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Jérémy Jacob, Nicolas Bossard, Thierry Bariac, Valery Terwilliger, Philippe Biron, et al.. Hydrogen isotopic fractionations during syntheses of lipid biomarkers in the seeds of broomcorn millet (*Panicum miliaceum* L.) under controlled environmental conditions. *Organic Geochemistry*, 2021, 154, pp.104221. 10.1016/j.orggeochem.2021.104221 . insu-03175971

HAL Id: insu-03175971

<https://insu.hal.science/insu-03175971v1>

Submitted on 22 Mar 2021

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Journal Pre-proofs

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PII: S0146-6380(21)00042-5
DOI: <https://doi.org/10.1016/j.orggeochem.2021.104221>
Reference: OG 104221

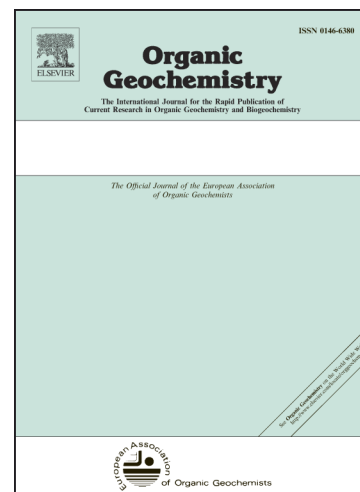
To appear in: *Organic Geochemistry*

Received Date: 1 October 2020
Revised Date: 10 March 2021
Accepted Date: 11 March 2021

Please cite this article as: Jacob, J., Bossard, N., Bariac, T., Terwilliger, V., Biron, P., Richard, P., Le Milbeau, C., Vergès, E., Hydrogen isotopic fractionations during syntheses of lipid biomarkers in the seeds of broomcorn millet (*Panicum miliaceum* L.) under controlled environmental conditions, *Organic Geochemistry* (2021), doi: <https://doi.org/10.1016/j.orggeochem.2021.104221>

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1 Hydrogen isotopic fractionations during syntheses of lipid biomarkers in the seeds of broomcorn
2 millet (*Panicum miliaceum* L.) under controlled environmental conditions

3

4 Jérémy Jacob^{a,b}, Nicolas Bossard^a, Thierry Bariac^c, Valery Terwilliger^{a,d,e,f}, Philippe Biron^c, Patricia
5 Richard^c, Claude Le Milbeau^a, Elisabeth Vergès^a.

6

7 ^a *Univ Orléans, CNRS, BRGM, Institut des Sciences de la Terre d'Orléans (ISTO), UMR*
8 *7327, 45071 Orléans, France*

9 ^b *Laboratoire des Sciences du Climat et de l'Environnement, CEA-CNRS-UVSQ, Université*
10 *Paris-Saclay, 91198, Gif-sur-Yvette, France*

11 ^c *Institut d'Ecologie et des Sciences de l'Environnement - Paris, UMR 7618 CNRS-SU-UPMC-IRD-UPEC,*
12 *78850 Thiverval-Grignon, France*

13 ^d *Le Studium, Loire Valley Institute for Advanced Studies, 1 rue Dupanloup, 45000 Orléans, France*

14 ^e *Department of Geography, University of Kansas, Lawrence, KS 66045, USA*

15 ^f *Department of Archaeology, Simon Fraser University, Burnaby, BC, V5A 1S6, Canada*

16

17 Highlights

- 18 - Broomcorn millet was grown under controlled condition to examine fractionation of
- 19 hydrogen isotopes during seeds lipid (miliacin and n-alkanes) synthesis
- 20 - Leaf water hydrogen isotopic composition is the primary driver of lipid $\delta^2\text{H}$ values
- 21 - Apparent and biosynthetic fractionation could be calculated for n-alkanes and miliacin
- 22 - Subtle differences in biosynthetic fractionation between n-alkanes could reflect the impact
- 23 of relative humidity, although both the experimental setup and the specific location of lipids
- 24 analyzed (seeds) could have affected this relationship

25

26

27 Abstract

28 Compound specific hydrogen isotopic analyses have the potential to reveal the biosynthetic
29 pathways of biomarkers and to reconstruct the effects of water stress in a plant, or in an ecosystem.
30 Although C4 graminoids are of great geological interest and are some of the world's leading crops,
31 there are few experimental studies of their biomarker responses to hydrological conditions. Here,
32 we study a C4 graminoid, broomcorn millet, and compare the effects of controlled changes in
33 environmental conditions on the distributions of *n*-alkane homologues and on the pentacyclic
34 triterpene, miliacin, which is a biomarker for broomcorn millet; both were measured in the seeds of
35 the graminoid. Broomcorn millet plants were propagated in hydroponic solutions with four different
36 $\delta^2\text{H}$ values for each of two growth chambers, differing in relative humidity (58 and 74 %). Analyses
37 of $\delta^2\text{H}$ values of the lipid compounds (miliacin and *n*-alkanes) in seeds and water extracted from
38 transpiring and non-transpiring organs allowed us to quantify the apparent (lipids vs. source water
39 to plant) and biosynthetic (lipids vs. leaf water) fractionations during miliacin and *n*-alkane
40 syntheses. Miliacin and *n*-alkane $\delta^2\text{H}$ values were linearly related to leaf water $\delta^2\text{H}$ values,
41 permitting credible biosynthetic fractionations to be calculated for *n*-alkanes (average $-149.5\% \pm$
42 11) and miliacin ($-118\% \pm 5$). These biosynthetic fractionations were within the range of published
43 values for compounds with their respective biosynthetic pathways, although a ^2H -enrichment of
44 miliacin compared to *n*-alkanes remains unexplained.

45 Whereas a 16% decrease in relative humidity had no significant impact on the biosynthetic
46 fractionation of miliacin, *n*-C₂₅ and *n*-C₂₇ alkanes, it led to a $\sim 25\%$ decrease in biosynthetic
47 fractionation for *n*-C₃₁ and *n*-C₃₃ alkanes. This could be the consequence of a contribution of more
48 depleted pools of hydrogen atoms in cytoplasmic water (compared to chloroplastic hydrogen pools)
49 during the *n*-alkyl lipid elongation process. This finding suggests that the respective influences of
50 source water $\delta^2\text{H}$ values and relative humidity on the $\delta^2\text{H}$ values of organic compounds may be
51 discretely inferred by examining the $\delta^2\text{H}$ values of compounds synthesized from distinct sources of

52 hydrogen in cells. It provides clues to the biosynthetic fractionations in a C₄ plant for compounds
53 derived from distinct pathways, but also highlights specific issues related to seed lipids that would
54 require further research.

55

56

57 Key words: Biosynthetic fractionation; hydrogen isotopes; *Panicum miliaceum*; Biogeochemical
58 chambers; compound-specific $\delta^2\text{H}$, miliacin, biomarker.

59

62 The hydrogen isotope ratios ($\delta^2\text{H}$) of lipids synthesised by autotrophs and preserved in
63 geological archives are increasingly used to reconstruct past climates (reviewed by Sachse et al.,
64 2012). These sedimentary lipids are produced by source organisms once living in the catchment and
65 then transported, accumulating over time in sediments. When the conditions of their preservation
66 are favorable, the isotope ratios of their C-bound hydrogen atoms may be unaltered over geological
67 time (Yang and Huang, 2003). The rationale for using lipid $\delta^2\text{H}$ values to reconstruct past climatic
68 conditions rests on their ability to record the climatic conditions that prevailed at the time of
69 biosynthesis (Sessions et al., 1999; Sauer et al., 2001; Huang et al., 2002; Gleixner and Mügler,
70 2007). An active area of research concerns the role that organism-level functional and biochemical
71 fractionation has on proper interpretations of climate from lipid $\delta^2\text{H}$ values in sedimentary archives.

72 Precipitation water $\delta^2\text{H}$ values are influenced by climatic variables; physical explanations
73 for these relationships have long been established (Craig, 1961; Dansgaard, 1964). As the original
74 source of hydrogen for the synthesis of lipids in autotrophs is precipitation water, the $\delta^2\text{H}$ values of
75 the lipids can potentially reflect the $\delta^2\text{H}$ of precipitation and thus provide a quantitative indicator of
76 climate variability (e.g., Gleixner and Mügler, 2007; Sachse et al., 2012).

77 Nevertheless, $\delta^2\text{H}$ values of lipids are shaped not only by the $\delta^2\text{H}$ values of precipitation, but
78 also by additional factors. The identification and quantification of these factors is not only
79 necessary to discern climatic information contributed by precipitation from $\delta^2\text{H}$ analyses of lipid
80 compounds, but may also lead to a wider range of climatic inferences. Understanding these factors
81 thus remains a very active area of study (Sachse et al., 2012; Terwilliger and Jacob, 2013; Sessions,
82 2016).

83 Categories of factors that can affect the $\delta^2\text{H}$ values of land-plant derived lipids include: (1)
84 physical fractionations to hydrogen in water on its journey from precipitation to the site where it
85 will be incorporated into organic compounds, and (2) fractionations during biosynthesis of
86 compounds. The largest of the physical fractionations to water occur during the change of state

87 from liquid to vapor, during which liquid water becomes enriched in molecules with heavy isotopes
88 because of the easier vaporization of water with light isotopes. The main state changes of relevance
89 to land plants are during evaporation of surface soil water and during transpiration from leaves (e.g.,
90 Gonfiantini et al., 1965; Dongman et al., 1974; Farris and Strain 1978; Allison and Leaney, 1982).
91 Water uptake by non-transpiring parts of plants remains isotopically similar to its sources in the soil
92 (e.g., Ehleringer and Dawson, 1992; Bariac et al., 1994a;b). Evaporation and transpiration rates are,
93 in turn, affected by environmental factors, such as temperature, relative humidity, net radiation and
94 wind speed. The $\delta^2\text{H}$ values of lipids synthesised by terrestrial higher plants thus integrate not only
95 the $\delta^2\text{H}$ values of precipitation, but also the climatic factors that drive rates of state changes to
96 precipitation prior to the use of its hydrogen atoms in organic compound synthesis in the plant (Hou
97 et al., 2007a,b; Gleixner and Mügler, 2007; Douglas et al., 2012; Sachse et al., 2012; Kahmen et al.,
98 2013a,b; Tipple et al., 2015).

99 The extent to which the water providing hydrogen for photosynthesis will have been
100 affected by the aforementioned physical fractionations may vary between plant types (i.e., Sachse et
101 al., 2012). As most of the evaporation of soil water occurs at the surface and the more evaporation
102 that occurs, the more difficult it is for plants to take up that water, the impact of evaporation on the
103 $\delta^2\text{H}$ of water will depend on root structure. Evapotranspiration causes ^2H enrichment of leaf water,
104 although there is strong evidence that exposure to evapotranspiration is heterogeneous (Yakir,
105 1992). At present, evidence favors the hypothesis that for leaves of C4 grasses, water is more
106 protected from evaporation in basal bundle sheath cells and mixed with non-evaporated xylem
107 water, compared to water in mesophyll cells (Zhou et al., 2016; Gamara et al., 2016). Within cells,
108 cytoplasmic water is more affected by evaporation than chloroplastic water (reviewed by Yakir,
109 1992).

110 Hydrogen fractionations during biosynthesis of compounds in a plant primarily involve
111 enzyme preferences for specific hydrogen isotopes and bond positions during kinetic reactions
112 (Estep and Hoering, 1980; Sessions 2016). Although the first source of hydrogen for synthesis of a

113 compound will be the water at the site of photosynthesis, hydrogen can also originate from
114 intermediates (e.g., NADPH and metabolites) produced elsewhere in the plant, at a different time to
115 that of the final compound synthesised, with distinct $\delta^2\text{H}$ values, and in varying proportions (Sachse
116 et al., 2012; Cormier et al., 2018). Thus, the exact climate information in the $\delta^2\text{H}$ value of a
117 compound will depend on where enzymatic reactions leading to its production take place in a plant.

118 Efforts to identify the precise imprints of specific climatic factors on specific lipid
119 compounds have largely centred on studies of *n*-alkyl lipids (e.g., *n*-alkanes, fatty acids), which are
120 major components in the leaf waxes of many land plants (Eglinton and Hamilton, 1967; Diefendorf
121 et al., 2011; Diefendorf and Freimuth, 2017). One approach has been to analyse the $\delta^2\text{H}$ values of
122 leaf waxes in plants and sediments over natural environmental gradients (e.g., Huang et al., 2002;
123 Sachse et al., 2004; Sachse et al., 2006; Jia et al., 2008; Feakins and Sessions., 2010; Bai et al.,
124 2011; Douglas et al., 2012; Garcin et al., 2012; Kahmen et al., 2013a). Another approach has been
125 to extract specific *n*-alkyl lipids from leaf waxes of plant species grown under controlled
126 environmental conditions (e.g., Zhou et al., 2011; Kahmen et al., 2013b; Gao et al., 2014; Tipple et
127 al., 2015; Gamarra et al., 2016; Zhou et al., 2016). As these compounds are found in differing
128 proportions across many different plant types, the results are limited in the extent to which they can
129 be extrapolated to reconstruct climatic changes in systems where vegetation may also have changed
130 (Hou et al., 2007b; Smith and Freeman, 2006; Liu and Yang, 2008; Polissar and Freeman, 2010).

131 Our study reports the effects of growing broomcorn millet plants at different $\delta^2\text{H}$ values of
132 hydroponic solutions (HS) and at different relative humidities, on the $\delta^2\text{H}$ values of *n*-alkanes and
133 of miliacin, both extracted from seeds. Miliacin (olean-18-en-3 β -ol ME; Fig. 1), a pentacyclic
134 triterpene methyl ether, is a specific biomarker of broomcorn millet (*Panicum miliaceum*), which is
135 a C4 plant. This biomarker was found in the sediments of lake Le Bourget dated as prior to the
136 Bronze Age (Jacob et al., 2008a,b; 2009; Bossard et al., 2011; Bossard, 2013). It was also used as a
137 tracer of C4 grasses in tropical soils (Schwab et al., 2015). The first objective was to determine the
138 apparent (lipid vs. water source to plant; ϵ_{app}) and biosynthetic (lipid vs. leaf water; ϵ_{bio})

139 fractionations of two classes of lipids produced by distinct biosynthetic pathways in a single plant
140 species. The second objective of our report was to quantify the effects of relative humidity on the
141 apparent and biosynthetic fractionations of miliacin and *n*-alkanes. Our results suggest minute
142 differences in the relationships of humidity to isotopic compositions between individual compounds
143 extracted from the seeds of one C₄ species.

144

145 2. MATERIALS AND METHODS

146 2.1. Experimental design

147 We quantified the impacts of two factors that contribute to the $\delta^2\text{H}$ values of plant lipids: source
148 water $\delta^2\text{H}$ values (water taken up by plants) and relative humidity of the atmosphere surrounding
149 plants. Millet plants were cultivated in two controlled climate growth chambers built for
150 experiments on the isotopic consequences of the environment on plant physiology at the Institut
151 d'Ecologie et des Sciences de l'Environnement (UMR 7618, Paris, France): RUBIC I (described in
152 Rothfuss et al., 2010) and RUBIC V (Longchamp et al., 2015). RUBIC stands for *Reactor Used for*
153 *Continental Isotopic Biogeochemistry*. We maintained relative humidities of 58 % in the 0.5 m³
154 RUBIC I and of 74 % in the 9 m³ RUBIC V (Fig. 2). The relative humidity of the air was precisely
155 regulated in the different chambers by controlling the condensation temperature in a heat exchanger.
156 The excess moisture condensed and was then discharged to the outside, thus providing excellent
157 stability of the relative humidity of the air. We sampled the condensate at the end of each day to
158 analyse its isotopic composition. The air temperature was regulated by the volume of air flowing
159 through the heat exchanger. One of the particularities of the RUBIC chambers is that the vapor they
160 contain is not only a driver of transpiration (through a vapor pressure deficit), but is also a resulting
161 effect of condensation (heat exchanger). Otherwise, the environmental conditions were the same in
162 the two chambers: a photoperiod of 12 hours per 24 (at 600 $\mu\text{mol}/\text{m}^2/\text{s}$ photosynthetic photon flux
163 density), a temperature of 18°C (night) and 25°C (day), and a slow wind speed (0.1 m s⁻¹) to

164 homogenize the chamber air. Twenty plants per chamber were germinated on vermiculite from
165 seeds (provided by Parard-Sévin, Mézières-lez-Cléry, France). Then, 5 plants were grown in 4 tanks
166 with different $\delta^2\text{H}$ values of water in hydroponic solutions. Four tanks (and hence 20 plants) were
167 placed in each chamber. Hydroponic solutions were prepared with 8 stock solutions (4 per chamber)
168 of $^2\text{H}_2\text{O}$ and tap water of -50‰ (V-SMOW; Vienna Standard Mean Ocean Water). The tanks
169 containing hydroponic solutions were covered with plastic plates drilled with a single hole for each
170 plant. These holes were plugged with rubber stoppers filled with Teroson® (sticky putty) to limit
171 exchange between the hydroponic solution and surrounding air. In addition, each hydroponic
172 solution was replaced weekly with stock solution to ensure that its isotopic composition remained
173 constant (as described below).

174 2.2. Water $\delta^2\text{H}$ and $\delta^{18}\text{O}$ analyses

175 $\delta^2\text{H}$ determination of water in hydroponic solutions and in plant organs allowed us to calculate
176 apparent and biosynthetic fractionations. In addition, we determined $\delta^{18}\text{O}$ values of the same waters
177 to characterize the effects of transpiration. Water was extracted from one plant per treatment
178 (relative humidity and $\delta^2\text{H}$ value of the hydroponic solution) one week before seed harvest, which
179 was when seeds were fully developed, but not dry. This was done to obtain enough water for
180 isotopic ratio measurements. Water was extracted from the following organs of each plant: roots,
181 first internode (collet) and other internodes, leaves and panicles (i.e., stems and spikelets composed
182 of glumes, paleas and lemmas; Fig. 2). Water was extracted from the plant organs by cryogenic
183 distillation and analyzed for $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values (Araguas-Araguas et al., 1995). $\delta^{18}\text{O}$ values were
184 measured (analytical precision: $\pm 0.1\text{‰}$) on an isotope ratio mass spectrometer (IRMS; Isoprime)
185 coupled to an Aquaprep system and for $\delta^2\text{H}$ on an IRMS PyrOH (Isoprime, analytical precision: \pm
186 1‰) coupled to an elemental analyzer (Eurovector). $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of hydroponic solutions

187 were determined prior to cultivation and before weekly replacements with the same device. No
188 discernible evolution of the isotopic composition of the hydroponic solutions was noted (Table S1).

189 2.3. Extraction and purification of compounds

190 We analyzed lipids from the seeds because Bossard et al. (2013) showed that miliacin is
191 very abundant in *P. miliaceum* seeds, but has negligible concentrations elsewhere in the plant. In
192 addition to miliacin, seeds contain other lipids, such as *n*-alkanes. Seeds were harvested when they
193 were fully developed and the plants had become dry. The seeds from one to three plants that had
194 sufficient seeds to yield enough of the compounds for analysis were sampled per treatment ($\delta^2\text{H}$
195 value and relative humidity) for replication. For each plant, about 40 seeds of the main panicle were
196 ground to a powder and weighed. Lipids were extracted by immersing each seed sample in
197 dichloromethane:isopropanol (2:1 v:v) and sonicating for 5 minutes, 3 times, before centrifugation.
198 For each plant, the lipid extracts were combined and dried under a stream of N_2 gas. The total
199 extract was separated into neutral, acidic and polar fractions by ion exchange chromatography on
200 aminopropyl-bonded silica, as per Jacob et al. (2005). The different classes of compounds (*n*-
201 alkanes and miliacin) were purified from the neutral fraction by flash chromatography on activated
202 silica using solvents of increasing polarity (as per Bossard et al., 2011). Aliphatic hydrocarbons,
203 such as *n*-alkanes, were first eluted with 2 mL of heptane, then aromatic hydrocarbons were eluted
204 with 1 mL heptane and then 2 mL heptane:toluene 2:1. Miliacin was eluted with 2 mL
205 hexane:toluene (1:1) and 2 mL hexane/ethyl acetate (19:1). The different fractions were dried under
206 a stream of tank nitrogen and stored at 4 °C until analysis.

207 2.3.1. Compound quantitation and purity assessment

208 The abundance and purity of miliacin were assessed by gas chromatography-mass
209 spectrometry (GC-MS) on a Trace gas chromatograph coupled to a Polaris GCQ ion trap mass
210 spectrometer (both from Thermo Scientific, Bremen) according to the protocol of Jacob et al.
211 (2005). 5α -cholestane was added prior to analysis by GC-MS, for quantification. The gas

212 chromatograph was fitted with a RTX-5 MS capillary column (5 m column guard, 30 m, 0.25 mm
213 i.d., 0.25 μm film thickness; Restek, Bellefonte, PA, USA). The column temperature was held at
214 40°C for 1 min, and then increased from 40 to 300°C at a rate of 20°C min^{-1} , with a final isothermal
215 hold at 300°C for 30min. The sample was dissolved in toluene and injected splitless in a 2 μL
216 volume with injector temperature set at 280°C. The carrier gas was helium at a flow rate of 1.4 mL
217 min^{-1} . The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV ionization
218 energy and scanned from m/z 50 to 650.

219 2.3.2. Compound-specific $\delta^2\text{H}$ measurements

220 $\delta^2\text{H}$ values of miliacin and *n*-alkanes were determined by gas chromatography-stable isotope
221 ratio mass spectrometry (GC-irMS) on a TraceGC chromatograph equipped with a Triplus
222 Autosampler coupled to a DeltaV Advantage IRMS through a GC-Isolink interface and a Conflo IV
223 dilution system (all from Thermo Scientific). The chromatographic conditions were the same as
224 those used for the GC-MS analyses, except that the GC column used (J&W DB5, 30 m, 0.25 mm
225 i.d., 0.25 μm film thickness; Agilent, Palo Alto, CA, USA) was slightly different. 1 μL of the
226 sample, dissolved in toluene, was injected at least 3 times. We used a standard consisting of 1 μL of
227 a mixture of 15 *n*-alkanes (*n*-C₁₆ to *n*-C₃₀) with $\delta^2\text{H}$ values ranging from -46.3 to -242.3‰ V-
228 SMOW as determined off-line (Mixture A4, A. Schimmelmann, Indiana University, USA) to
229 calibrate our values over the V-SMOW isotopic scale. For miliacin, 1 μL of the standard mixture
230 was co-injected with 1 μL of each sample. No coelution was observed. Data were then normalized
231 to the V-SMOW isotopic scale by using the $\delta^2\text{H}$ values of the *n*-C₂₅ and *n*-C₂₇ alkanes as references
232 (Sessions et al., 1990).

233 For *n*-alkanes, the standard A4 mixture was injected after every 3 samples, to calibrate
234 reference gas prior to analyses. Then, each sample *n*-alkane $\delta^2\text{H}$ value was normalized to the V-
235 SMOW (Vienna Standard Mean Ocean Water) isotopic scale using the reference gas $\delta^2\text{H}$ value.
236 Over the period of analysis, measured $\delta^2\text{H}$ values for the *n*-alkane A4 mixture were in good
237 agreement with those measured off-line ($r^2 \geq 0.99$). The overall precision for the *n*-alkane standard

238 was greater than 6%. 1 μL of each sample was injected at least three times, or until the precision
239 was better than 6%. Replicates were injected randomly in order to ensure that the reproducibility
240 was independent of the order of the analyses. All isotopic values are expressed in ‰ V-SMOW.
241 The H_3^+ factor (Sessions et al., 2001) was determined on a daily basis and was 7.26 ± 0.17 .

242 2.3.3. Statistical analyses

243 Simple linear regressions were used to establish the significance of relationships between unpooled
244 values of variables within humidity treatments (e.g., of $\delta^2\text{H}$ vs. $\delta^{18}\text{O}$ values of water from organs
245 affected by transpiration). Differences in the slopes and intercepts of relationships were established
246 visually as there were not enough samples for more robust statistical comparisons via analysis of
247 covariance. If the resulting regression equation was not significant, because the y-intercept value
248 did not significantly differ from zero, another regression was run with a forced intercept of zero
249 (regression through the origin).

250 With one $\delta^2\text{H}$ value for each compound per hydroponic solution treatment, and thus four \mathcal{E}_{app}
251 values per compound in each of the two chambers, our most robust statistical options for comparing
252 \mathcal{E} values between compounds and humidities were non-parametric. We did not correct for possibly
253 inflated type I error from multiple comparisons as the small sample sizes already made the
254 likelihood of any rejection of null hypotheses very conservative. We compared \mathcal{E} values between
255 compounds using Wilcoxon paired-sample tests (Tables S3 and S4). Only unidirectional differences
256 were examined (via one-tailed tests) where Wilcoxon tests were performed to compare compounds
257 within chambers, because sample comparison numbers were too small (4 per compound) to detect
258 overall inequalities in \mathcal{E} value pairs (two-tailed tests). For comparisons of a compound between
259 humidity chambers, we performed Mann Whitney U tests of possible inequalities in \mathcal{E} values within
260 chambers (Table S5). \mathcal{E}_{bio} values were pooled into medians per plant for non-parametric analyses.

261 All statistical analyses were performed using Minitab (version 13, State College, PA, USA) and
262 were considered to be significant at $p \leq 0.05$.

263

264

265 3. RESULTS

266 3.1. $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of water

267 3.1.1. $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of the source water (hydroponic solutions)

268 The range in $\delta^2\text{H}$ values of water in hydroponic solution waters (HS) at 58% relative
269 humidity (RH) was 175‰, where Tank 1 = -15, Tank 2 = 15, Tank 3 = 105 and Tank 4 = 160‰
270 (Fig. 3; Table 1). $\delta^{18}\text{O}$ values were -1.09, -2.24, -2.44 and -3.45‰. The hydroponic solution $\delta^2\text{H}$
271 values at 74% RH spanned a range of 223‰, with specific values of -36, -10, 95, and 187‰ for
272 tanks 1 through 4, respectively. The corresponding $\delta^{18}\text{O}$ values for these tanks were -4.84, -6.58, -
273 6.58, -6.79‰ (Table 1). The slight decreases in $\delta^{18}\text{O}$ values with increasing $\delta^2\text{H}$ values at both 58
274 and 74% relative humidity were probably caused by the higher $\delta^{18}\text{O}$ values of $^2\text{H}_2\text{O}$ than of the tap
275 water used for the preparation of stock solutions. The parameters of regression lines between $\delta^{18}\text{O}$
276 and $\delta^2\text{H}$ values for each tank and each relative humidity are given in Table 2a.

277 3.1.2. Water $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of non-transpiring organs

278 The $\delta^2\text{H}$ values of water in non-transpiring organs were similar to one another in a given
279 tank and relative humidity (Table S2). At 58% RH, water $\delta^2\text{H}$ values of non-transpiring organs,
280 such as roots, first internode and other internodes, ranged from -23 to -13‰ for a hydroponic
281 solution at -15‰ (Tank 1), from 14 to 18‰ for a hydroponic solution at 15‰ (Tank 2), from 104 to
282 111‰ for a hydroponic solution at 105‰ (Tank 3), and from 151 to 164‰ for a hydroponic
283 solution at 160‰ (Tank 4) (Figure 3 and Table 1; Table S2 contains further details). At 74% RH,
284 water $\delta^2\text{H}$ values of non-transpiring organs ranged from -39 to -35‰ for a hydroponic solution at -

285 50‰ (Tank 1), from -10 to -9‰ for a hydroponic solution at -9‰ (Tank 2), from 92 to 95‰ for a
286 hydroponic solution at 95‰ (Tank 3), and from 186 to 189‰ for a hydroponic solution at 187‰
287 (Tank 4). Furthermore, the $\delta^2\text{H}$ values of non-transpiring organ waters (y) were nearly identical to
288 those of their respective hydroponic solution waters (x), as evidenced by their close to 1:1
289 relationships and y-intercepts close to, or in the case of 58% RH, not significantly different from, 0
290 (Fig. 4; Table 2b).

291 Water $\delta^{18}\text{O}$ values in non-transpiring organs were also very close to the $\delta^{18}\text{O}$ values of water
292 in their corresponding hydroponic solutions (Fig. 3; Table 1 and Table S2 contain details). At 58%
293 RH, water $\delta^{18}\text{O}$ values in non-transpiring organs ranged from -1.4 to -0.8‰ for a hydroponic
294 solution at -1.1‰ (Tank 1), from -2.8 to -2.1‰ for a hydroponic solution at -2.2‰ (Tank 2), from -
295 2.8 to -2‰ for a hydroponic solution at -2.4‰ (Tank 3), and from -3.6 to -3‰ for a hydroponic
296 solution at -3.4‰ (Tank 4). At 74% RH, the $\delta^{18}\text{O}$ values of water in non-transpiring organs ranged
297 from -5.5 to -4.7‰ for a hydroponic solution at -4.8‰ (Tank 1), from -7 to -6.4‰ for a hydroponic
298 solution at -6.6‰ (Tank 2), from -6.7 to -6‰ for a hydroponic solution at -6.6‰ (Tank 3), and
299 from -7 to -6.1‰ for a hydroponic solution at -6.8‰ (Tank 4).

300 3.1.3. Water $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of transpiring organs

301 The $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of water extracted from panicles were similar to, or did not differ
302 systematically from, their respective values in leaf waters (Fig. 4; Table S2). Therefore, we pooled
303 their δ values into a single “transpiring organs” category, comprising panicles and leaves, in further
304 analyses (Table 1 and Fig. 4). The $\delta^2\text{H}$ values of water extracted from transpiring organs ranged
305 from 26 to 129‰ at 58 % RH, and from -9 to +117‰ at 74 % RH (Fig. 3; Table 1). At both relative
306 humidities, the waters of transpiring organs were enriched in deuterium compared to their
307 hydroponic solution waters when the $\delta^2\text{H}$ values of the hydroponic solution were low (Tanks 1 and
308 2), but were depleted in deuterium when the $\delta^2\text{H}$ values of the hydroponic solution were high
309 (Tanks 3 and 4). In contrast, the $\delta^{18}\text{O}$ values of the transpiring organs (TO) were always higher than
310 their respective hydroponic solution $\delta^{18}\text{O}$ values (Fig. 3; Table 1).

311 As observed for non-transpiring organs, the slopes of the relationships of $\delta^2\text{H}$ values of
312 transpiring organ waters (y) to $\delta^2\text{H}$ values of hydroponic solution waters (x) were similar for the
313 two relative humidities (Fig. 4 and Table 2c). In contrast to what was observed for non-transpiring
314 organs however, the relationship of $\delta^2\text{H}$ values of water in transpiring organs to hydroponic solution
315 was not 1:1 but close to 0.5 at the two relative humidities (Fig. 4 and Table 2c). In addition, for a
316 given hydroponic solution $\delta^2\text{H}$ value, the water in transpiring organs had higher $\delta^2\text{H}$ values at 58%
317 RH than at 74% RH (Fig. 4 and Table 2c).

318 For technical reasons, the vapor isotopic composition (δ_v) was not monitored in the
319 chambers during experiments. Instead, we used the condensate isotopic composition (δ_C , averaged
320 along the experiment) to examine the water vapor in isotopic equilibrium with the condensates. In
321 steady state conditions, the isotopic compositions of the condensates (RUBIC I: $\delta^{18}\text{O}_C = -3.5\text{‰}$ and
322 $\delta^2\text{H}_C = 52\text{‰}$ and RUBIC V: $\delta^{18}\text{O}_C = -7.0\text{‰}$ and $\delta^2\text{H}_C = 57\text{‰}$) were about the same as the average
323 of the isotopic compositions of the 4 hydroponic solutions (RUBIC I: $\delta^{18}\text{O}_{\text{HS}} = -2.3\text{‰}$ and $\delta^2\text{H}_{\text{HS}} =$
324 66‰ ; RUBIC V: $\delta^{18}\text{O}_{\text{HS}} = -6.2\text{‰}$ and $\delta^2\text{H}_{\text{HS}} = 59\text{‰}$). Small observed differences between the
325 measured and theoretical values were related to the mixing of isotopic transient state values (more
326 depleted in heavy isotopes) during the first hours of the day and steady state values in the afternoon.
327 This explains the difference between the average of the hydroponic solution and the average of the
328 condensate isotopic compositions.

329 330 3.1.4. Lipid $\delta^2\text{H}$ values

331 We analysed the $\delta^2\text{H}$ values of the *n*-C₂₅, *n*-C₂₇, *n*-C₃₁ and *n*-C₃₃ alkanes in the seeds because
332 this is the only plant organ where miliacin is reliably abundant enough for $\delta^2\text{H}$ value determination
333 (e.g., Bossard et al., 2011). The *n*-C₂₉ alkane was also abundant, but unfortunately co-eluted with
334 the 5 α -cholestane used as a quantitation standard. Consequently, we were unable to obtain reliable
335 $\delta^2\text{H}$ values for *n*-C₂₉. The data reported in Table 3 correspond to $\delta^2\text{H}$ values of miliacin and all
336 alkanes except *n*-C₂₉ alkane, averaged per tank.

337 The $\delta^2\text{H}$ values of miliacin and the *n*-alkanes analysed all increased with increasing $\delta^2\text{H}$
338 values of the hydroponic solutions (Figure 4). Miliacin $\delta^2\text{H}$ values at 58 % RH ranged from -77 (at
339 $\delta^2\text{H}_{\text{HS}} = -15\text{‰}$) to -17‰ (at $\delta^2\text{H}_{\text{HS}} = 160\text{‰}$) and from -122 (for $\delta^2\text{H}_{\text{HS}} = -36\text{‰}$) to -31‰ (for $\delta^2\text{H}_{\text{HS}}$
340 $= 187\text{‰}$) at 74 % RH (Figure 4; Table 3). Miliacin $\delta^2\text{H}$ values thus spanned 60‰ at 58% RH and
341 91‰ at 74% RH, over a range of $\delta^2\text{H}_{\text{HS}}$ values of 175 and 223‰ at the two relative humidities. $\delta^2\text{H}$
342 values of *n*-alkanes ranged from -119 to -59‰ (58% RH) and from -155 to -61‰ (74% RH) for *n*-
343 C_{25} , from -96 to -57‰ (58% RH) and from -170 to -66‰ (74% RH) for *n*- C_{27} , from -97 to -32‰
344 (58% RH) and from -160 to -71‰ (74% RH) for *n*- C_{31} and from -102 to -31‰ (58% RH) and from
345 -179 to -70‰ (74% RH) for *n*- C_{33} (Figure 4; Table 3).

346 Where significant, the slopes of the relationships between the $\delta^2\text{H}$ values of each lipid and
347 the $\delta^2\text{H}$ values of the hydroponic solution were all similar to the slopes of the relationship between
348 $\delta^2\text{H}$ values of water in transpiring organs and $\delta^2\text{H}$ values of the hydroponic solution (Table 2b; Fig.
349 4). The slopes of the relationships (where significant) between the $\delta^2\text{H}$ values of each lipid and the
350 $\delta^2\text{H}$ values of water in transpiring organs, although not 1:1, were all higher than those of the $\delta^2\text{H}$
351 values of each lipid vs. the $\delta^2\text{H}$ value of the hydroponic solution (Table 2c; Fig. 5).

352

353 4. DISCUSSION

354 In order to evaluate the respective impacts of source water $\delta^2\text{H}$ values and transpiration on
355 the $\delta^2\text{H}$ values of *n*-alkanes and miliacin, we first discuss the impacts of water uptake and
356 transpiration on $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of water in non-transpiring and transpiring organs. Then we
357 examine the apparent and biosynthetic fractionations for *n*-alkanes and miliacin and compare our
358 results to literature data. Finally, we inspect the extent to which relative humidity impacts the
359 biosynthetic fractionation of each compound and propose an explanation for the distinct behaviour
360 of *n*-alkanes.

361 4.1. water uptake and transpiration

362 4.1.1. *Water uptake*

363 $\delta^{18}\text{O}$ and $\delta^2\text{H}$ values of water in non-transpiring organs (roots, first and other internodes) are
 364 close to those of their hydroponic solutions (HS; Table 1 and Fig. 3). In addition, the regression
 365 lines of $\delta^2\text{H}$ values of non-transpiring organ waters vs. their corresponding hydroponic solution
 366 have nearly 1:1 slopes (58% RH: 1.0067; 74% RH: 1.0056; Table 2b and Fig. 4) and y-intercepts
 367 that are not different from (at 58% RH), or that are close to, zero (-0.87 at 74% RH; Table 2b and
 368 Fig. 4). These two results suggest that there is no discernible isotopic fractionation during water
 369 uptake and are consistent with previous studies on suberized plant tissues in which $\delta^2\text{H}$ values of
 370 water in non-transpiring organs are the same as those of the source water (Ehleringer and Dawson,
 371 1992; Bariac et al., 1994a,b; Terwilliger and DeNiro, 1995; Sachse et al., 2006; Gleixner and
 372 Mügler, 2007).

373 4.1.2. *Transpiration*

374 Our results suggest that the experiments at 58% RH and at 74% RH are realized under a
 375 permanent hydric regime. RUBIC I and RUBIC V are designed to provoke a permanent flow
 376 regime and isotopic steady states (hydroponic solution; δ_{HS} , water vapor; δ_{v} , water vapor
 377 transpiration; δ_{E} , and condensate; δ_{C}).

$$378 \quad \phi_{\text{HS}} = \phi_{\text{E}} = \phi_{\text{C}}$$

379 where ϕ_{HS} is the flow of the source water, ϕ_{E} is the flow of transpiration/evaporation and ϕ_{HS}
 380 is the flow of condensates.

381 δ_{v} is substantially constant during the day in the reactors, as are RH and δ_{HS} for each
 382 treatment. It is therefore possible to reach an isotopic steady state in the leaf water at the end of the
 383 day. In steady state, conditions in the 3 water pools are as follows:

$$384 \quad \delta_{\text{HS}} \times \phi_{\text{HS}} = \delta_{\text{E}} \times \phi_{\text{E}} = \delta_{\text{C}} \times \phi_{\text{C}}$$

385 So:

386 $\delta_{HS} = \delta_E = \delta_C$

387 There is an isotopic equilibrium between the condensate and the vapor and between the
 388 hydroponic solution and the vapor in the reactor, with no exchange between the latter two pools. In
 389 this experiment, the plants of each treatment were supplied with differently labelled hydroponic
 390 solutions. The isotopic composition of the vapor is a mixing of vapor transpiration from plants
 391 cultivated in the four hydroponic solutions. Because of this mixing, there is no longer an isotopic
 392 equilibrium between the hydroponic solutions and the water vapor. Instead, there is an isotopic
 393 exchange between the leaf water and a vapor whose isotopic composition is very different from that
 394 encountered under natural conditions.

395 In contrast to non-transpiring organs, the δ^2H values of water extracted from transpiring
 396 organs (leaves and panicles) are different from the δ^2H values of their hydroponic solution source
 397 waters (Fig. 3). In 2H -depleted hydroponic solutions, the δ^2H values of waters in transpiring organs
 398 are enriched in 2H compared to the corresponding values of the hydroponic solution. In contrast, in
 399 2H -enriched hydroponic solutions, the opposite trend occurs and transpiring organs are depleted in
 400 2H compared to the hydroponic solution (Fig. 3).

401 This shift in behaviour of water δ^2H values in transpiring organs with respect to their source
 402 water 2H enrichment is consistent with the Craig and Gordon model (1965; Eq.1) revisited by
 403 Dongmann et al. (1974) and extensively used in the literature (e.g., Bariac et al., 1989, 1990;
 404 Barbour et al., 2017). The isotopic compositions (δ^2H or $\delta^{18}O$) of water vapor (δ_v) are constant
 405 during the day in the reactors, as are the δ values of the hydroponic solution (δ_{HS}) and RH (relative
 406 humidity). It is therefore possible to reach an isotopic steady state in the leaf water (with δ_L) at the
 407 end of the day.

$$408 \quad \delta_L = \xi (\delta_{HS} + 1) + RH (\delta_v + 1) (\epsilon_k + 1) - 1 \quad (\text{Eq.1})$$

409 With:

$$410 \quad \xi = (1 - RH) (1 + \epsilon_e) (1 + \epsilon_k)$$

411 Where:

- 412 • δ_L is the isotopic composition of leaf water,
- 413 • h is the relative humidity of air at the leaf temperature,
- 414 • ϵ_e and ϵ_k are respectively the isotopic enrichment factor at equilibrium and the kinetic
415 enrichment factor between liquid and vapor,
- 416 • δ_V is the isotopic composition of the ambient water vapor,
- 417 • δ_{HS} is the isotopic composition of the hydroponic solution.

418 Relative humidity appears to be an important factor in the isotopic variations of leaf water
419 (Ferhi et al., 1983) via the terms " $\delta_{HS} (1 - h)$ " and " $(h \delta_V)$ ". When relative humidity increases in the
420 reactor, the influence of δ_V also increases. As mentioned by Farquhar and Cernusak (2005) and
421 Farquhar et al. (2007), it means that at a relative humidity (h) greater than 50%, more water enters
422 the leaf from the air.

423 Some minor differences in water δ^2H values are observed within a category of transpiring
424 organs. Leaf water enrichment (both in 2H and ^{18}O) is less for lower (and senescent) than for higher
425 leaves, which thus exhibit an intermediate enrichment between non-transpiring organs and leaves
426 (Figure 3; further details are given in Table S2). Water extracted from panicles also shows an
427 enrichment in both 2H and ^{18}O , compared to hydroponic solution waters. In most cases, the range of
428 δ^2H and $\delta^{18}O$ values of water in panicles falls within the range found for transpiring organs (Fig. 3).
429 This is the reason why leaves and panicles were considered together as transpiring organs in the
430 previous sections. Panicles have glumes (Fig. 2) which are photosynthetically active and it is
431 possible that accompanying transpiration accounts for this observed enrichment (Lu and Lu, 2004;
432 Zhang et al., 2008). As observed for leaves, some panicle δ^2H values were closer to those of non-
433 transpiring organ δ^2H values than others. This may have resulted from more introduction of water
434 from non-transpiring panicle stems in some samples than others. Alternatively, differences in
435 proportional inputs of more water from senescing glumes that were no longer transpiring may have
436 produced the observed variation in δ^2H values among the panicle samples.

437 4.2. Apparent and biosynthetic fractionations

438 As expected, all lipids (*n*-alkanes and miliacin) are systematically depleted in deuterium
 439 compared to their corresponding hydroponic solutions and transpiring organ waters (Figure 4; Table
 440 3). This depletion could be due to the preferential selection of protium over deuterium during
 441 biosynthesis and may be a result of high NADPH selectivity towards deuterium (Schmidt et al.,
 442 2003; Chikaraishi et al., 2009). Apparent fractionation (ϵ_{app}) is a measure of the difference in $\delta^2\text{H}$
 443 values between lipid compounds and hydroponic solution, the original source of hydrogens for
 444 organic compound synthesis in a plant. Biosynthetic fractionation (ϵ_{bio}) reflects the difference in
 445 $\delta^2\text{H}$ values between lipid compounds and water in transpiring organs, the source of hydrogens for
 446 the production of photosynthates that will eventually be involved in the synthesis of other
 447 compounds (Gleixner and Mügler, 2007; Chikaraishi et al., 2009; Kahmen et al., 2013b).

448 Here, we calculate apparent fractionation as (sensu Sessions et al., 1999; Chikaraishi and
 449 Narakoa 2003):

$$450 \quad \epsilon_{\text{app}} = 1000[(\delta^2\text{H compound} + 1000) / (\delta^2\text{H}_{\text{HS}} + 1000) - 1] \quad (\text{Eq. 2})$$

451 and biosynthetic fractionation as:

$$452 \quad \epsilon_{\text{bio}} = 1000[(\delta^2\text{H compound} + 1000) / (\delta^2\text{H}_{\text{TO}} + 1000) - 1] \quad (\text{Eq. 3})$$

453 ϵ_{bio} values are medians per tank calculated from water $\delta^2\text{H}$ values in transpiring organs (TO:
 454 panicles and leaves; Table 3).

455

456 4.2.1. *n*-Alkanes

457 As discussed above, the relationship between $\delta^2\text{H}$ values of leaf water and hydroponic
 458 solutions (slope ≈ 0.46) and between non-transpiring organs and hydroponic solutions (slope ≈ 1.0)
 459 differed in a manner consistent with an explanation of transpiration influences (Fig. 4). ϵ_{app} values
 460 did not differ significantly between the two humidity levels for any of the *n*-alkanes (*p* values in
 461 Table S3) and so our analyses do not take humidity into consideration. The overall (combined for

462 58 and 74% RH) ranges of average apparent fractionations (ϵ_{app}) between *n*-alkanes and
463 hydroponic solution $\delta^2\text{H}$ values were consistently highly variable, with a range from -209 to -106‰
464 for *n*-C₂₅, -213 to -82‰ for *n*-C₂₇, -217 to -83‰ for *n*-C₃₁ and -217 to -88‰ for *n*-C₃₃ (Table 4).
465 The highly variable ranges of ϵ_{app} values are in agreement with the deviation of leaf water $\delta^2\text{H}$
466 values from $\delta^2\text{H}_{\text{HS}}$ values that are thought to be caused by transpiration (as described above).

467 For comparison purposes with the ϵ_{app} values given in the literature, here we only consider
468 the most realistic water isotopic compositions for millet growing in natural conditions (with $\delta^2\text{H}_{\text{HS}}$
469 values at -15 at 58% RH and -36 and -10‰ at 74% RH). This gives a range of ϵ_{app} values from -
470 125 to -106‰ for *n*-C₂₅, -153 to -82‰ for *n*-C₂₇, -131 to -83‰ for *n*-C₃₁ and -148 to -88‰ for *n*-
471 C₃₃. Our ϵ_{app} values for *n*-alkanes produced by *P. miliaceum* are consistent with ranges published in
472 the literature for other species. In C3 plants, ϵ_{app} between *n*-alkanes and growth water ranged
473 between -230 and -22‰ (Sessions et al., 1999; Sessions, 2006, Sachse et al., 2006; 2009; 2010;
474 Hou et al., 2007b; Feakins and Sessions 2010; Kahmen et al., 2011, 2013a;b; Garcin et al., 2012;
475 Tipple et al., 2013; Tipple et al., 2015; Gamarra et al., 2015). For C4 plants, ϵ_{app} values ranged
476 from -150 to -100‰ (Chikaraishi and Naraoka 2003; Chikaraishi et al., 2004a; Smith and Freeman,
477 2006; McInerney et al., 2011; Gamarra et al., 2016). To the best of our knowledge, only Smith and
478 Freeman (2006) have determined ϵ_{app} for a *Panicum* species (*P. virgatum*: $\epsilon_{\text{app}} = -151\text{‰}$).

479 $\delta^2\text{H}$ values of *n*-alkanes are related to the $\delta^2\text{H}$ values of water in transpiring organs (Table 3
480 and Fig. 5). This is also illustrated by the range of ϵ_{bio} , which is narrower than that of ϵ_{app} ($\epsilon_{\text{bio}} = -$
481 $151 \pm 5\text{‰}$ for *n*-C₂₅, $-158 \pm 14\text{‰}$ for *n*-C₂₇, $-145 \pm 14\text{‰}$ for *n*-C₃₁ and $-144 \pm 19\text{‰}$ for *n*-C₃₃; mean
482 of all values at both relative humidities; Table 4). This is consistent with an explanation of $\delta^2\text{H}$

483 values of biogenicity primarily reflecting leaf water $\delta^2\text{H}$ values (Kahmen et al., 2013b; Sachse et
484 al., 2010; Tipple et al., 2015).

485 As with the ϵ_{app} values, our ϵ_{bio} values of *n*-alkanes (from -163 to -130‰) fall within the
486 range of values reported by other authors. Feakins and Sessions (2010) reported ϵ_{bio} of $-147 \pm 18\text{‰}$
487 for *n*-alkanes extracted from plants thriving in an entire ecosystem. For trees, ϵ_{bio} values ranged
488 from -120 to -170‰ (Sachse et al., 2009; Tipple et al., 2013; Kahmen et al., 2011). ϵ_{bio} values
489 calculated from *n*-C₂₉ and *n*-C₃₁ alkanes and leaf water $\delta^2\text{H}$ values in dicotyledonous plant species
490 ranged from -136 to -241‰ (Kahmen et al., 2013b). For C4 plants, Zhou et al. (2010) and Smith
491 and Freeman (2006) reported ϵ_{bio} of -180 and -150‰ (the difference being attributed to
492 temperature) and -157‰, respectively. ϵ_{bio} values calculated from Kahmen et al. (2013b) ranged
493 between -181 and -158‰ for *n*-C₂₉ and *n*-C₃₁ alkanes in maize. Finally, Gamarra et al. (2016)
494 reported a mean ϵ_{bio} of -164‰ for *n*-alkanes produced by C4 grasses, and more specifically of -
495 181‰ for *Panicum virgatum*. The range of ϵ_{bio} for *n*-alkanes in these studies is narrower than that
496 of ϵ_{app} and independent of the plant type. Our study provides the first values for *n*-alkane ϵ_{bio} in
497 *Panicum miliaceum*.

498 4.2.2. Miliacin

499 Like *n*-alkanes, miliacin $\delta^2\text{H}$ values are related to $\delta^2\text{H}$ values of water in transpiring organs
500 with 0.725 (RH = 58%) and 0.784 (RH = 74%) slopes (Figure 5, Table 2) and an average ϵ_{bio} of -
501 $118 \pm 5\text{‰}$ (Table 4). Conversely, the slopes of the miliacin $\delta^2\text{H}$ values vs. $\delta^2\text{H}_{\text{HS}}$ values are 0.329

502 (RH = 58%) and 0.389 (TH = 74%), which explains the large standard deviation (44‰) of ϵ_{app}
503 around an average value of -119‰ (Table 4), as illustrated in Fig. 4.

504 Bossard et al. (2011) reported a mean $\delta^2\text{H}$ value of -120‰ for miliacin extracted from millet
505 seeds grown in the field, with a meteoric source water of -50‰ (Millot et al., 2010). This
506 corresponds to an ϵ_{app} of -73.8‰ which is lower than the ϵ_{app} values we obtained (-104‰ on
507 average at 58% RH and -133‰ on average at 74% RH).

508 Miliacin $\delta^2\text{H}$ values ranged from -122 to -18‰. In the literature, $\delta^2\text{H}$ values of pentacyclic
509 triterpenes are found in the following ranges: -171 to -142‰ in *Spartina alterniflora* and -252 to -
510 226‰ in *Daucus carota* (Sessions et al., 1999); -235 to -177‰ in taraxerol produced by
511 *Rhizophora sp.* trees in mangroves (Ladd and Sachs, 2015a); -192 to -154‰ in taraxerol produced
512 by *Rhizophora stylosa* (Ladd and Sachs, 2015b) and -276 to -185‰ (for β -amyrin), -272 to -181‰
513 (for lupeol) and -252 to -174‰ (for taraxerol) in greenhouse-grown *Rhizophora mangle* (Park et al.,
514 2019).

515 The ϵ_{app} of miliacin is about -119‰ on average. The ϵ_{app} of triterpenes range from -191 to -
516 122‰ for *Spartina alterniflora* (Sessions, 2006), from -226 to -150‰ in *Rhizophora spp.* (Ladd and
517 Sachs 2015a; 2015b), from -241 to -231‰ in *Bruguiera gymnorhiza* (Ladd and Sachs, 2017), and is
518 -150‰ for sedimentary triterpenols (Sauer et al., 2001), the latter identical to that reported for
519 miliacin in Cameroon soil samples (Schwab et al., 2015). The authors reported a 20‰ increase in
520 ϵ_{app} for C4 graminoid-derived pentacyclic triterpene methyl ethers (PTMEs) for a 35 % decrease in
521 RH, in agreement with a 19‰ increase in ϵ_{app} for miliacin with a 16% decrease in relative
522 humidity.

523 ϵ_{bio} values obtained for miliacin ($-118 \pm 5\%$) indicated less fractionation than that reported
524 for taraxerol from *Rhizophora* spp. (from -229 to -148% ; Ladd and Sachs 2015a; 2015b), or for
525 lupeol in *Bruguiera gymnorhiza* (from -239 to -223% ; Ladd and Sachs, 2017).

526

527 4.2.3. Relationships between compound-specific, leaf water and $\delta^2\text{H}_{\text{HS}}$ values

528 As explained earlier, the $\delta^2\text{H}$ values of miliacin and *n*-alkanes correlate with leaf water $\delta^2\text{H}$
529 values, with slopes close to each other (Fig. 5). Several studies have concluded that the $\delta^2\text{H}$ values
530 of biochemicals are influenced by the $\delta^2\text{H}$ values of leaf water, which integrates the $\delta^2\text{H}$ values of
531 meteoric water and fractionations caused by transpiration processes (Sessions et al., 1999;
532 Chikaraishi and Narakoa, 2003; Hou et al., 2007b; McInerney et al., 2011; Gamarra et al., 2016).
533 Our results clearly suggest that the $\delta^2\text{H}$ values of *n*-alkanes and miliacin are more closely related to
534 leaf water $\delta^2\text{H}$ values than to $\delta^2\text{H}_{\text{HS}}$ values (Table 3 and Fig. 4 and 5). This result suggests that
535 transpiration processes that affect $\delta^2\text{H}$ values of leaf waters will have a strong impact on the $\delta^2\text{H}$
536 values of biological compounds synthesised in C4 plant leaves, i.e., the factors controlling the
537 transpiration of the plants will strongly influence the $\delta^2\text{H}$ values of biochemicals (Gleixner and
538 Mügler, 2007; Sessions et al., 1999; Hou et al., 2007a,b).

539

540 4.2.4. Differences in ϵ_{bio} between miliacin and *n*-alkanes

541 The overall average ϵ_{bio} value was $-118 \pm 5\%$ for miliacin, whereas it reached -151 ± 7 , -
542 158 ± 14 , -145 ± 14 and $-144 \pm 19\%$ for the *n*-C₂₅, *n*-C₂₇, *n*-C₃₁ and *n*-C₃₃ alkanes, respectively
543 (Table 4). This constitutes a significant (Table S4 shows Wilcoxon paired sample tests between the
544 ϵ_{bio} values of each compound) enrichment of this C-3 oxygenated pentacyclic triterpene compared
545 to *n*-alkanes. This is in contrast to previous studies in which pentacyclic triterpenes were depleted

546 by ~100‰ in *Daucus carota* (Sessions et al., 1999) and by 40 to 50‰ in *Knizopnora* spp. (Ladd
547 and Sachs, 2015a), compared to *n*-alkanes. This depletion is the same as that observed for sterols
548 compared to *n*-alkyl lipids (Sessions et al., 1999; Zhou et al., 2016; Chikaraishi et al., 2004a;
549 2004b) and is classically attributed to the distinct metabolic pathways leading to these compounds,
550 and the source of their hydrogen atoms. *n*-Alkanes are produced via the acetogenic pathway from
551 palmitic acid produced in the chloroplast and then by the decarboxylation and elongation that occur
552 in the cytosol. Their hydrogen atoms are thought to derive from intracellular water (50%), from
553 carbohydrate precursors (25%) and from NADPH (25%; Sachse et al., 2012). C-3 oxygenated
554 pentacyclic triterpenes, as well as tetracyclic triterpenes (sterols), are produced in the cytosol via the
555 mevalonic acid pathway (MVA; Sessions et al., 1999; Sessions, 2006; Chikaraishi et al., 2009;
556 Zhou et al., 2011) from isopentenyl diphosphate, a precursor that is produced in both the plastid and
557 the cytosol, with uncertainties on how much can be transported through the plastid membrane.
558 These separate pathways not only involve distinct enzymatic processes but also different pools of
559 water (in the cytosol and in the plastid) with which C-bound hydrogen atoms can be exchanged.
560 They also involve different pools of NADPH produced through distinct processes and with distinct
561 isotopic values (Cormier et al., 2018) that can significantly modify the isotopic composition of the
562 final product; for example, during hydrogenation and dehydrogenation (Chikaraishi et al., 2009).
563 According to Sachse et al. (2012), the ²H-depletion of isoprenoid lipids produced through the MVA
564 pathway compared to *n*-alkyl lipids is due to hydrogen atoms transferred from NADPH to terpene
565 intermediates during the synthesis of mevalonate.

566 Our data show the reverse, with miliacin being enriched compared to *n*-alkanes. This pattern
567 has already been encountered in *Spartina alterniflora* (a C4 Poaceae), where there was either no
568 difference (Sessions et al., 1999), or a ~20‰ enrichment (Sessions, 2006) of pentacyclic triterpenes
569 compared to *n*-alkanes. Hence, an enrichment of pentacyclic triterpenes compared to *n*-alkanes
570 could be specific to C4 grasses, probably related to the compartmentation of photosynthesis in C4
571 plants into mesophyll and bundle sheath cells. Water in bundle sheath cells at the base of C4

572 grasses leaves is a mix between xylem (non-evaporated water) and leaf water (submitted to
573 evaporation). Organic compounds produced in bundle sheath cells are hence ^2H -depleted compared
574 to compounds produced in the rest of the leaves, because they derive from waters less subjected to
575 evaporation (Zhou et al., 2016; Gamara et al., 2016). In our case, the ^2H -enrichment of miliacin
576 compared to *n*-alkanes could result from the preferential synthesis of *n*-alkanes in basal cells,
577 whereas miliacin could be produced in mesophyll cells. This hypothesis is contradicted by
578 Chikaraishi et al. (2004) who reported depleted sterols compared to *n*-alkanes in C4 grasses and,
579 more clearly, by Zhou et al. (2016) who reported an ϵ_{bio} of -155‰ for *n*-alkanes and of -200‰ for
580 sterols in *Panicum coloratum*. Therefore, it is unclear whether the ^2H -enrichment observed for
581 miliacin compared to *n*-alkanes results from a specificity of miliacin synthesis, or a peculiarity of *P.*
582 *miliaceum*.

583 Specific attention should be paid to the plant location from which we extracted lipids. We
584 observed a good correlation between the $\delta^2\text{H}$ values of lipids and water in transpiring organs (leaves
585 and panicles) but this relationship could mask more complex mechanisms. It is unclear whether, in
586 *P. miliaceum*, lipids can be directly photosynthesized in seeds; i.e., whether hydrogen atoms could
587 originate from seed water, this water possibly having a distinct isotopic composition compared to
588 that of leaves (Sanchez-Bragado et al., 2019). Alternatively, lipids in seeds could be synthesised
589 from intermediates that were formed during pre-anthesis by photosynthesis in leaves, and then
590 translocated to seeds during grain-filling, with additional fractionation. Although such heterotrophy
591 is also observed in leaves, it could be more prevalent for lipids produced in seeds, and could be a
592 more important shaper of lipid $\delta^2\text{H}$ values than the organ water isotopic composition. In addition,
593 lipids could also be transferred directly from leaves to seeds in late stages of maturation, as
594 suggested for mustard (Mukherjee, 1983). There is currently no information available for the time at
595 which these processes occur in *P. miliaceum*. The extent to which all these factors more or less
596 affect $\delta^2\text{H}$ values of short chain *n*-alkanes, long chain *n*-alkanes and miliacin, remains to be
597 elucidated in order to better understand the significance of ϵ_{bio} values.

598

599 4.3. Impact of relative humidity

600 4.3.1. Impact of relative humidity on leaf water δ^2H values

601 The impact of transpiration is visible from the data shown in Figure 6 with δ^2H values of
602 water in transpiring organs systematically $\sim 29\%$ higher at 58% than at 74 % relative humidity
603 (calculated from the intercepts of respective regression lines displayed in Table 2). Eq. (1) shows
604 that, depending on the isotopic compositions of the hydroponic solutions, there is a theoretical
605 maximum difference of 6.5‰ for ^{18}O and 35‰ for 2H , when passing from 58% to 74% relative
606 humidity. This supports the idea that leaf water δ^2H values are not only influenced by the δ^2H_{HS}
607 values, but also by relative humidity. This illustrates the impact of relative humidity on water flux
608 control in stomata (Manzoni et al., 2013), attested by increasing the weight of the term “ $\delta_{HS} (1 - h)$ ”
609 and decreasing the weight of the term “ $h (\delta_v - \epsilon_k)$ ” with decreasing humidity in Eq. (1) (Craig and
610 Gordon, 1965).

611 4.3.2. Impact of relative humidity on ϵ_{bio} values

612 As described above, the first parameter that controls miliacin and *n*-alkane δ^2H values is leaf
613 water δ^2H . For a given relative humidity, ϵ_{bio} values of each compound are rather homogeneous,
614 regardless of the δ^2H_{HS} values. This result is consistent with some previous studies (i.e., Hou et al.,
615 2007b; Chikaraishi et al., 2009; Kahmen et al., 2013a). Sachse et al. (2010) reported that lipid δ^2H
616 values are mainly controlled by the leaf water δ^2H value which, in turn, is influenced by the δ^2H_{HS}
617 value. However, the leaf water enrichment compared to the hydroponic solution is mainly due to the
618 humidity that partly controls transpiration (Kahmen et al., 2013a,b).

619 For miliacin, the difference in ϵ_{bio} between 58 (from -124 to -112‰) and 74% RH (from -
620 130 to -120‰) is not significant (Table 4; Table S5). It thus appears that relative humidity has no or

621 little impact on miliacin ϵ_{bio} values. Conversely, minor, but significant differences between n -
622 alkane ϵ_{bio} values with respect to RH are noted. If confirmed, this could provide clues about
623 variations in the respective impacts of source water $\delta^2\text{H}$ values and relative humidity on the $\delta^2\text{H}$
624 values of biochemicals. ϵ_{bio} values for the $n\text{-C}_{25}$ alkane are similar at both relative humidities (from
625 -161 to -142‰ at 58% and from -163 to -146‰ at 74 H RH; Table 4; Fig. 7; Mann Whitney U test
626 statistics are summarised in Table S5). The same applies to $n\text{-C}_{27}$, with no significant difference in
627 ϵ_{bio} values between 58% RH (from -169 to -130‰) and 74% RH (from -175 to -149‰). For longer
628 chain n -alkanes ($n\text{-C}_{31}$ and $n\text{-C}_{33}$), the difference in ϵ_{bio} between the two relative humidity levels,
629 although small, is significant at $p=0.03$ (Table 4 and Fig. 7; Mann Whitney U test statistics are
630 provided in Table S5). There is more biosynthetic fractionation at 74% RH, compared to 58% RH,
631 for $n\text{-C}_{31}$ (from -166 to -150‰ and from -145 to -131‰, respectively) and for $n\text{-C}_{33}$ (from -184 to -
632 142‰ and from -136 to -122‰). This constitutes a 23 (for $n\text{-C}_{31}$) and a 28‰ (for $n\text{-C}_{33}$) additional
633 fractionation of hydrogen isotopes for a 16% increase in RH. Such a distinct behaviour of n -alkane
634 biosynthetic fractionation depending on carbon chain-length was reported by Kahmen et al.
635 (2013b). In their case, less fractionation was observed for the $n\text{-C}_{31}$ alkane under drier conditions
636 for *Populus balsamifera* and *Helianthus annuus*. The reverse was observed for *Zea mays* and
637 *Triticum aestivum* (Figure 7). There is as yet no clear pattern of ϵ_{bio} changes with relative humidity.
638 The small difference in behaviour of the ϵ_{bio} values of n -alkanes towards humidity suggested by our
639 results could be related to different proportions of hydrogen atoms derived from the chloroplast and
640 from the cytosol, depending on chain length. During n -alkane elongation beyond 16 carbon atoms,
641 they integrate a larger proportion of cytoplasmic hydrogen atoms (derived from water or from
642 NADPH) compared to chloroplastic hydrogen atoms. The contribution of hydrogens originating
643 from the chloroplast thus decreases with increasing chain length. As humidity increases, longer

644 chain *n*-alkanes may integrate hydrogen atoms that are more ²H-depleted from water in the cytosol,
645 but not from cytosolic NADPH that is considered ²H-enriched compared to plastidic NADPH
646 (Cormier et al., 2018). This would imply a preferential input of water H atoms compared to
647 NADPH during *n*-alkane elongation in the cytosol, for which we have no explanation. Conversely,
648 because the synthesis of miliacin only occurs in the cytosol (except for a potential contribution of
649 the isopentenyl diphosphate precursor from the plastid; Hemmerlin et al., 2012), ϵ_{bio} does not
650 appear to be affected by relative humidity. The small relative humidity difference between the two
651 chambers (16%), when compared to similar studies (34% for Tipple et al., 2015; 35.2% for Kahmen
652 et al., 2013b) may have been insufficient to reveal differences in miliacin ϵ_{bio} values.

653 Our results could also have been affected by the specific experimental setting we used,
654 implying transpiration of millet plants growing over a very large range of $\delta^2\text{H}$ values of the
655 hydroponic solution in the same chamber. Through the backflow of water vapor in the leaf, this may
656 have modified the expected isotopic composition of leaf water and could have masked or altered the
657 impacts of relative humidity.

658 These findings indicate that studies to more precisely discern the isotopic contributions of
659 source water $\delta^2\text{H}$ values and to back-calculate relative humidity are now needed to maximize the
660 rigor of the palaeoclimatic interpretation of $\delta^2\text{H}$ values from sedimentary biomarkers. Evaluating
661 the extent to which minute differences in $\delta^2\text{H}$ values of *n*-alkanes in a single C4 plant may reflect
662 relative humidity is a promising study strategy. Our results also suggest that the relationships
663 between the $\delta^2\text{H}$ values of lipids and climate factors should be compared between leaves and seeds,
664 especially as the latter can be an important source of sedimentary biomarkers.

665

666 5. CONCLUSIONS

667 The quality of interpretation of compound-specific isotope analysis in sedimentary archives
668 for paleoclimatic studies, and the potential to obtain quantitative estimates of variables that define

669 the hydrological cycle, are contingent upon our understanding of the main parameters that affect
670 these $\delta^2\text{H}$ values. Our contribution was conducted on *Panicum miliaceum* plants cultivated under
671 controlled conditions over hydroponic solutions of varying $\delta^2\text{H}$ values, and under two relative
672 humidity levels.

673 $\delta^2\text{H}$ values of *n*-alkanes and miliacin showed a strong correlation with leaf water $\delta^2\text{H}$ values
674 as indicated by relatively constant biosynthetic fractionation when compared to apparent
675 fractionations. Our results thus confirm that leaf water $\delta^2\text{H}$ values (which integrate source water
676 $\delta^2\text{H}$ values and transpiration) are the main control that shapes the $\delta^2\text{H}$ values of biochemicals.

677 The difference in biosynthetic fractionations between two humidity levels is non-significant
678 for miliacin, *n*-C₂₅ and *n*-C₂₇ alkanes, but becomes significant for longer chain *n*-alkanes. We
679 suspect that the respective impacts of humidity and source water $\delta^2\text{H}$ values could be discriminated
680 by comparing $\delta^2\text{H}$ values of compounds made for hydrogen atoms originating from distinct
681 compartments and sources within photosynthetic cells. Nonetheless, our results could also be
682 affected both by the specific experimental design, which may have muted any relationship between
683 lipid $\delta^2\text{H}$ values and relative humidity, and by the organ from which lipids were extracted, since
684 there is currently a lack of knowledge on the source of their hydrogen atoms and the timing of their
685 synthesis. The hypotheses drawn here should thus be confirmed by additional experiments.

686

687

688 ACKNOWLEDGMENTS

689 The authors wish to thank S. Feakins (as an Associate Editor for a first version of the
690 manuscript, and as a reviewer), N. Ladd and three anonymous reviewers for their impressive work
691 and constructive comments on an earlier version of this manuscript. This study was part of the
692 PalHydroMil Project, supported by an Agence Nationale de la Recherche grant (ANR-2010-
693 JCJC607-1). N. Bossard received a PhD grant provided by the Ministère de la Recherche et de
694 l'Enseignement Supérieur (Action Thématique Prioritaire, 2009-2012). V. J. Terwilliger was a

695 senior research fellow at ISTO under the auspices of Le Studium® (Loire Institute for Advanced
696 Studies). E. Rowley-Jolivet is acknowledged for English language editing of the manuscript.

Journal Pre-proofs

- 699 Allison, G.B., Leaney, F.W., 1982. Estimation of isotopic exchange parameters, using constant-feed
700 pans. *Journal of Hydrology* 55, 151-161.
- 701 Araguás-Araguás, L., Rozanski, K., Gonfiantini, R., Louvat, D., 1995. Isotope effects
702 accompanying vacuum extraction of soil water for stable isotope analyses. *Journal of*
703 *Hydrology* 168, 159-171.
- 704 Bai, Y., Fang, X., Gleixner, G., Mügler, I., 2011. Effect of precipitation regime on $\delta^2\text{H}$ values of
705 soil n-alkanes from elevation gradients - Implications for the study of paleo-elevation.
706 *Organic Geochemistry* 42, 838-845.
- 707 Barbour, M.M., Farquhar, G.D., Buckley, T.N., 2017. Leaf water stable isotopes and water transport
708 outside the xylem. *Plant Cell & Environment*. 40, 914-92.
- 709 Bariac, T., Rambal, S.A., Jusserand, C., Berger A., 1989. Evaluating water flux of field-grown
710 alfalfa from diurnal observations of natural isotope concentrations, energy budget and
711 ecophysiological parameters. *Agricultural and Forest Meteorology* 48, 263-283.
- 712 Bariac, T., Jusserand, C., Mariotti, A., 1990. Evolution spatio-temporelle de la composition
713 isotopique de l'eau dans le continuum sol-plante-atmosphère. *Geochimica et Cosmochimica*
714 *Acta* 54, 13-424.
- 715 Bariac T., Gonzalez-Dunia J., Katerji N., Béthenod O., Bertolini J.M., Mariotti, A. (1994a) Spatial
716 variation of the isotopic composition of water (^{18}O , ^2H) in the soil-plant-atmosphere system,
717 2. Assesment under field conditions. *Chemical Geology* 115, 317-333.
- 718 Bariac, T., Gonzalez-Dunia, J., Tardieu, F., Tessier, D., Mariotti, A., 1994b. Spatial variation of the
719 isotopic composition of water (^{18}O , ^2H) in organs of aerophytic plants: 1. Assessment under
720 laboratory conditions. *Chemical Geology* 115, 307-315.
- 721 Bossard, N., Jacob, J., Le Milbeau, C., Lallier-Verges, E., Terwilliger, V.J., Boscardin R., 2011.
722 Variation in δD values of a single, species-specific molecular biomarker: a study of miliacin

- 723 throughout a field of broomcorn millet (*Panicum miliaceum* L.). *Rapport Communications in*
724 *Mass Spectrometry* 25, 1-9.
- 725 Bossard, N., Jacob, J., Le Milbeau, C., Sauze, J., Terwilliger, V.J., Poissonnier, B., Verges, E.,
726 2013. Distribution of miliacin (olean-18-en-3 β -ol methyl ether) and related compounds in
727 broomcorn millet (*Panicum miliaceum*) and other reputed sources: Implications for the use of
728 sedimentary miliacin as a tracer of millet. *Organic Geochemistry* 63, 48-55.
- 729 Bossard, N., 2013. Pertinence et calibration d'un nouveau marqueur paléohydrologique : Le rapport
730 isotopique de l'hydrogène mesuré sur la miliacine. Ph.D. thesis, Université d'Orléans,
731 Orléans, France, 187 pp.
- 732 Chikaraishi, Y., Naraoka, H., 2003. Compound-specific δD - $\delta^{13}C$ analyses of *n*-alkanes extracted
733 from terrestrial and aquatic plants. *Phytochemistry* 63, 361-371.
- 734 Chikaraishi, Y., Naraoka, H., Poulson, S.R., 2004. Hydrogen and carbon isotopic fractionations of
735 lipid biosynthesis among terrestrial (C3, C4 and CAM) and aquatic plants. *Phytochemistry*
736 65, 1369-1381.
- 737 Chikaraishi, Y., Tanaka, R., Tanaka, A., Ohkouchi, N., 2009. Fractionation of hydrogen isotopes
738 during phytol biosynthesis. *Organic Geochemistry* 40, 569-573.
- 739 Cormier, M.A., Werner, R.A., Sauer, P.E. Gröcke, D.R., Leuenberger, M.C., Wieloch, T.,
740 Schleucher, J., Kahmen A., 2018. 2H -fractionations during the biosynthesis of carbohydrates
741 and lipids imprint a metabolic signal on the δ^2H values of plant organic compounds. *New*
742 *Phytologist* 218, 479-491.
- 743 Craig, H., 1961. Isotopic variations in meteoric waters. *Science* 133, 1702-1703.
- 744 Craig, H., Gordon, L.I., 1965. Deuterium and oxygen 18 variations in the ocean and the marine
745 atmosphere, In: Tongiorgi, E. (Eds.), *Stable Isotopes in Oceanographic Studies and*
746 *Paleotemperatures*. Consiglio nazionale delle ricerche, Laboratorio de geologia nucleare,
747 Spoleto, Italy. pp. 9-131.
- 748 Dansgaard, W., 1964. Stable isotopes in precipitation. *Tellus* 16, 436-468.

- 749 Diefendorf, A.F., Freeman, K.H., Wing, S.L., Granam H.V., 2011. Production of *n*-alkyl lipids in
750 living plants and implications for the geologic past. *Geochimica et Cosmochimica Acta* 75,
751 7472-7485.
- 752 Diefendorf, A.F., Freimuth E.J., 2017. Extracting the most from terrestrial plant-derived *n*-alkyl
753 lipids and their carbon isotopes from the sedimentary record: A review. *Organic*
754 *Geochemistry* 103, 1-21.
- 755 Dongman, G., Nürnberg, H.W., Förstel, H., Wagener, K., 1974. On the enrichment of H₂¹⁸O in the
756 leaves of transpiring plants. *Radiation and Environmental Biophysics* 11, 41-52.
- 757 Douglas, P.M.J., Pagani, M., Brenner, M., Hodell, D.A., Curtis, J. H., 2012. Aridity and vegetation
758 composition are important determinants of leaf-wax δ²H values in southeastern Mexico and
759 Central America. *Geochimica et Cosmochimica Acta* 97, 24-45.
- 760 Eglinton, G., Hamilton, R.J., 1967. Leaf epicuticular waxes. *Science* 156, 1322-1335.
- 761 Ehleringer, J.R., Dawson, T. E., 1992. Water uptake by plants: perspectives from stable isotope
762 composition. *Plant, Cell & Environment* 15, 1073-1082.
- 763 Estep, M., Hoering, T., 1980. Biogeochemistry of the stable hydrogen isotopes. *Geochimica et*
764 *Cosmochimica Acta* 44, 1197-1206.
- 765 Farris, R., Strain, B., 1978. The effect of water-stress on leaf H₂¹⁸O enrichment. *Radiation and*
766 *Environmental Biophysics* 15,167-202.
- 767 Farquhar, G.D., Cernusak, L.A., 2005. On the isotopic composition of leaf water in the non-steady
768 state. *Functional Plant Biology* 32, 293-303.
- 769 Farquhar, G.D., Cernusak, L.A., Barnes, B., 2007. Heavy water fractionation during transpiration.
770 *Plant Physiology* 143, 11-18.
- 771 Feakins, S.J., Sessions, A.L., 2010. Controls on the D/H ratios of plant leaf waxes in an arid
772 ecosystem. *Geochimica et Cosmochimica Acta* 74, 2128-2141.

- 773 Ferni, A., Bariac, T., Jusserand, C., Letolle, R., 1983. An integrated method for isotopic analysis of
774 oxygen from organic compounds, air water vapor and leaf water. *The International Journal of*
775 *Applied Radiation and Isotopes* 34, 1451-1457.
- 776 Gamarra, B., Sachse, D., Kahmen, A., 2016. Effects of leaf water evaporative ^2H -enrichment and
777 biosynthetic fractionation on leaf wax *n*-alkane $\delta^2\text{H}$ values in C3 and C4 grasses. *Plant, Cell*
778 *& Environment* 39, 2390-2403.
- 779 Gao, L., Edwards, E.J., Zeng, Y., Huang, Y., 2014. Major evolutionary trends in hydrogen isotope
780 fractionation of vascular plant leaf waxes. *PLoS ONE* 9, e112610.
- 781 Garcin, Y., Schwab, V.F., Gleixner, G., Kahmen, A., Todou, G., Séné, O., Onana, J.M.,
782 Achoundong, G., Sachse, D., 2012. Hydrogen isotope ratios of lacustrine sedimentary *n*-
783 alkanes as proxies of tropical African hydrology: Insights from a calibration transect across
784 Cameroon. *Geochimica et Cosmochimica Acta* 79, 106-126.
- 785 Gleixner, G., Mügler, I., 2007. Compound-specific hydrogen isotope ratios of biomarkers: tracing
786 climatic changes in the past, In: Dawson, T.E., Siegwolf, R.T.W. (Eds.), *Stable isotopes as*
787 *indicators of ecological change*. Oxford Academic Press, Oxford, pp. 249-266.
- 788 Gonfiantini, R., Gratsiu, S., Tongiorgi, E., 1965. Oxygen isotopic composition of water in leaves. In
789 *Isotopes and Radiation in Soil Plant Nutrition Studies*. IAEA, Vienna. pp. 405-410.
- 790 Hemmerlin, A., Harwood, J.L., Bach, T.J., 2012. A raison d'être for two distinct pathways in the
791 early steps of plant isoprenoid biosynthesis? *Progress in Lipid Research* 51, 95-148.
- 792 Hou, J., D'Andrea, W.J., Mac Donald, D., Huang, Y., 2007a. Evidence for water use efficiency as
793 an important factor in determining the $\delta^2\text{H}$ values of tree leaf waxes. *Organic Geochemistry*
794 38, 1251-1255.
- 795 Hou, J., D'Andrea, W.J., McDonald, D., Huang, Y., 2007b. Hydrogen isotopic variability in leaf
796 waxes among terrestrial and aquatic plants around Blood Pond, Massachusetts (USA).
797 *Organic Geochemistry* 38, 977-984.

- 798 Huang, Y., Snuman, B., wang, Y., webb, I., 2002. Hydrogen isotope ratios of palmitic acid in
799 lacustrine sediments record late Quaternary climate variations. *Geology* 30, 1103-1106.
- 800 Jacob, J., Disnar, J.-R., Boussafir, M., Spadano Albuquerque, A.L., Sifeddine, A., Turcq, B., 2005.
801 Pentacyclic triterpene methyl ethers in recent lacustrine sediments (Lagoa do Caço, Brazil).
802 *Organic Geochemistry* 36, 449-461.
- 803 Jacob, J., Disnar, J.R., Arnaud, F., Chapron, E., Debret, M., Lallier-Vergès, E., Desmet, M., Revel-
804 Rolland, M., 2008a. Millet cultivation history in the French Alps as evidenced by a
805 sedimentary molecule. *Journal of Archaeological Science* 35, 814-820.
- 806 Jacob, J., Disnar, J.R., Bardoux, G., 2008b. Carbon isotope evidence for sedimentary miliacin as a
807 tracer of *Panicum miliaceum* (broomcorn millet) in the sediments of Lake le Bourget (French
808 Alps). *Organic Geochemistry* 39, 1077-1080.
- 809 Jacob, J., Disnar, J.R., Arnaud, F., Gauthier, E., Billaud, Y., Chapron, E., Bardoux, G., 2009.
810 Impacts of new agricultural practices on soil erosion during the Bronze Age in French
811 Prealps. *The Holocene* 19, 241-249.
- 812 Jia, G., Wei, K., Chen, F., Peng, P., 2008. Soil *n*-alkane $\delta^2\text{H}$ vs. altitude gradients along Mount
813 Gongga, China. *Geochimica et Cosmochimica Acta* 72, 5165–5174.
- 814 Kahmen, A., Dawson, T.E., Vieth, A., Sachse, D., 2011. Leaf wax *n*-alkane $\delta^2\text{H}$ values are
815 determined early in the ontogeny of *Populus trichocarpa* leaves when grown under controlled
816 environmental conditions. *Plant, Cell & Environment* 34, 1639-1651.
- 817 Kahmen, A., Hoffmann, B., Schefuss, E., Arndt, S.K., Cernusak, L.A., West, J.B., Sachse, D.,
818 2013a. Leaf water deuterium enrichment shapes leaf wax *n*-alkane $\delta^2\text{H}$ values of angiosperm
819 plants II: Observational evidence and global implications. *Geochimica et Cosmochimica Acta*
820 111, 50–63.
- 821 Kahmen, A., Schefuss, E., Sachse, D., 2013b. Leaf water deuterium enrichment shapes leaf wax *n*-
822 alkane $\delta^2\text{H}$ values of angiosperm plants I: Experimental evidence and mechanistic insights.
823 *Geochimica et Cosmochimica Acta* 11, 39-49.

- 824 Ladd, N.S., Sachs, J.P., 2015a. Influence of salinity on hydrogen isotope fractionation in
825 *Rhizophora* mangroves from Micronesia. *Geochimica et Cosmochimica Acta* 168, 206-221.
- 826 Ladd, N.S., Sachs, J.P., 2015b. Hydrogen isotope response to changing salinity and rainfall in
827 Australian mangroves. *Plant, Cell & Environment* 38, 2674-2687.
- 828 Ladd, S.N., Sachs, J.P., 2017. $^2\text{H}/^1\text{H}$ fractionation in lipids of the mangrove *Bruguiera gymnorhiza*
829 increases with salinity in marine lakes of Palau. *Geochimica et Cosmochimica Acta* 204, 300-
830 312.
- 831 Liu, W., Yang, H., 2008. Multiple controls for the variability of hydrogen isotopic compositions in
832 higher plant *n*-alkanes from modern ecosystems. *Global Change Biology* 14, 2166-2177.
- 833 Longchamp, M., Castrec-Rouelle, M., Biron, P., Bariac, T., 2015. Variations in the accumulation,
834 localization and rate of metabolization of selenium in mature *Zea mays* plants supplied with
835 selenite or selenate. *Food Chemistry* 182, 128-135.
- 836 Lu, H., Zhang, J., Wu, N., Liu, K.B., Xu, D., Li, Q., 2009. Phytoliths analysis for the discrimination
837 of foxtail millet (*Setaria italica*) and common millet (*Panicum miliaceum*). *PLoS ONE* 4,
838 e4448.
- 839 Lu, Q., Lu, C., 2004. Photosynthetic pigment composition and photosystem II photochemistry of
840 wheat ears. *Plant Physiology and Biochemistry* 42, 395-402.
- 841 Manzoni, S., Vico, G., Katul, G., Porporato, A., 2013. Biological constraints on water transport in
842 the soil–plant–atmosphere system. *Advances in Water Resources* 51, 292-304.
- 843 McInerney, F.A., Helliker, B.R., Freeman, K.H., 2011. Hydrogen isotope ratios of leaf wax *n*-
844 alkanes in grasses are insensitive to transpiration. *Geochimica et Cosmochimica Acta* 75, 541-
845 554.
- 846 Millot, R., Petelet-Giraud, E., Guerrot, C., Négrel, P., 2010. Multi-isotopic composition ($\delta^7\text{Li}$ - $\delta^{11}\text{B}$ -
847 δD - $\delta^{18}\text{O}$) of rain waters in France: Origin and spatio-temporal characterization. *Applied*
848 *Geochemistry* 25, 1510-1524.

- 849 Muknerjee, K.D., 1983. Lipid biosynthesis in developing mustard seed. *Plant Physiology* 13, 929-
850 934.
- 851 Park, W.J., Ladd, N.S., Sachs, J.P., 2019. Hydrogen and carbon isotope responses to salinity in
852 greenhouse-cultivated mangroves. *Organic Geochemistry* 132, 23-36.
- 853 Polissar, P.J., Freeman, K.H., 2010. Effects of aridity and vegetation on plant-wax $\delta^2\text{H}$ in modern
854 lake sediments. *Geochimica et Cosmochimica Acta* 74, 5785-5797.
- 855 Rothfuss, Y., Biron, P., Braud, I., Canale, L., Durand, J.L., Gaudet, J.P., Richard, P., Vauclin, M.,
856 Bariac, T., 2010. Partitioning evapotranspiration fluxes into soil evaporation and plant
857 transpiration using water stable isotopes under controlled conditions. *Hydrological Processes*
858 24, 3177-3194.
- 859 Sachse, D., Radke, J., Gleixner, G., 2004. Hydrogen isotope ratios of recent lacustrine sedimentary
860 *n*-alkanes record modern climate variability. *Geochimica et Cosmochimica Acta* 68, 4877-
861 4889.
- 862 Sachse, D., Radke, J., Gleixner, G., 2006. δD values of individual *n*-alkanes from terrestrial plants
863 along a climatic gradient – Implications for the sedimentary biomarker record. *Organic*
864 *Geochemistry* 37, 469-483.
- 865 Sachse, D., Kahmen, A., Gleixner, G., 2009. Significant seasonal variation in the hydrogen isotopic
866 composition of leaf-wax lipids for two deciduous tree ecosystems (*Fagus sylvatica* and *Acer*
867 *pseudoplatanus*). *Organic Geochemistry* 40, 732-742.
- 868 Sachse, D., Gleixner, G., Wilkes, H., Kahmen, A., 2010. Leaf wax *n*-alkane $\delta^2\text{H}$ values of
869 fieldgrown barley reflect leaf water $\delta^2\text{H}$ values at the time of leaf formation. *Geochimica et*
870 *Cosmochimica Acta* 74, 6741-6750.
- 871 Sachse, D., Billault, I., Bowen, G.J., Chikaraishi, Y., Dawson, T.E., Feakins, S.J., Freeman, K.H.,
872 Magill, C.R., McNerney, F.A., van der Meer, M.T.J., Polissar, P., Robins, R., Sachs, J.P.,
873 Schmidt, H.-L., Sessions, A.L., White, J.W.C., West, J.B., Kahmen, A., 2012. Molecular

- 874 paleontology: interpreting the hydrogen-isotopic composition of lipid biomarkers from
875 photosynthetic organisms. *Annual Reviews of Earth and Planetary Sciences* 40, 221-249.
- 876 Sanchez-Bragado, R., Serret, M.D., Marimon, R.M., Bort, J. and Araus, J.L., 2019. The hydrogen
877 isotope composition $\delta^2\text{H}$ reflects plant performance. *Plant Physiology* 180, 793-812.
- 878 Sauer, P.E., Eglinton, T.I., Hayes, J.M., Schiimmelmann, A., Sessions, A.L., 2001. Compound-
879 specific D/H ratios of lipid biomarkers from sediments as a proxy for environmental and
880 climatic conditions. *Geochimica et Cosmochimica Acta* 65, 213-222.
- 881 Schmidt, H.-L., Werner, R.A., Eisenreich, W., 2003. Systematics of ^2H patterns in natural
882 compounds and its importance for the elucidation of biosynthetic pathways. *Phytochemical*
883 *Reviews* 2, 61-85.
- 884 Schwab, V.F., Garcin, Y., Sachse, D., Todou, G., Séné, O., Onana, J.-M., Achoundong, G.,
885 Gleixner, G., 2015. Effect of aridity on $\delta^{13}\text{C}$ and δD values of C₃ plant- and C₄ graminoid-
886 derived leaf wax lipids from soils along an environmental gradient in Cameroon (Western
887 Central Africa). *Organic Geochemistry* 78, 99-109.
- 888 Sessions, A.L., Burgoyne, T.W., Schimmelmann, A., Hayes, J.M., 1999. Fractionation of hydrogen
889 isotopes in lipid biosynthesis. *Organic Geochemistry* 30, 1193-1200.
- 890 Sessions, A.L., Burgoyne, T.W., Hayes, J.M., 2001. Correction of H_3^+ contributions in hydrogen
891 isotope ratio monitoring mass spectrometry. *Analytical Chemistry* 73, 192-199.
- 892 Sessions, A.L., 2006. Seasonal changes in D/H fractionation accompanying lipid biosynthesis in
893 *Spartina alterniflora*. *Geochimica et Cosmochimica Acta* 70, 2153-2162.
- 894 Sessions, A.L., 2016. Factors controlling the deuterium contents of sedimentary hydrocarbons.
895 *Organic Geochemistry* 96, 43-64.
- 896 Smith, F.A., Freeman, K.H., 2006. Influence of physiology and climate δD of leaf wax *n*-alkanes
897 from C₃ and C₄ grasses. *Geochimica et Cosmochimica Acta* 70, 1172-1187.

- 898 Terwilliger, V.J., Deniro, M.J., 1995. Hydrogen isotope fractionation in wood-producing avocado
899 seedlings: Biological constraints to paleoclimatic interpretations of δD values in tree ring
900 cellulose nitrate. *Geochimica et Cosmochimica Acta* 59, 5199-5207.
- 901 Terwilliger, V.J., Jacob, J., 2013. Introduction: Hydrogen isotopes as environmental recorders.
902 *Geochimica et Cosmochimica Acta* 111, 1-4.
- 903 Tipple, B.J., Pagani, M., 2013. Environmental control on eastern broadleaf forest species' leaf wax
904 distributions and D/H ratios. *Geochimica et Cosmochimica Acta* 111, 64-77.
- 905 Tipple, B.J., Berke, M.A., Hambach, B., Roden, J.S., Ehleringer, J.R., 2015. Predicting leaf wax *n*-
906 alkane $^2H/^1H$ ratios: controlled water source and humidity experiments with hydroponically
907 grown trees confirm predictions of Craig-Gordon model. *Plant, Cell & Environment* 38,
908 1035-1047.
- 909 Yakir, D., 1992. Water compartmentation in plant tissue: isotopic evidence, In: Somero, S.G.N.,
910 Osmond, C.B., Bolis, C.L. (Eds.), *Water and Life*. Springer-Verlag, Berlin, pp. 205-222.
- 911 Yang, H., Huang, Y., 2003. Preservation of lipid hydrogen isotope ratios in Miocene lacustrine
912 sediments and plant fossils at Clarkia, northern Idaho, USA. *Organic Geochemistry* 34, 413-
913 423.
- 914 Zhang, Y.H., Wang, Z.M., Huang, Q., Shu, W., 2008. Phosphoenolpyruvate carboxylase activity in
915 ear organs is related to protein concentration in grains of winter wheat. *Journal of Cereal
916 Science* 47, 386-391.
- 917 Zhou, Y., Grice, K., Stuart-Williams, H., Farquhar, G. D., Hocart, C.H., Lu, H., Liu, W., 2010.
918 Biosynthetic origin of the saw-toothed profile in $\delta^{13}C$ and δ^2H of *n*-alkanes and systematic
919 isotopic differences between *n*-, iso- and anteiso-alkanes in leaf waxes of land plants.
920 *Phytochemistry* 71, 388-403.
- 921 Zhou, Y., Grice, K., Chikaraishi, Y., Stuart-Williams, H., Farquhar, G.D., Ohkouchi, N., 2011.
922 Temperature effect on leaf water deuterium enrichment and isotopic fractionation during leaf

- 923 lipid biosynthesis: Results from controlled growth of C3 and C4 land plants. *Phytochemistry*
924 72, 207-213.
- 925 Zhou, Y., Grice, K., Stuart-Williams, H., Hocart, C.H., Gessler, A., Farquhar, G.D., 2016.
926 Hydrogen isotopic differences between C3 and C4 land plant lipids: consequences of
927 compartmentation in C4 photosynthetic chemistry and C3 photorespiration. *Plant, Cell &*
928 *Environment* 39, 2676–2690.

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929 **Figure Captions**

930 Fig. 1: Structure of miliacin.

931 Fig. 2: (a) Experimental design. $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of the hydroponic solution are those reported
932 in Section 2.1. (b) Sketch of a *Panicum miliaceum* plant with reference to organs sampled for this
933 study and details of a spikelet (redrawn from Lu et al., 2009).

934 Fig. 3: Water $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values in different organs for distinct hydroponic solution $\delta^2\text{H}$ values at
935 58 and 74% RH (Table S2). Considering the analytical precision ($\pm 0.1\text{‰}$ for $\delta^{18}\text{O}$ values and $\pm 1\text{‰}$
936 for $\delta^2\text{H}$ values) and reproducibility (cf. Table S1), error bars are not displayed.

937 Fig. 4: $\delta^2\text{H}$ values of water in non-transpiring organs (NTO), transpiring organs (TO), panicles (P),
938 miliacin and *n*-alkanes (averaged) versus $\delta^2\text{H}$ values of the hydroponic solution (HS) at 58%
939 relative humidity (a) and at 74% relative humidity (b). Considering the analytical precision ($\pm 0.1\text{‰}$
940 for $\delta^{18}\text{O}$ values and $\pm 1\text{‰}$ for $\delta^2\text{H}$ values) and reproducibility (cf. Table S1), error bars are not
941 displayed for water $\delta^2\text{H}$ values. Error bars for compound $\delta^2\text{H}$ values correspond to the standard
942 deviation reported in Table 3.

943 Fig. 5: $\delta^2\text{H}$ values of miliacin and individual *n*-alkanes versus $\delta^2\text{H}$ values of water in transpiring
944 organs (TO) at 58% relative humidity (a) and at 74% relative humidity (b). Considering the
945 analytical precision ($\pm 0.1\text{‰}$ for $\delta^{18}\text{O}$ values and $\pm 1\text{‰}$ for $\delta^2\text{H}$ values) and reproducibility (cf.
946 Table S1), error bars are not displayed for water $\delta^2\text{H}$ values in transpiring organs. Error bars for
947 compound $\delta^2\text{H}$ values correspond to the standard deviation reported in Table 3.

948 Fig. 6: Evolution of water $\delta^2\text{H}$ values in transpiring organs (TO) versus hydroponic solution (HS)
949 $\delta^2\text{H}$ values at 58% relative humidity and at 74 % relative humidity. Considering the analytical
950 precision ($\pm 0.1\text{‰}$ for $\delta^{18}\text{O}$ values and $\pm 1\text{‰}$ for $\delta^2\text{H}$ values) and reproducibility (cf. Table S1),
951 error bars are not displayed for water $\delta^2\text{H}$ values.

952 Fig. 7: a- ϵ_{bio} values of *n*-alkanes and miliacin at 58 and 74% relative humidity in *Panicum*
953 *miliaceum*. Each value corresponds to the median, minimum and maximum of ϵ_{bio} values (Table 4)
954 calculated by tank 1 to 4 (from left to right) from $\delta^2\text{H}$ values of compounds (Table 3) compared to

955 $\delta\text{-H}$ values of water in the corresponding individual transpiring organs (Table S2); $\delta\text{-}\epsilon_{\text{bio}}$ values of
956 $n\text{-C}_{31}$ alkane at 36 and 71.2% relative humidity in various plants (data from Kahmen et al., 2013).

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Table 1: $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of water from hydroponic solutions, and non-transpiring (roots and internodes) and transpiring (leaves and panicles) organs in growth chambers at 58% and 74% relative humidities. δ values are averages where replicates exist or are otherwise individual values. Table S2 contains the data for all individual values. HS = hydroponic solution, NTO = non-transpiring organs, TO = transpiring organs.

58% RH	$\delta^{18}\text{O}$ (‰ V-SMOW)		$\delta^2\text{H}$ (‰ V-SMOW)		74% RH	$\delta^{18}\text{O}$ (‰ V-SMOW)		$\delta^2\text{H}$ (‰ V-SMOW)			
	Average	Stdev	Average	Stdev		Average	Stdev	Average	Stdev		
Tank 1	HS	-1.1		-15		Tank 1	HS	-4.8		-36	
	NTO	-1.1	0.2	-18	4.6		NTO	-4.9	0.3	-37	1.4
	TO	8.7	1.6	38	7.2		TO	7.5	4.0	0	9.8
Tank 2	HS	-2.2		15		Tank 2	HS	-6.6		-10	
	NTO	-2.5	0.2	16	1.2		NTO	-6.7	0.3	-10	0.3
	TO	7.4	2.0	51	4.8		TO	6.1	1.6	12	2.1
Tank 3	HS	-2.4		105		Tank 3	HS	-6.6		95	
	NTO	-2.3	0.3	109	2.5		NTO	-6.3	0.3	94	1.0
	TO	6.3	3.7	88	6.2		TO	7.1	1.9	52	3.2
Tank 4	HS	-3.5		160		Tank 4	HS	-6.8		187	
	NTO	-3.3	0.2	157	5.8		NTO	-6.7	0.3	187	1.2
	TO	4.3	1.0	118	12.3		TO	6.5	0.7	114	2.7

Table 2: Regression statistics for (a) Figure 3 (tank and plant water $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values); (b) Figure 4 ($\delta^2\text{H}$ values of water in HS, organs, miliacin and *n*-alkanes); (c) Figure 5 ($\delta^2\text{H}$ values of miliacin and *n*-alkanes versus $\delta^2\text{H}$ values of water in transpiring organs); (d) Figure 6 ($\delta^2\text{H}$ values of water in HS and in transpiring organs at two RH). a is the slope, b is the intercept.

(a) Figure 3	RH = 58 %				RH = 74 %			
	a	b	r ²	p	a	b	r ²	p
Tank 1	5.6075	-11.462	0.9902	<0.0005	2.9528	-22.337	0.9911	<0.0005
Tank 2	3.4749	24.872	0.9856	<0.0005	1.6498	1.3168	0.9943	<0.0005
Tank 3	-2.1234	102.23	0.8824	<0.0005	-3.0654	74.186	0.983	<0.0005
Tank 4	-5.0907	140.57	0.8549	<0.0005	-5.5574	149.94	0.9939	<0.0005

(b) Figure 4	RH = 58 %				RH = 74 %			
	a	b	r ²	p	a	b	r ²	p
NTO	1.0067	0	0.997	<0.0005	1.0056	-0.87	1	<0.0005
TO	0.45	43.8	0.95	<0.0005	0.48	15.5	0.963	<0.0005
Miliacin	0.329	-69.412	0.99	0.005	0.389	-108	0.981	0.01
<i>n</i> -C ₂₅	0.307	-111	0.953	0.024	0.404	-136	0.99	0.005
<i>n</i> -C ₂₇	n.s.	n.s.	n.s.	0.18	0.477	-154	0.997	0.001
<i>n</i> -C ₃₁	0.326	-92	0.907	0.048	0.376	-142	0.987	0.006
<i>n</i> -C ₃₃	0.375	-91.11	0.968	0.016	n.s.	n.s.	0.898	0.052

(c) Figure 5	RH = 58 %				RH = 74 %			
	a	b	r ²	p	a	b	r ²	p
Miliacin	0.725	-101	0.983	0.009	0.784	-120	0.998	0.001
<i>n</i> -C ₂₅	0.683	-141	0.964	0.003	0.8	-148	0.971	0.015
<i>n</i> -C ₂₇	n.s.	n.s.	n.s.	0.175	0.94	-167	0.967	0.017
<i>n</i> -C ₃₁	0.735	-125	0.97	0.015	0.748	-153	0.846	0.013
<i>n</i> -C ₃₃	0.83	-127	0.97	0.03	n.s.	n.s.	n.s.	0.081

(d) Figure 6	a	b	r ²	p
RH = 58 %	0.448	43.824	0.947	<0.0005
RH = 74 %	0.482	15.456	0.963	<0.0005

957 Table 3: $\delta^2\text{H}$ values of hydroponic solution (HS) and transpiring organ (TO) (averaged if >1 value per tank) waters and miliacin and *n*-alkanes
 958 extracted from *P. miliaceum* seeds collected after cultivation at 58 and at 74% relative humidity in climatic chambers. n corresponds to the number of
 959 replicates for each treatment (seeds from 1 to 3 plants per tank) and can vary between compounds depending on the concentration of each compound in
 960 the seed extract considered.
 961

	HS	TO	Miliacin $\delta^2\text{H}$			<i>n</i> -C ₂₅ alkane $\delta^2\text{H}$			<i>n</i> -C ₂₇ alkane $\delta^2\text{H}$			<i>n</i> -C ₃₁ alkane $\delta^2\text{H}$			<i>n</i> -C ₃₃ alkane $\delta^2\text{H}$			
	Tank	(‰ V-SMOW)	(‰ V-SMOW)	(‰ V-SMOW)	Stdev	n	(‰ V-SMOW)	Stdev	n	(‰ V-SMOW)	Stdev	n	(‰ V-SMOW)	Stdev	n	(‰ V-SMOW)	Stdev	n
58%	1	-15	38	-77	6	3	-119		1	-96		1	-97		1	-102		1
	2	15	51	-61		1	-99		1	-128		1	-83		1	-78		1
	3	105	88	-34	1	2	-83	12	2	-81	0	2	-70	18	2	-54	10	2
	4	160	118	-18		1	-59		1	-57		1	-32		1	-31		1
74%	1	-36	0	-122	7	3	-155	2	2	-170	6	3	-160	3	3	-179	14	3
	2	-10	12	-109	2	2	-134		1	-161		1	-140		1	-133		1
	3	95	52	-80	7	2	-98		1	-105		1	-108		1	-98		1
	4	187	114	-31	1	3	-61	10	2	-66	1	2	-71	11	2	-70	2	2

HS=hydroponic solution; TO=transpiring organs (averaged according to Table S2).

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965 Table 4: Apparent (ϵ_{app}) and biosynthetic (ϵ_{bio}) fractionation for miliacin and *n*-alkanes, calculated from equation 2 and equation 3, at 58% and at 74%
 966 relative humidity. ϵ_{bio} values are calculated from $\delta^2\text{H}$ values of miliacin and *n*-alkanes (Table 3) and $\delta^2\text{H}$ values of water in individual transpiring
 967 organs (TO; leaves and panicles, Table S2).
 968

Tank	ϵ_{app} (‰)					ϵ_{bio} (‰)															
	Miliacin	<i>n</i> -C ₂₅	<i>n</i> -C ₂₇	<i>n</i> -C ₃₁	<i>n</i> -C ₃₃	Miliacin			<i>n</i> -C ₂₅			<i>n</i> -C ₂₇			<i>n</i> -C ₃₁			<i>n</i> -C ₃₃			
						Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max	
58% RH	1	-63	-106	-82	-83	-88	-112	-116	-100	-153	-157	-141	-130	-134	-119	-131	-135	-119	-136	-140	-125
	2	-75	-112	-141	-97	-92	-106	-114	-103	-142	-150	-139	-169	-177	-166	-127	-134	-124	-122	-130	-119
	3	-126	-170	-168	-158	-144	-112	-118	-105	-157	-163	-151	-155	-169	-149	-145	-150	-138	-131	-136	-124
	4	-153	-189	-187	-166	-165	-124	-130	-108	-161	-167	-146	-159	-164	-144	-137	-143	-122	-135	-141	-120
74% RH	1	-89	-123	-139	-129	-148	-127	-131	-112	-160	-163	-145	-175	-178	-160	-164	-168	-150	-184	-187	-169
	2	-100	-125	-153	-131	-124	-120	-121	-117	-145	-146	-142	-171	-172	-168	-150	-151	-147	-144	-145	-141
	3	-160	-176	-183	-185	-176	-125	-128	-121	-143	-146	-139	-149	-152	-145	-152	-155	-147	-142	-145	-138
	4	-184	-209	-213	-217	-217	-130	-132	-128	-156	-158	-155	-161	-163	-160	-166	-167	-164	-165	-167	-163
Overall average	-119	-151	-158	-146	-144	-118				-151			-158			-145			-144		
Overall Stdev	44	39	40	45	43	5				7			14			14			19		

Declaration of interests

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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Figure 1

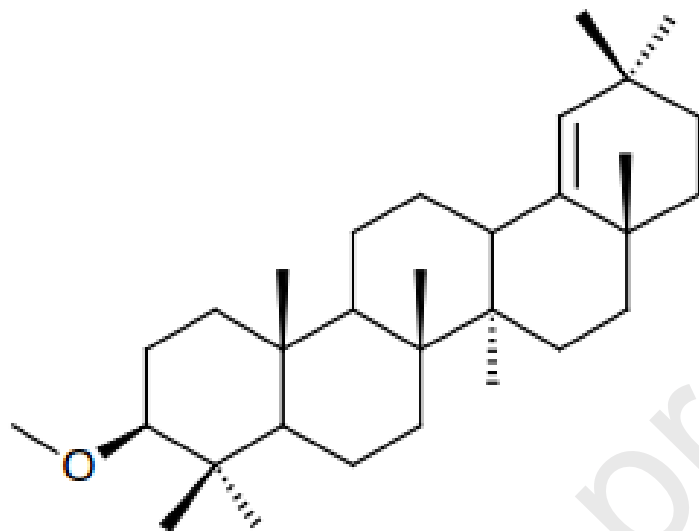


Figure 2

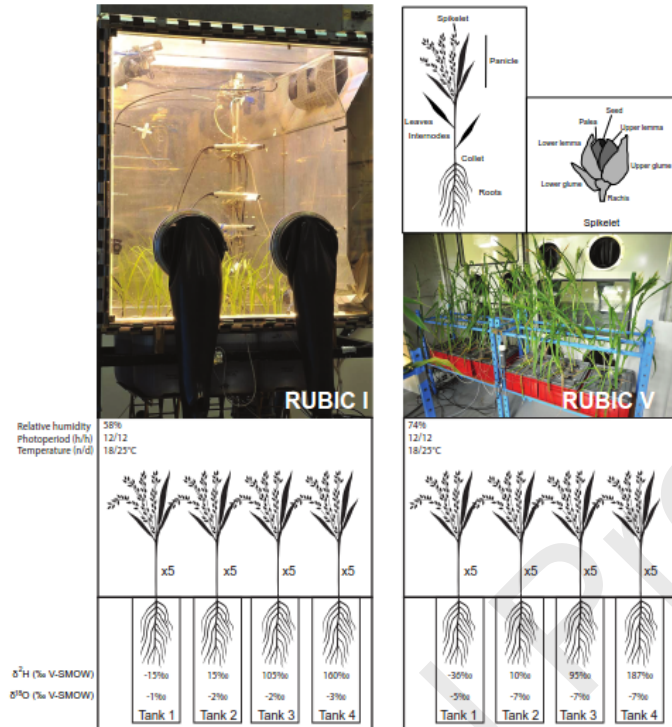
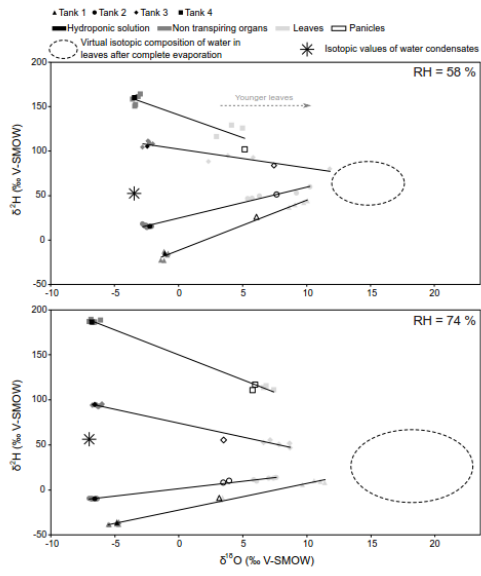
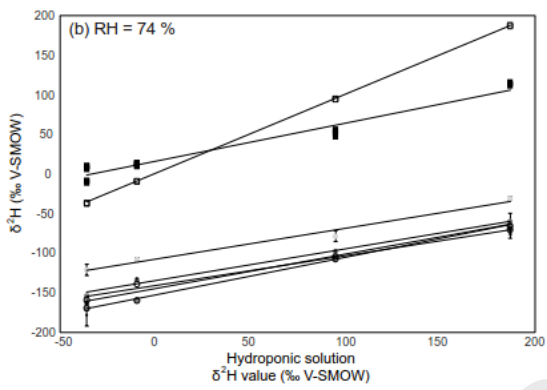
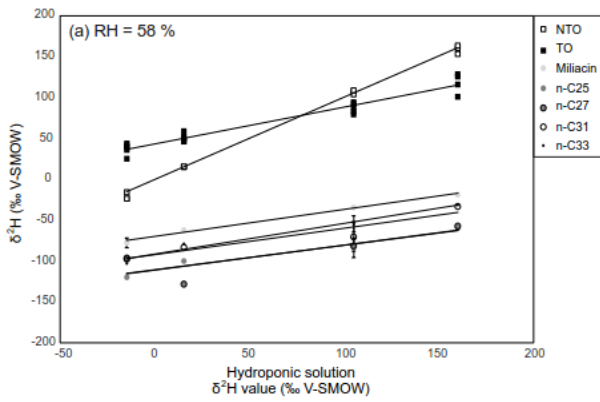
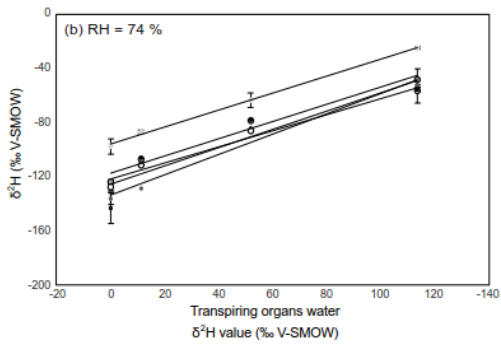
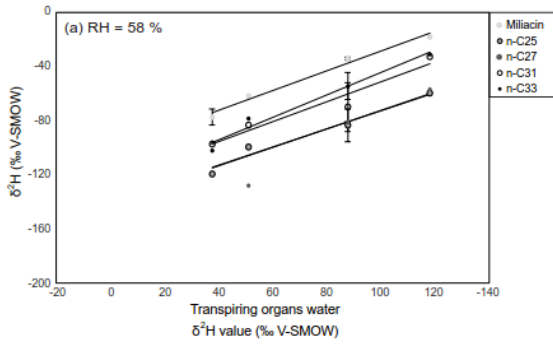


Figure 3







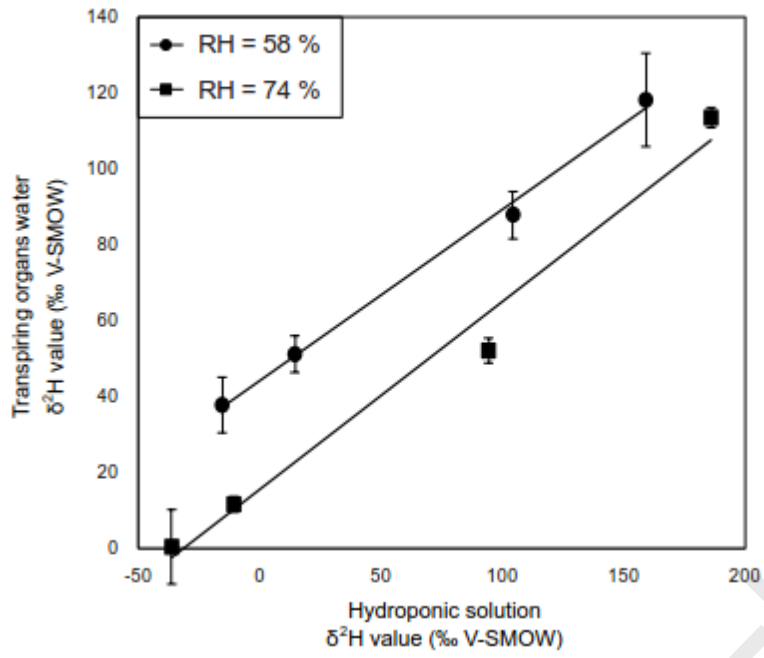


Figure 7

