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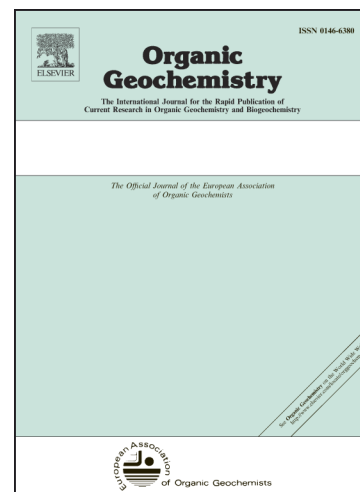
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2 millet (*Panicum miliaceum* L.) under controlled environmental conditions

3

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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  $^2\text{H}$ -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*-C<sub>25</sub> and *n*-C<sub>27</sub> alkanes, it led to a  $\sim 25\%$  decrease in biosynthetic  
47 fractionation for *n*-C<sub>31</sub> and *n*-C<sub>33</sub> 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 C<sub>4</sub> 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  $^2\text{H}$  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 $\beta$ -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;  $\epsilon_{\text{app}}$ ) and biosynthetic (lipid vs. leaf water;  $\epsilon_{\text{bio}}$ )

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 m<sup>3</sup>  
154 RUBIC I and of 74 % in the 9 m<sup>3</sup> 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<sup>-1</sup>) 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  $1\text{‰}$ ) 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  $\text{N}_2$  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).  $5\alpha$ -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  $\mu\text{m}$  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  $\text{min}^{-1}$ , with a final isothermal  
215 hold at 300°C for 30min. The sample was dissolved in toluene and injected splitless in a 2  $\mu\text{L}$   
216 volume with injector temperature set at 280°C. The carrier gas was helium at a flow rate of 1.4 mL  
217  $\text{min}^{-1}$ . 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 miltiacin 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  $\mu\text{m}$  film thickness; Agilent, Palo Alto, CA, USA) was slightly different. 1  $\mu\text{L}$  of the  
226 sample, dissolved in toluene, was injected at least 3 times. We used a standard consisting of 1  $\mu\text{L}$  of  
227 a mixture of 15 *n*-alkanes (*n*-C<sub>16</sub> to *n*-C<sub>30</sub>) 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 miltiacin, 1  $\mu\text{L}$  of the standard mixture  
230 was co-injected with 1  $\mu\text{L}$  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*-C<sub>25</sub> and *n*-C<sub>27</sub> 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  $\mu\text{L}$  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  $\text{H}_3^+$  factor (Sessions et al., 2001) was determined on a daily basis and was  $7.26 \pm 0.17$ .

### 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  $\mathcal{E}_{\text{app}}$   
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).  $\mathcal{E}_{\text{bio}}$  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  $\delta$  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 ( $\delta_v$ ) was not monitored in the  
319 chambers during experiments. Instead, we used the condensate isotopic composition ( $\delta_C$ , 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:  $\delta^{18}\text{O}_C = -3.5\%$  and  
322  $\delta^2\text{H}_C = 52\%$  and RUBIC V:  $\delta^{18}\text{O}_C = -7.0\%$  and  $\delta^2\text{H}_C = 57\%$ ) were about the same as the average  
323 of the isotopic compositions of the 4 hydroponic solutions (RUBIC I:  $\delta^{18}\text{O}_{\text{HS}} = -2.3\%$  and  $\delta^2\text{H}_{\text{HS}} =$   
324  $66\%$ ; RUBIC V:  $\delta^{18}\text{O}_{\text{HS}} = -6.2\%$  and  $\delta^2\text{H}_{\text{HS}} = 59\%$ ). 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*-C<sub>25</sub>, *n*-C<sub>27</sub>, *n*-C<sub>31</sub> and *n*-C<sub>33</sub> 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*-C<sub>29</sub> alkane was also abundant, but unfortunately co-eluted with  
334 the 5 $\alpha$ -cholestane used as a quantitation standard. Consequently, we were unable to obtain reliable  
335  $\delta^2\text{H}$  values for *n*-C<sub>29</sub>. The data reported in Table 3 correspond to  $\delta^2\text{H}$  values of miliacin and all  
336 alkanes except *n*-C<sub>29</sub> 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  $\text{C}_{25}$ , from -96 to -57‰ (58% RH) and from -170 to -66‰ (74% RH) for *n*- $\text{C}_{27}$ , from -97 to -32‰  
344 (58% RH) and from -160 to -71‰ (74% RH) for *n*- $\text{C}_{31}$  and from -102 to -31‰ (58% RH) and from  
345 -179 to -70‰ (74% RH) for *n*- $\text{C}_{33}$  (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;  $\delta_{\text{HS}}$ , water vapor;  $\delta_{\text{v}}$ , water vapor  
 377 transpiration;  $\delta_{\text{E}}$ , and condensate;  $\delta_{\text{C}}$ ).

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

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

381  $\delta_{\text{v}}$  is substantially constant during the day in the reactors, as are RH and  $\delta_{\text{HS}}$  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  $\delta^2H$  values of water extracted from transpiring  
 396 organs (leaves and panicles) are different from the  $\delta^2H$  values of their hydroponic solution source  
 397 waters (Fig. 3). In  $^2H$ -depleted hydroponic solutions, the  $\delta^2H$  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  $\delta^2H$  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 ( $\delta^2H$  or  $\delta^{18}O$ ) of water vapor ( $\delta_v$ ) are constant  
 405 during the day in the reactors, as are the  $\delta$  values of the hydroponic solution ( $\delta_{HS}$ ) and RH (relative  
 406 humidity). It is therefore possible to reach an isotopic steady state in the leaf water (with  $\delta_L$ ) 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 •  $\delta_L$  is the isotopic composition of leaf water,
- 413 •  $h$  is the relative humidity of air at the leaf temperature,
- 414 •  $\epsilon_e$  and  $\epsilon_k$  are respectively the isotopic enrichment factor at equilibrium and the kinetic
- 415 enrichment factor between liquid and vapor,
- 416 •  $\delta_V$  is the isotopic composition of the ambient water vapor,
- 417 •  $\delta_{HS}$  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  $\delta_V$  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  $\delta^2H$  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  $\delta^2H$  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  $\delta^2H$  values were closer to those of non-  
433 transpiring organ  $\delta^2H$  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  $\delta^2H$  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 ( $\epsilon_{app}$ ) 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 ( $\epsilon_{bio}$ ) 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):

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

451 and biosynthetic fractionation as:

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

453  $\epsilon_{bio}$  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  $\approx 0.46$ ) and between non-transpiring organs and hydroponic solutions (slope  $\approx 1.0$ )  
 459 differed in a manner consistent with an explanation of transpiration influences (Fig. 4).  $\epsilon_{app}$  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 ( $\epsilon_{\text{app}}$ ) 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*-C<sub>25</sub>, -213 to -82‰ for *n*-C<sub>27</sub>, -217 to -83‰ for *n*-C<sub>31</sub> and -217 to -88‰ for *n*-C<sub>33</sub> (Table 4).  
465 The highly variable ranges of  $\epsilon_{\text{app}}$  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  $\epsilon_{\text{app}}$  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  $\epsilon_{\text{app}}$  values from -  
470 125 to -106‰ for *n*-C<sub>25</sub>, -153 to -82‰ for *n*-C<sub>27</sub>, -131 to -83‰ for *n*-C<sub>31</sub> and -148 to -88‰ for *n*-  
471 C<sub>33</sub>. Our  $\epsilon_{\text{app}}$  values for *n*-alkanes produced by *P. miliaceum* are consistent with ranges published in  
472 the literature for other species. In C3 plants,  $\epsilon_{\text{app}}$  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,  $\epsilon_{\text{app}}$  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  $\epsilon_{\text{app}}$  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  $\epsilon_{\text{bio}}$ , which is narrower than that of  $\epsilon_{\text{app}}$  ( $\epsilon_{\text{bio}} = -$   
481  $151 \pm 5\text{‰}$  for *n*-C<sub>25</sub>,  $-158 \pm 14\text{‰}$  for *n*-C<sub>27</sub>,  $-145 \pm 14\text{‰}$  for *n*-C<sub>31</sub> and  $-144 \pm 19\text{‰}$  for *n*-C<sub>33</sub>; 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  $\epsilon_{\text{app}}$  values, our  $\epsilon_{\text{bio}}$  values of *n*-alkanes (from -163 to -130‰) fall within the  
486 range of values reported by other authors. Feakins and Sessions (2010) reported  $\epsilon_{\text{bio}}$  of  $-147 \pm 18\%$   
487 for *n*-alkanes extracted from plants thriving in an entire ecosystem. For trees,  $\epsilon_{\text{bio}}$  values ranged  
488 from -120 to -170‰ (Sachse et al., 2009; Tipple et al., 2013; Kahmen et al., 2011).  $\epsilon_{\text{bio}}$  values  
489 calculated from *n*-C<sub>29</sub> and *n*-C<sub>31</sub> 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  $\epsilon_{\text{bio}}$  of -180 and -150‰ (the difference being attributed to  
492 temperature) and -157‰, respectively.  $\epsilon_{\text{bio}}$  values calculated from Kahmen et al. (2013b) ranged  
493 between -181 and -158‰ for *n*-C<sub>29</sub> and *n*-C<sub>31</sub> alkanes in maize. Finally, Gamarra et al. (2016)  
494 reported a mean  $\epsilon_{\text{bio}}$  of -164‰ for *n*-alkanes produced by C4 grasses, and more specifically of -  
495 181‰ for *Panicum virgatum*. The range of  $\epsilon_{\text{bio}}$  for *n*-alkanes in these studies is narrower than that  
496 of  $\epsilon_{\text{app}}$  and independent of the plant type. Our study provides the first values for *n*-alkane  $\epsilon_{\text{bio}}$  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  $\epsilon_{\text{bio}}$  of -  
501  $118 \pm 5\%$  (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  $\epsilon_{\text{app}}$   
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  $\epsilon_{\text{app}}$  of -73.8‰ which is lower than the  $\epsilon_{\text{app}}$  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  $\beta$ -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  $\epsilon_{\text{app}}$  of miliacin is about -119‰ on average. The  $\epsilon_{\text{app}}$  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  $\epsilon_{\text{app}}$  for C4 graminoid-derived pentacyclic triterpene methyl ethers (PTMEs) for a 35 % decrease in  
521 RH, in agreement with a 19‰ increase in  $\epsilon_{\text{app}}$  for miliacin with a 16% decrease in relative  
522 humidity.

523  $\epsilon_{\text{bio}}$  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 $\delta^2H_{\text{HS}}$ values

528 As explained earlier, the  $\delta^2H$  values of miliacin and *n*-alkanes correlate with leaf water  $\delta^2H$   
529 values, with slopes close to each other (Fig. 5). Several studies have concluded that the  $\delta^2H$  values  
530 of biochemicals are influenced by the  $\delta^2H$  values of leaf water, which integrates the  $\delta^2H$  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  $\delta^2H$  values of *n*-alkanes and miliacin are more closely related to  
534 leaf water  $\delta^2H$  values than to  $\delta^2H_{\text{HS}}$  values (Table 3 and Fig. 4 and 5). This result suggests that  
535 transpiration processes that affect  $\delta^2H$  values of leaf waters will have a strong impact on the  $\delta^2H$   
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  $\delta^2H$  values of biochemicals (Gleixner and  
538 Mügler, 2007; Sessions et al., 1999; Hou et al., 2007a,b).

539

#### 540 4.2.4. Differences in $\epsilon_{\text{bio}}$ between miliacin and *n*-alkanes

541 The overall average  $\epsilon_{\text{bio}}$  value was  $-118 \pm 5\%$  for miliacin, whereas it reached  $-151 \pm 7$ , -  
542  $158 \pm 14$ ,  $-145 \pm 14$  and  $-144 \pm 19\%$  for the *n*-C<sub>25</sub>, *n*-C<sub>27</sub>, *n*-C<sub>31</sub> and *n*-C<sub>33</sub> alkanes, respectively  
543 (Table 4). This constitutes a significant (Table S4 shows Wilcoxon paired sample tests between the  
544  $\epsilon_{\text{bio}}$  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 <sup>2</sup>H-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  $^2\text{H}$ -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  $^2\text{H}$ -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  $\epsilon_{\text{bio}}$  of -155‰ for *n*-alkanes and of -200‰ for  
580 sterols in *Panicum coloratum*. Therefore, it is unclear whether the  $^2\text{H}$ -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  $\epsilon_{\text{bio}}$  values.

598

## 599 4.3. Impact of relative humidity

600 4.3.1. Impact of relative humidity on leaf water  $\delta^2H$  values

601 The impact of transpiration is visible from the data shown in Figure 6 with  $\delta^2H$  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  $\delta^2H$  values are not only influenced by the  $\delta^2H_{HS}$   
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  $\epsilon_{bio}$  values

612 As described above, the first parameter that controls miliacin and *n*-alkane  $\delta^2H$  values is leaf  
613 water  $\delta^2H$ . For a given relative humidity,  $\epsilon_{bio}$  values of each compound are rather homogeneous,  
614 regardless of the  $\delta^2H_{HS}$  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  $\delta^2H$   
616 values are mainly controlled by the leaf water  $\delta^2H$  value which, in turn, is influenced by the  $\delta^2H_{HS}$   
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  $\epsilon_{bio}$  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  $\epsilon_{\text{bio}}$  values. Conversely, minor, but significant differences between  $n$ -  
622 alkane  $\epsilon_{\text{bio}}$  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.  $\epsilon_{\text{bio}}$  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  $\epsilon_{\text{bio}}$  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  $\epsilon_{\text{bio}}$  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  $\epsilon_{\text{bio}}$  changes with relative humidity.  
638 The small difference in behaviour of the  $\epsilon_{\text{bio}}$  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 <sup>2</sup>H-depleted from water in the cytosol,  
645 but not from cytosolic NADPH that is considered <sup>2</sup>H-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),  $\epsilon_{\text{bio}}$  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  $\epsilon_{\text{bio}}$  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*-C<sub>25</sub> and *n*-C<sub>27</sub> 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

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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-  $\epsilon_{\text{bio}}$  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  $\epsilon_{\text{bio}}$  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.  $\delta$  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 <sup>2</sup>	p	a	b	r <sup>2</sup>	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 <sup>2</sup>	p	a	b	r <sup>2</sup>	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 <sub>25</sub>	0.307	-111	0.953	0.024	0.404	-136	0.99	0.005
<i>n</i> -C <sub>27</sub>	n.s.	n.s.	n.s.	0.18	0.477	-154	0.997	0.001
<i>n</i> -C <sub>31</sub>	0.326	-92	0.907	0.048	0.376	-142	0.987	0.006
<i>n</i> -C <sub>33</sub>	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 <sup>2</sup>	p	a	b	r <sup>2</sup>	p
Miliacin	0.725	-101	0.983	0.009	0.784	-120	0.998	0.001
<i>n</i> -C <sub>25</sub>	0.683	-141	0.964	0.003	0.8	-148	0.971	0.015
<i>n</i> -C <sub>27</sub>	n.s.	n.s.	n.s.	0.175	0.94	-167	0.967	0.017
<i>n</i> -C <sub>31</sub>	0.735	-125	0.97	0.015	0.748	-153	0.846	0.013
<i>n</i> -C <sub>33</sub>	0.83	-127	0.97	0.03	n.s.	n.s.	n.s.	0.081

(d) Figure 6	a	b	r <sup>2</sup>	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 <sub>25</sub> alkane $\delta^2\text{H}$			<i>n</i> -C <sub>27</sub> alkane $\delta^2\text{H}$			<i>n</i> -C <sub>31</sub> alkane $\delta^2\text{H}$			<i>n</i> -C <sub>33</sub> 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 ( $\epsilon_{\text{app}}$ ) and biosynthetic ( $\epsilon_{\text{bio}}$ ) fractionation for miliacin and *n*-alkanes, calculated from equation 2 and equation 3, at 58% and at 74%  
 966 relative humidity.  $\epsilon_{\text{bio}}$  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	$\epsilon_{\text{app}}$ (‰)					$\epsilon_{\text{bio}}$ (‰)															
	Miliacin	<i>n</i> -C <sub>25</sub>	<i>n</i> -C <sub>27</sub>	<i>n</i> -C <sub>31</sub>	<i>n</i> -C <sub>33</sub>	Miliacin			<i>n</i> -C <sub>25</sub>			<i>n</i> -C <sub>27</sub>			<i>n</i> -C <sub>31</sub>			<i>n</i> -C <sub>33</sub>			
						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:

Journal Pre-proofs

Figure 1

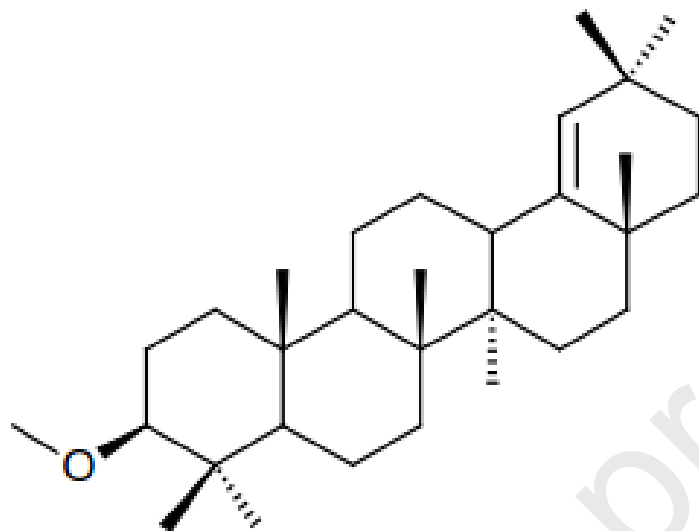


Figure 2

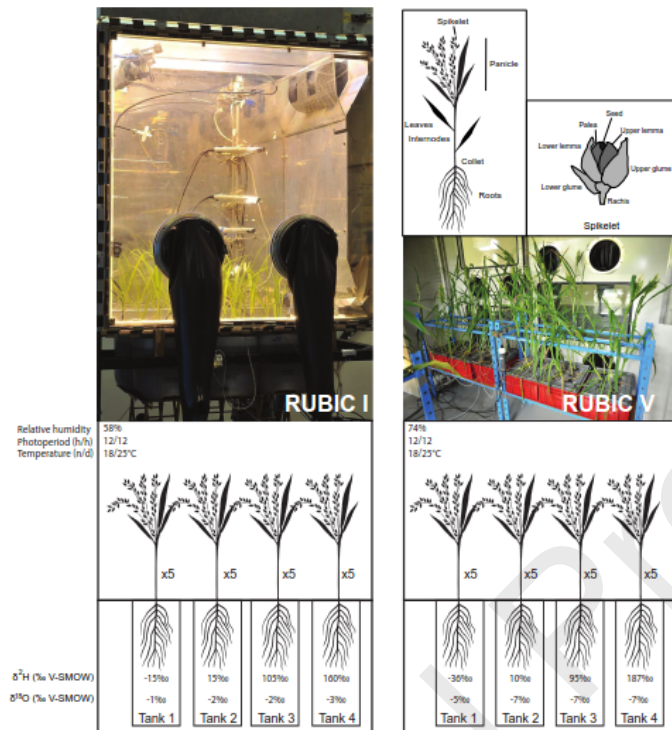


Figure 3

