2H -enriched C-H incorporated into the α position of the ester bonds during hydrolysis.

3.4 | Isotopic composition differences between α -cellulose recovered from bleaching in isotopically contrasting media

As shown above, when pure α -cellulose products were obtained after 2 h bleaching (indicated by the stabilized weight of the recovered α -CLC), species- and organ-dependent significant $\delta^2\text{H}$ value differences (corrected for the exchangeable Hs, Figure 2)

FIGURE 6 Oxidation of the hydroxymethyl ($-\text{CH}_2\text{OH}$) group and replacement of C-H (although both H-2 and H-3 are isotopically affected under the proposed free radical mechanism, for clarity only H-2 is color-coded) in the glucose unit (of α -cellulose) and xylose unit (of hemicellulose) with H from the bleaching media [Color figure can be viewed at wileyonlinelibrary.com]

between the ^2H -enriched and unenriched treatments were observed. Such differences must be in the C-H rather than in the -OH and -COOH (exchangeable Hs) of the α -celluloses as the latter was equilibrated with the same moisture under the same conditions. This implies that H from the medium must have been incorporated into the C-H of the glucose unit or non-glucose (probably xylose) units of the residual hemicellulose (if any). There are two known mechanisms (Figure 6) that can break or weaken the C-H bond in the (α)-cellulose, viz. oxidation of the hydroxymethyl group (-CH₂OH) according to Pagliaro²⁶ and free radicalization of the C-Hs at C-2 and C-3 of the glucose or xylose unit in the cellulose as proposed by Liszkay et al.²⁷ and Fry et al.¹⁷ The anion free radical $\text{O}_2^{\bullet -}$ that effects the loosening of the C-H bond is from ClO_2^- (Maia and Moura²⁸). Since the first one does not incorporate H from the medium into the α -cellulose products (although it does bring about the same isotopic effect to the C-Hs of celluloses recovered from both ^2H -enriched and unenriched bleaching solutions), the second one must be responsible for the observed isotopic difference between the α -cellulose products recovered from the ^2H -enriched and unenriched processing media.

3.5 | Implications

In our study, the maximum difference observed in the $\delta^2\text{H}$ values of pure α -cellulose samples recovered from ^2H -enriched and unenriched bleaching media is 25.9 mUr (for *E. longifolia* stem) (average of the three $\delta^2\text{H}$ values obtained after more than 2 h of bleaching, see Table S1, supporting information, for data). Using the isotopic difference between the two processing media (272.9 mUr), the maximum proportion p of C-H in α -cellulose exchanged with the processing medium due to bleaching is calculated to be 9.37% (= 25.6/272.9). Since the natural variation of the water $\delta^2\text{H}$ value from the equator to high latitude is usually less than 50 mUr, the maximum error associated with the processing medium must be less than 4.7 mUr ($\approx 50 \times 9.37\%$ mUr), which is only slightly greater than the instrumental error of 2.5 mUr. Therefore, the currently adopted acidified NaClO_2 bleaching method is considered acceptable in terms of preserving the isotopic fidelity if isotopically "normal" water is used to prepare the bleaching solution.

Nevertheless, two issues need to be taken into account for reliable interpretation of the $\delta^2\text{H}$ value of α -cellulose. First, the maximum $\delta^2\text{H}$ value difference in α -cellulose ($\Delta\delta^2\text{H}_{\alpha\text{-cel_C-H}} = 25.9$ mUr) between ^2H -enriched and unenriched treatments is calculated assuming $f = 72\%$ (slightly greater than the theoretical maximum of 70%) when making correction for the contribution of the exchangeable Hs in the α -cellulose products. In some plant species where the microcrystalline component dominates the α -cellulose, the value of f can be as high as 87%.²⁹ According to Equation (1), the value of $\Delta\delta^2\text{H}_{\alpha\text{-cel_C-H}}$ can be significantly magnified if a similarly high value of f is applied to the *E. longifolia* stem, making the measured $\delta^2\text{H}$ value unreliable. Therefore, it is

highly recommended that experiments should be designed to characterize the crystallinity of α -cellulose (in particular those samples extracted from newly explored species/organs) and to obtain a realistic value for f if reliable correction for the contribution of exchangeable H is to be made. Secondly, as shown in Figure 2, the $\delta^2\text{H}$ value of the recovered α -cellulose showed an overall increasing trend when bleached for longer than 2 h. A plausible explanation for this is that bleaching preferentially removes lignin. When the lignin is exhausted, the C-Hs in the α -cellulose (and the minor hemicellulose contaminants) may be increasingly susceptible to exchange. Therefore, the period of bleaching needs to match the quantity of lignin needing to be removed in order to minimize exchange of the C-Hs with the processing medium.

4 | CONCLUDING REMARKS

The $^2\text{H}/^1\text{H}$ ratio of carbon-bound hydrogen (C-H) in α -cellulose, and other organic molecules of biological origin such as *n*-alkanes,^{18,30,31} has long been employed for plant physiological and climatic studies as it is an integrator of prevailing climate that largely controls the $^2\text{H}/^1\text{H}$ of the water utilized by plant growth, the leaf evaporative conditions that control the $^2\text{H}/^1\text{H}$ of leaf water used for photosynthesis, and the metabolism that incorporates leaf and stem waters. Methods for extracting α -cellulose for H isotopic analysis must preserve its isotopic fidelity before such isotopic signals can be applied for climatic, environmental and metabolic studies.

A common feature of the current methods for extracting α -cellulose is that an oxidant such as ClO_2^- must be used and an unavoidable chemistry involved is the cleavage of the possible chemical bonds between lignin and cellulose and the breakdown of the lignin network. Nevertheless, the implicit assumption that there is no breakup and change of the C-H (and therefore no isotope effect) in the final α -cellulose product is untested. The simple approach that we took to test this assumption showed that H in the bleaching medium does appear in the C-H of the α -cellulose product. This isotope effect is species- and organ-dependent and small if isotopically "normal" water is used to prepare the bleaching solution. Nevertheless, it is still a concern for α -cellulose extraction for H isotopic analysis, as such replacement of the H in the C-H bond with H from the processing medium increases with bleaching time. It is therefore recommended that samples (for a particular project) be processed with bleaching solutions of the same isotopic composition (and close to the isotopic composition of the C-H in the cellulose) and that the bleaching time be limited. As the fraction $(1-f)$ of exchangeable H in α -cellulose varies with species, organs, age and perhaps the processing methods, if an equilibration method,³² rather than a nitration method,³³ is to be used to obtain the $\delta^2\text{H}$ value of C-H in α -cellulose, it is also highly recommended that efforts be made to characterize the crystallinity of the α -cellulose³⁴ so that a reliable value of f can be obtained for the correction.

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ORCID

Zhenyu Zhu <https://orcid.org/0000-0002-8750-8987>

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SUPPORTING INFORMATION

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

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