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## Hydraulic plasticity and water use regulation act to maintain the hydraulic safety margins of Mediterranean trees in rainfall exclusion experiments

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1 Hydraulic plasticity and water use regulation act to maintain the  
2 hydraulic safety margins of Mediterranean trees in rainfall  
3 exclusion experiments

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5

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9

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21 Key words: hydraulic failure, hydraulic adjustment, phenotypic plasticity, stomatal control, cuticular  
22 conductance, turgor, throughfall exclusion, evergreen and deciduous trees.

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## 32 Abstract:

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34 Hydraulic failure due to xylem embolism has been identified as one of the main mechanisms involved  
35 in drought-induced forest decline. The tree vulnerability to hydraulic failure depends on the hydraulic  
36 safety margin (HSM), which can be computed as the difference between the highest water stress  
37 experienced under drought or turgor loss point and the water stress causing hydraulic failure. While  
38 it has been shown that HSM globally converge between tree species and biomes, there is still limited  
39 knowledge regarding how HSM can adjust to locally varying drought conditions within species. In  
40 this study, we relied on three long-term partial rainfall exclusion experiments in Southern France (15,  
41 9 and 6 years of rainfall exclusion treatment respectively for Puéchabon, Font-blanche and O3HP  
42 sites) to investigate the plasticity of hydraulic traits and HSM under intensified drought conditions  
43 for three Mediterranean tree species (*Quercus ilex* L., *Quercus pubescens* Willd., and *Pinus halepensis*  
44 *Mill.*). Our findings show, for all species, a similar HSM in trees submitted to intensified drought and  
45 trees in the control treatments, despite reduced precipitation and, hence soil water availability. This  
46 homeostasis of HSM in response to rainfall reduction is achieved through different mechanisms,  
47 which appear to be related to the water use strategies of the three species. For *Q. ilex*, our results  
48 indicate that the convergence in HSM is attributed to the adjustment of two plant hydraulic traits,  
49 namely the turgor loss point ( $\Psi_{\text{tlp}}$ ) and the water potential at which 50% of xylem conductivity is lost  
50 due to embolism (P50). In contrast, both *P. halepensis* and *Q. pubescens* exhibited similar minimal  
51 water potentials under control and drought treatments, due to isohydric behavior for the first and  
52 probably other traits adjustment for the latter. Thus, the plasticity of the hydraulic vulnerability was  
53 not required to maintain their HSM in these two species. Our results suggest that trees exposed to  
54 moderately drier conditions over several years may be able to maintain plastically their HSM.  
55 However, it remains to be seen whether this acclimatization of HSM to drier conditions will be  
56 sufficient to withstand the more extreme droughts expected in the Mediterranean region.

57

## 58 Introduction:

59

60 Over the last decades, many studies have been conducted to better understand the mechanisms  
61 responsible for tree dieback following severe water stress (Barigah et al., 2013; Choat et al., 2018;  
62 Sperry et al., 2002; Tyree & Sperry, 1988). Among the different processes involved in plant mortality  
63 under drought (Allen et al., 2015; Anderegg et al., 2015), hydraulic failure has been identified as one  
64 of the key triggers of tree death (Adams et al., 2017; Anderegg et al., 2016; Arend et al., 2021;

65 McDowell et al., 2008). During drought, soil and atmospheric drying result in an increase in xylem  
66 water tension (i.e., a decrease in plant water potential). Excessive water tension promotes the  
67 occurrence of xylem cavitation, which cause the rupture of water columns between roots and leaves  
68 by embolism. Hydraulic failure occurs when the water transport in the plant is severely impaired by  
69 embolism (Tyree & Sperry, 1989), causing different damages to living tissues that may prevent the  
70 recovery of the tree when drought ends, and eventually kill the tree (Mantova et al., 2022).

71 The timing and likelihood of hydraulic failure during drought is influenced by various  
72 physiological characteristics that control plant desiccation resistance (Choat et al., 2018; Duursma et  
73 al., 2019; Ruffault et al., 2022). The xylem vulnerability to cavitation is a crucial trait that define the  
74 rate of spread of embolism with water potential decrease. It is derived from vulnerability curves  
75 relating increased xylem embolism (e.g. the loss of hydraulic conductance) with decreasing xylem  
76 water potentials (Cochard, 2002; Cochard et al., 2008, 2013). The main parameter of this vulnerability  
77 curve is the water potential causing 50 % of hydraulic conductance loss (P50), which is negatively  
78 correlated with survival time during drought and the dryness of species habitat (Lens et al., 2016;  
79 Martin-StPaul et al., 2017). The rate of water potential decline during drought (i.e. plant desiccation  
80 dynamics resistance) depends, among other factors, on water loss regulation through stomatal control  
81 (Martin-StPaul et al., 2017). Thus, the earlier the stomata close after the drought onset, the more the  
82 plants are able to limit the decrease in their water potential, and preserve xylem conduits from  
83 cavitation. The water potential at turgor loss point ( $\Psi_{\text{tlp}}$ ) can be used as a surrogate of the water  
84 potential at which stomata close (Brodribb & Holbrook, 2003; Martin-StPaul et al., 2017), given that  
85 stomata close once guard cells lose turgidity. In addition, plant residual transpiration, occurring  
86 through incompletely closed stomata or the cuticle (Machado et al., 2021), also determine the rate of  
87 the plant-drying process and the hydraulic risk (Duursma et al., 2019).

88 The hydraulic safety margin (HSM) is an integrative trait used to evaluate the risk of hydraulic  
89 failure under drought, with a higher HSM indicating a lower risk. Two versions of HSM have been  
90 proposed. The first one corresponds to the difference between the minimum xylem water potential  
91 (e.g.  $\Psi_{\text{mid min}}$ ) reached by a plant and the threshold water potential causing severe xylem embolism  
92 (e.g. P50) (Choat et al., 2012). According to this definition, HSM represents the hydraulic risk  
93 associated to the water stress actually experienced by the plant. In the second definition,  $\Psi_{\text{mid min}}$  is  
94 replaced by the water potential causing stomatal closure (Martin-StPaul et al., 2017). This second  
95 version of HSM integrates the degree to which stomatal control can prevent hydraulic risk. Since the  
96 minimum potential achieved by a plant depends on stomatal control, the two definitions of HSM are  
97 closely linked. The HSM has been shown to be conserved across forest tree species worldwide (Choat

98 et al., 2012), as a result of the coordination of multiple traits optimizing the xylem safety-efficiency  
99 trade-off with species habitat (Franklin et al., 2022; Guillemot et al., 2022; Martin-StPaul et al., 2017;  
100 Pivovarov et al., 2018). However, there has been little investigation into the intraspecific variations  
101 of the HSM in response to drier conditions. To our knowledge, the only publication focusing on this  
102 topic report narrower HSM for Mediterranean species occurring at the dry edge of their geographical  
103 distribution (Alon et al. 2023).

104         Understanding the impact of plant trait plasticity on plant desiccation dynamics is therefore  
105 crucial for predicting hydraulic risk in the current context of rapid climate change (Jinagool et al.,  
106 2018). Yet, limited research has thus far investigated the adjustment of multiple traits after long  
107 periods of increased drought. Some studies suggested that leaf traits, such as  $\Psi_{tp}$  change more in  
108 response to sustained drought than the xylem vulnerability to cavitation (Bartlett et al., 2012; Torres-  
109 Ruiz et al., 2019), which is sometimes invariant (Lobo et al., 2018; Rosas et al., 2019) and sometimes  
110 plastic (Bert et al., 2021; Herbette et al., 2021; Lemaire et al., 2021). For the Mediterranean species  
111 *Quercus ilex*, past studies have reported limited plasticity of xylem vulnerability to cavitation  
112 (Limousin et al., 2022; Limousin et al., 2010; Martin-StPaul et al., 2013). Additionally, it is important  
113 to note that, until recently, the methods used to measure xylem vulnerability to cavitation in long-  
114 vessel branches, such as those of *Q. ilex*, were affected by a bias due to open vessel methodological  
115 artefact (Martin-StPaul et al., 2014; Torres-Ruiz et al., 2014). Similarly, the methods used to estimate  
116 leaf hydraulic vulnerability curves based on leaf hydraulic conductance were also biased by the  
117 integration of an extra-xylary water pathway (Li et al., 2020; Limousin et al., 2022; Trifiló et al.,  
118 2016). Hence, it is important to revisit the impact of plant trait plasticity on plant desiccation dynamics  
119 with up-to-date and unbiased methods, such as Cavitron (Cochard et al. 2002) or optical vulnerability  
120 (Brodribb et al. 2016) techniques.

121 In this study, we aimed to assess whether, and by which mechanisms, the HSM of a given species can  
122 change with increasing drought conditions. We focused on three of the most widespread tree species  
123 in the Mediterranean basin, namely *Pinus halepensis* Mill., *Quercus ilex* L. and *Quercus pubescens*  
124 Willd. Those species are being monitored in three long term partial rainfall exclusion experiments set  
125 up in natural forests in southern France: the Puéchabon experimental site (a *Q. ilex* forest), the Oak  
126 Observatory at the Haute-Provence Observatory (a *Q. pubescens* forest), and the Font-blanche site (a  
127 mixed forest of *P. halepensis* and *Q. ilex*). In those three sites, we measured and compared hydraulic  
128 traits in adults of the three species under control and aggravated drought conditions.

129

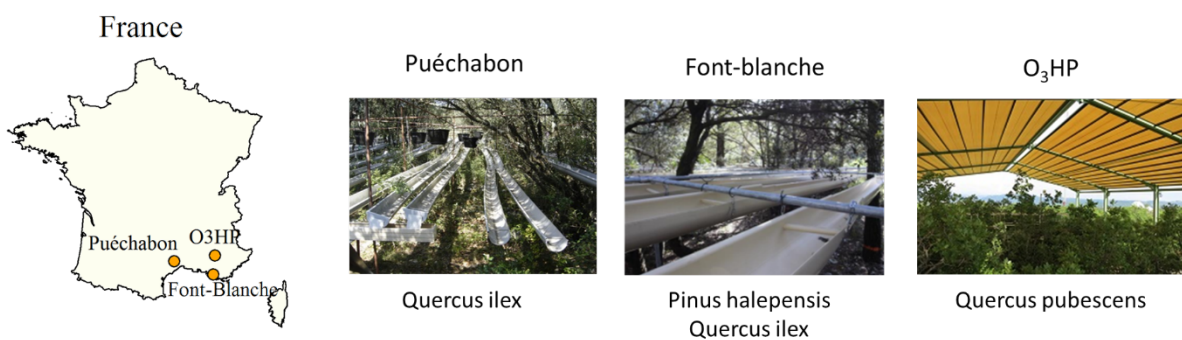
130 **Materials and Methods:**

131

132 **Sites description**

133

134 The study was conducted on three experimental forest sites located in the French Mediterranean  
 135 region (Figure 1A) that stand out by their vegetation types: the Font-blanche (FB hereafter) forest is  
 136 composed of *P. halepensis* in the overstory and *Q. ilex* in the understory, the oak observatory at the  
 137 Haute-Provence Observatory (O<sub>3</sub>HP hereafter) is dominated by *Q. pubescens*, and the Puéchabon site  
 138 (P hereafter) by *Q. ilex*. The climate of the sites is Mediterranean, with wet, mild winters and dry, hot  
 139 summers (Fig. S1). The subsoil of all sites is composed by a hard limestone bedrock. The soils of the  
 140 FB and P sites are similar and classified as silty clay loam, while the O<sub>3</sub>HP site is a clay-loam soil.  
 141 They have a high proportion of rocks and soil depth varies between 35 to 50 in FB and P and 30-40  
 142 cm in O<sub>3</sub>HP. On all sites, a rainfall exclusion experiment has been implemented for for several years  
 143 (15, 9 and 6 years respectively for Puéchabon, Font-blanche and O<sub>3</sub>HP sites at the time of this study  
 144 (more details provided in Table 1) excluding approximately 30 % of the precipitation reaching the  
 145 ground. At FB and P, the system excluding precipitation is passive, with PVC gutters hung under the  
 146 tree canopies. At O<sub>3</sub>HP, rainfall exclusion is achieved with a mobile rainout-shelter deployed  
 147 manually only during some rainfall events from spring to autumn (Fig. 1). Table 1 provide more  
 148 details on sites characteristics and rainfall exclusion treatments. On each site, meteorological  
 149 variables are monitored (including half-hourly precipitation, radiation, air temperature and humidity).



150

151 Figure 1. Locations of studied sites and pictures of the rainfall exclusion systems. PVC gutters placed under  
 152 the canopy were used to collect around 30 % of precipitation at Puéchabon and Font-blanche sites, while in  
 153 the O<sub>3</sub>HP site, a mobile shelter placed above the canopy is deployed manually.

154 Table 1: Characteristics of studied sites.

	<b>Font-Blanche</b>	<b>Puéchabon</b>	<b>O<sub>3</sub>HP</b>
<b>Location</b>	43°14'27"N, 5°40'45"E	43°44'29"N, 3°35'45"E	43°56'115" N, 05°42'642" E

<b>Precipitation (mm) / temperature (°C) (annual mean value between 2016-2019)</b>	615 /14.2	1033 /14.0	871 /12.9
<b>Soil characteristics: type, depth (cm), available soil water (mm)</b>	Silty clay loam, 20 to 50, 160	Silty clay loam, 30 to 70 ,140	Clay-loam, 30 to 40, NA
<b>Vegetation type</b>	Evergreen <i>P. halepensis</i> and <i>Q. ilex</i> forest	Evergreen <i>Q. ilex</i> forest	Deciduous <i>Q. pubescens</i> forest
<b>Basal area (m<sup>2</sup>/ha) (mean 2018)</b>	29.7	27.2	21
<b>Tree height (mean 2018)</b>	13.5 for <i>P. halepensis</i> 6.5 for <i>Q. ilex</i>	5.5	5
<b>Rainfall exclusion device, treatments area (m<sup>2</sup>), year of the start of the exclusion</b>	Gutters, 625, 2009	Gutters, 140*4 replicates, 2003	mobile rainout-shelter, 300, 2012
<b>Precipitation exclusion ratio</b>	≈ 30 %	≈ 30 %	≈ 30 %
<b>References</b>	(Moreno et al., 2021)	(Limousin et al., 2009)	(Genard-Zielinski et al., 2018)

155 Water potentials in the field  
156

157 Water potentials data were taken from Moreno et al. (2021) for *P. halepensis* and *Q. ilex* at FB, from  
158 Limousin et al. (2022) for *Q. ilex* at P, and from Genard-Zielinski et al. (2018) for *Q. pubescens* at  
159 O<sub>3</sub>HP. Predawn ( $\Psi_{pd}$ ) and midday ( $\Psi_{midd}$ ) water potentials were measured from 2015 to 2019 in each  
160 site (Table 2) during summer using Scholander pressure chambers (different brands depending on  
161 year and site). The samplings were performed at several dates from the onset to the end of summer  
162 (between two to six measurements per year depending on year and site). At each site and date,  
163 measurements were made on twigs (FB and P sites) or leaves (O<sub>3</sub>HP site) of at least three trees per  
164 species and per treatment (control/ rainfall exclusion). One to three samples per tree were measured  
165 depending on site and date during the two hours preceding sunrise for  $\Psi_{pd}$ , and between 2 to 4 pm for  
166  $\Psi_{midd}$ .  $\Psi_{midd}$  were measured on transpiring twigs/leaves. For  $\Psi_{pd}$ , we have selected the data from the

167 date when the lowest values were recorded per species for each year to compute the extreme annual  
168 predawn water potentials ( $\Psi_{pd\ min}$ ). For  $\Psi_{midd}$ , we have selected the data from the date when minimum  
169 values were observed, combining all years, to compute the extreme midday water potentials ( $\Psi_{midd}$   
170  $min$ ).

171

## 172 Vulnerability to cavitation

173

174 Depending on the length of xylem conduits of each species, two techniques were used to  
175 measure the xylem vulnerability to cavitation. For *P. halepensis*, a gymnosperm species with only  
176 tracheids, we used the cavitron technique (Cochard, 2002). In March 2018 and October 2019, south  
177 facing branches, approximately 50 cm long, were sampled from the top of the canopy of a total of  
178 17 control trees and 10 trees submitted to rainfall exclusion of the FB site (Table S1: no significant  
179 difference of P50 between sampling date, Fig. S2). Once cut, branches were placed under water and  
180 the basal 10 cm recut to eliminate cavitated tracheids due to conduits left open during the first cut.  
181 Then, they were placed in humidified black bags in order to limit transpiration and prevent  
182 dehydration. At the laboratory, branches were placed under water, the foliage was removed, and a  
183 straight 30-cm-long section of the branch was recut. The sections were rolled into humidified  
184 absorbent paper, wrapped in plastic film, and placed in an icehouse for shipping them to the to the  
185 INRAE-PIAF laboratory at Clermont-Ferrand (central France). There, the bark was removed and the  
186 vulnerability to cavitation of the samples measured with the cavitron (Cochard, 2002). Tension in the  
187 xylem was gradually increased, with the rotation speed and the percent loss of conductivity (PLC)  
188 being calculated using Cavisoft (v.4.0; University of Bordeaux) as:

$$189 \quad PLC (\%) = 100 * (1 - K / K_{max}) \quad (1)$$

190 where K is the conductivity recorded at a given rotation speed and  $K_{max}$  the maximal  
191 conductivity recorded at the lowest rotation speed.

192 Xylem vulnerability curves were then constructed for each sample by plotting PLC as a  
193 function of the xylem tension caused by rotation. The following sigmoid function was fitted to data:

$$194 \quad PLC = 100 / (1 + \exp(s(P - P50))) \quad (2)$$

195 where s is the slope at the inflexion point and P50 is the water potential causing 50 % loss of  
196 hydraulic conductivity.

197 Because xylem vessel length in the two oaks species can reach up to 1m (Martínez-Vilalta et  
198 al., 2002), we were unable to employ the cavitron technique with oaks, due to the bias for long-vessel



199 species when branch samples are shorter than the vessel length (Beikircher et al., 2010; Cochard et  
200 al., 2013; Martin-StPaul et al., 2014; Sergent et al., 2020). Instead, we used the optical technique  
201 (Brodribb et al., 2016) to measure the xylem vulnerability to cavitation in the leaves. We opted for  
202 the leaf optical technique rather than the stem optical technique, as it does not require to remove tissue  
203 limiting potential bias caused by endomaged xylem if not well executed. All the branches used to  
204 measure VC were not flushed to remove native embolism, as such procedure can bias vulnerability  
205 curves (<https://doi.org/10.1093/treephys/tpad088>) due to open-vessels artifact. Drawing from the  
206 findings of Li et al., (2020), and our own observation of similar P50 using the optical method and  
207 respectively X-ray microtomography for *Q. ilex* stems (Sergent et al, 2020) and cavitron for *Q.*  
208 *pubescens* branches (personal data given by Delzon) (Fig. S3), we assumed that hydraulic  
209 segmentation did not occur in the two oak species. Anyway, despite the use of different hydraulic  
210 techniques across species, it is important to note that the aim of this study is to evaluate intraspecific  
211 plasticity in hydraulic traits, rather than interspecific drought tolerance.

212         During summer 2019, 4 to 5 branches more than 1 m long of *Q. ilex* and *Q. pubescens* sampled  
213 from different trees, were harvested in each control and rainfall exclusion treatments of the three  
214 studied sites. Branches were placed in humidified bags and stored in a dark cold room with the cut  
215 end in water for at least one night to rehydrate. Once fully rehydrated, branches were allowed to  
216 dehydrate progressively on the lab bench (bench dehydration technique). One or two mature leaves  
217 per branch, still attached to the branch, were placed on a flatbed light transmission scanner (Epson  
218 V850). The leaves were kept flat using microscope slides attached to the scanner glass with  
219 transparent tape. Leaf scans of a resolution of 2400 dpi were recorded in light transmission mode  
220 every 5 min while the branch was progressively dehydrating. Concomitantly, xylem water potential  
221 was measured on bagged leaves of the same branch using a Scholander pressure chamber (PMS  
222 instrument), approximatively every 3 hours throughout branch desiccation (along 3 to 5 days, a  
223 minimum period of 3 days being enough to reach 100% emboli in the xylem, Fig. S4).

224         Once the branch fully desiccated (and the xylem water potential was no longer measurable  
225 with Scholander pressure chamber), all images were analyzed to detect cavitation events as changes  
226 in light transmittance between successive scans (<http://www.opensourceov.org/>). Image J (FIJI,  
227 (Schindelin et al., 2012)) was used to reveal embolism events by overlapping successive leaf scans  
228 and calculating the pixel area of embolized vessels. Vulnerability curves were constructed for each  
229 leaf by plotting the relative cumulative embolized area (corresponding to the cumulative embolized  
230 area divided by the total embolized area at the end of leaf dehydration) as a function of leaf water  
231 potential, and fitting the same sigmoid function as for *P. halepensis*.

## 233 Foliar traits

234

235 Pressure–volume curves (hereafter PV curves) were used to characterize different foliar traits  
 236 related to the maintenance of leaf turgor and hydration (Bartlett et al., 2012; Tyree & Hammel, 1972).  
 237 For each species, two well-lit twigs per tree were collected from the outer crown of 4-6 trees in the  
 238 control and exclusion treatments. The sampling took place in March 2018 for *P. halepensis* and *Q.*  
 239 *ilex* at FB and P sites and in September 2019 for *Q. pubescens* at O<sub>3</sub>HP site. In all cases, once  
 240 collected, twigs were bagged and placed in a cooler at 4°C until reaching the laboratory. Once in the  
 241 lab, each twig end was re-cut under water with a razor blade, then put overnight in a cool chamber in  
 242 distilled water to rehydrate. Half of the samples were used to estimate the leaf mass area and the other  
 243 half to perform the PV curves

244 PV curves were established using the bench drying method proposed by Hinckley et al.,  
 245 (1980). Briefly, the weight and the water potential of the twigs were measured all along their  
 246 dehydration using a precision balance (FS-220, resolution 0.1 mg) and a Scholander pressure chamber  
 247 (PMS instrument), respectively. To overcome oversaturation of rehydrated twigs, the first  
 248 measurement of water potential and weight was removed, and full turgor weight was extrapolated  
 249 from the regression between twig weight and water potential before the turgor loss point. The osmotic  
 250 potential at full turgor ( $\pi_{100}$ ) and at the turgor loss point ( $\Psi_{t_{lp}}$ ), the relative water content at turgor loss  
 251 point ( $RWC_{t_{lp}}$ ), the symplastic water fraction at full turgor ( $F_s$ ), and the bulk modulus of tissue  
 252 elasticity ( $\epsilon$ ) were obtained by plotting the inverse of water potential ( $-1/\Psi$ , in MPa) against twig  
 253 water saturation deficit (1-Relative Water Content, in %). PV curves data used for *Q. ilex* are the same  
 254 as those reported in Limousin et al. (2022).

255 Minimum leaf conductance to water vapor ( $g_{min}$ ) was obtained considering the average twig  
 256 water loss (corresponding to sample transpiration) after stomatal closure. To this end, relative  
 257 humidity and temperature were recorded every second with a probe (PT100) to compute VPD, and  
 258 minimum leaf conductance was obtained as follows for each species:

$$259 \quad g_{min} = \frac{E}{VPD \times DA} \times Patm, (3)$$

260 with E being the transpiration after turgor loss, Patm the atmospheric pressure (set at 101 kPa), and  
 261 DA the developed leaf area. The developed leaf area (DA) was obtained from the projected leaf area  
 262 by using the ratio between leaf dry mass and leaf mass per area. For both oaks, with flat leaf surface,  
 263 DA was calculated as twice the projected leaf area. For *P. halepensis*, we calculated DA considering

264 that needles formed a perfect half-cylinder, which was confirmed by measurements of width and  
265 thickness at the top, middle and bottom of 15 needles (data not shown). We choose to express  $g_{min}$   
266 in function of developed leaf area to report more realistic value. Indeed,  $g_{min}$  does not result only  
267 from water loss through not fully close stomata but also by cuticular leaks, that can happen from all  
268 the leaf surface.

269

## 270 Hydraulic safety margins

271

272 Two types of Hydraulic safety margins (HSM) were computed at each site and for both treatments by  
273 subtracting either (i) the minimal midday water potentials ( $\Psi_{mid\ min}$ ) to P50, following the approach  
274 outlined by Choat et al. (2012), or (ii) the turgor loss point, as a surrogate as water potential causing  
275 stomatal closure to P50 as in Martin-StPaul et al. (2017).

276 To avoid damaging trees, the samples collected to construct hydraulic vulnerability curves were taken  
277 from different trees than those sampled for leaf water potential and turgor loss point measurements.  
278 Therefore, it was not possible to calculate the HSM at the tree level. Instead, we determined both  
279 HSM versions at the species and treatment levels using a nonparametric bootstrap approach. For each  
280 species and treatment (control and rainfall exclusion), we randomly sampled, with replacements, 5  
281 values of P50 and  $\Psi_{mid\ min}/\Psi_{tlp}$  and determined 5 HSM as the difference between these values. This  
282 process was repeated 1,500 times to obtain a distribution of HSM values for each site and treatment  
283 that was subsequently used for statistical comparisons.

284

## 285 Native stem xylem embolism measured by X-ray microtomography

286

287 Native stem xylem embolism was estimated for *Quercus ilex* and *Pinus halepensis* trees in  
288 both rainfall exclusion and control treatments at FB and P. The sampling took place in March 2018.  
289 Four to eight well-lit branches of more than 1m were collected depending on the sites, the treatments  
290 and the species considered. Once collected, branches were immediately recut under water to avoid  
291 cutting artefacts.

292 The cut surface of the branches was kept under water until reaching the laboratory. Then, short  
293 segments of branches (diameter < 0.7 cm and length < 4 cm) were cut under water, plunged in liquid  
294 paraffin, and stored in a cold chamber at 4°C until analysis. Measurements were performed following  
295 the protocol described in Cochard et al. (2015). Samples were inserted in an X-ray microtomograph  
296 (Nanotom 180 XS; GE, Wunstorf, Germany) and analyzed using a field of view of  $5 \times 5 \times 5 \text{ mm}^3$ , X-

297 ray voltage of 60 kV, current of 240  $\mu$ A, and a scan time of 21 min. The final spatial resolution of the  
 298 3D images was 2.5  $\mu$ m after 3D-reconstruction. For each sample, one transversal 2D slice was  
 299 extracted from the middle of the branch using VGStudio Max© software (Volume Graphics,  
 300 Heidelberg, Germany). The surface area of embolized conduits was estimated from slices using the  
 301 software ImageJ (Schneider et al., 2012).

302 As *Q. ilex* is a semi ring-porous species with log normal distribution of xylem conduit sizes,  
 303 we measured the surface area of large embolized vessels (contributing the most to hydraulic  
 304 conductivity) to estimate their mean diameter and their corresponding hydraulic conductivity. The  
 305 maximal hydraulic conductivity was estimated thanks to a second scan we performed on the same  
 306 branch sample once it was fully embolized. The level of embolism was calculated as the ratio between  
 307 estimated native hydraulic conductivity and maximum hydraulic conductivity.

308 Table 2. Summary of measurement periods according to sites and species. FB and P are the acronyms  
 309 for respectively Font-Blanche and Puéchabon sites.

310

Measurements	<i>P. halepensis</i>	<i>Q. pubescens</i>	<i>Q. ilex FB</i>	<i>Q. ilex P</i>
Trees water potentials $\Psi_{pd\ min}$ (MPa)	From 2013 to 2018	2014, 2015, 2018	From 2013 to 2018	From 2015 to 2018 2017
$\Psi_{midd\ min}$ (MPa)	2018	2015	2017	2017
Pressure volume curves: $\Psi_{tlp}$ (MPa), $\pi 100$ (MPa)	March 2018	September 2019	March 2018	March 2018
$g_{min}$ ( $mmol\ s^{-1}\ m^{-2}$ )	March 2018	September 2019	March 2018	March 2018
Xylem vulnerability to cavitation: P50 (MPa)	March 2018, October 2018	September 2019	Onset of summer 2019	Onset of summer 2019
Native stem xylem embolism (%)	March 2018	/	March 2018	March 2018

311

## 312 Statistics

313

314 To assess whether plant and leaf traits were different between treatments we used t-test for all traits  
 315 measured. For the HSM, we made a Student t-test for each of the 1500 subsamples obtained via  
 316 bootstrapping. We considered that rainfall exclusion and control treatments were significantly  
 317 different if the probability of having a  $P\_value > 0.05$  among the 1500 t-tests realized was lower than  
 318 0.05. Statistical analyses were performed with the R software (3,5,2, R Development Core Team  
 319 2018). Differences in parameters derived from xylem vulnerability curve (slope and P50; Table S2-  
 320 3, Fig S5),  $\pi 100$ ,  $\Psi_{tlp}$  or  $g_{min}$  between rainfall exclusion and control treatments were found to be  
 321 similar for *Q. ilex* in both Font-blanche and Puéchabon sites (Limousin et al., 2022). Hence, to

322 increase the statistical power in testing for a rainfall exclusion effect on traits values, as in Limousin  
323 et al. (2022) we decided to combine the data from both sites for this particular species. All Statistical  
324 analyses were performed with R software (3,5,2, R Development Core Team 2018)  
325

## 326 Results

327

### 328 Rainfall exclusion impacts on tree water status

329

330 Variations in the response of  $\Psi_{pd\ min}$  to rainfall exclusion treatments were observed among different  
331 tree species and sites (Fig. 2). For *Q. pubescens* at O<sub>3</sub>HP,  $\Psi_{pd\ min}$  tended to be more negative for trees  
332 growing in the rainfall exclusion treatments (P\_value = 0.005) until 2015 (two years after the onset  
333 of the rainfall exclusion). After that,  $\Psi_{pd\ min}$  was similar to that of control trees (P\_value > 0.1). For  
334 *P. halepensis* at FB,  $\Psi_{pd\ min}$  were similar across years, except in 2015 (P\_value = 0.05), when they  
335 were slightly more negative in the rainfall exclusion treatments (Fig. 2). It should be noted, however,  
336 that if we consider the whole water potential dynamic rather than the extreme values, significantly  
337 more negative potentials in rainfall exclusions appear between treatments at lower levels of water  
338 stress (Genard-Zielinski et al., 2018; Moreno et al., 2021). Moreover, soil water content taken at  
339 shallow ground level (< 50 cm), tended to be more negative in the rainfall exclusion treatment than  
340 in the control (Genard-Zielinski et al., 2018; Moreno et al., 2021). All of these findings attest to the  
341 effectiveness of the exclusion treatments for these two species.

342 Concerning *Q. ilex* at both FB and P sites,  $\Psi_{pd\ min}$  tended to be more negative in the rainfall exclusion  
343 treatments (Fig. 2, P\_value < 0.05 in FB site until 2015; P\_value < 0.006 in P site until 2016, then  
344 P\_value < 0.05).

345

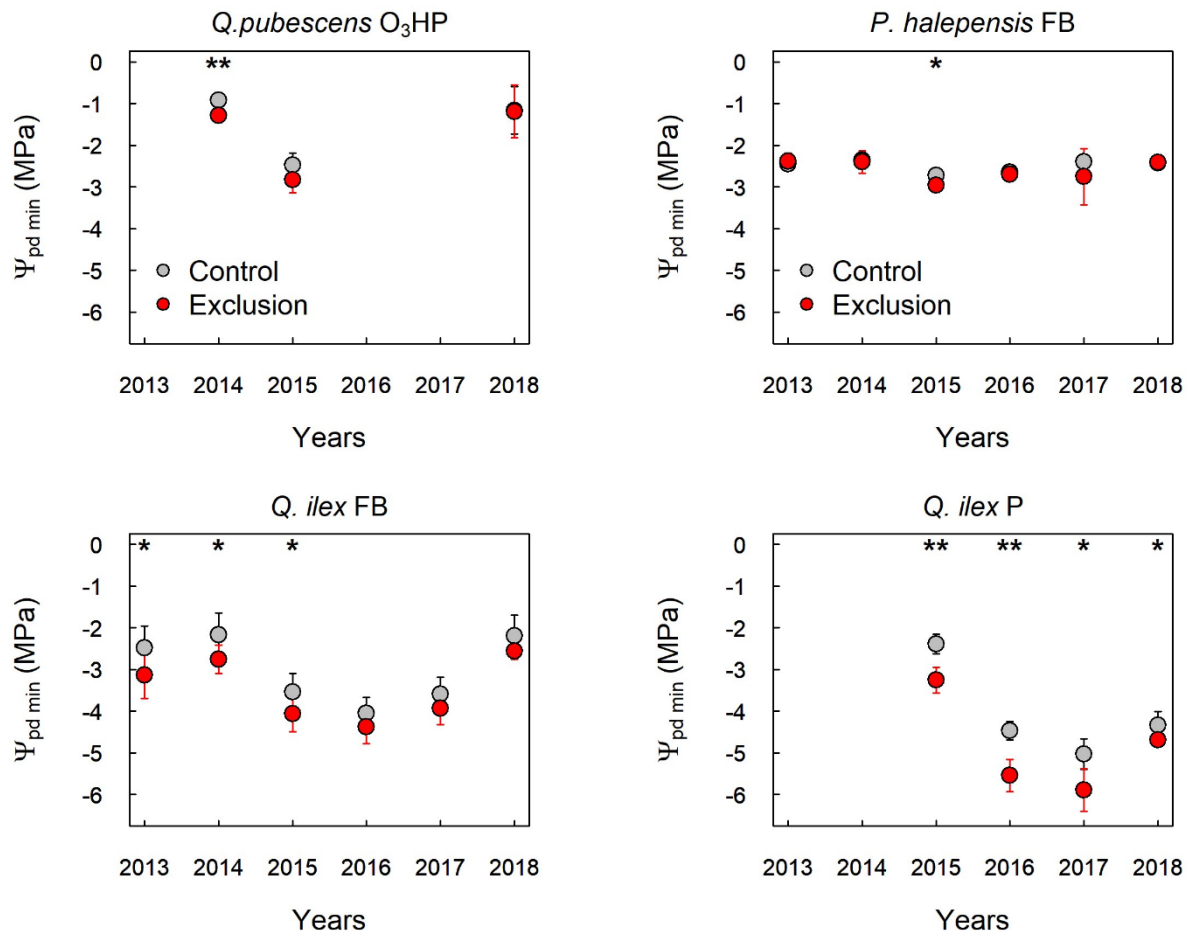


Figure 2. Average minimum predawn water potentials ( $\Psi_{pd\ min}$ ) measured for each species in both control and rainfall exclusion treatments according to year and site. Bars represent standard deviations associated to the mean. Significant differences between treatments (t-test) are indicated by asterisks (\*,  $0.01 \leq P\_value < 0.05$ ; \*\*,  $0.001 \leq P\_value < 0.01$ ; \*\*\*,  $P\_value < 0.001$ ).

346

### 347 Effects of rainfall exclusion on hydraulic and leaf traits

348

349 No significant differences in  $P_{50}$ ,  $\pi_{100}$ ,  $\Psi_{tlp}$  nor  $g_{min}$ , were found between the rainfall exclusion and  
 350 control treatments for *Q. pubescens* and *P. halepensis* (Fig. 3,  $P\_value > 0.2$  for each of these traits).

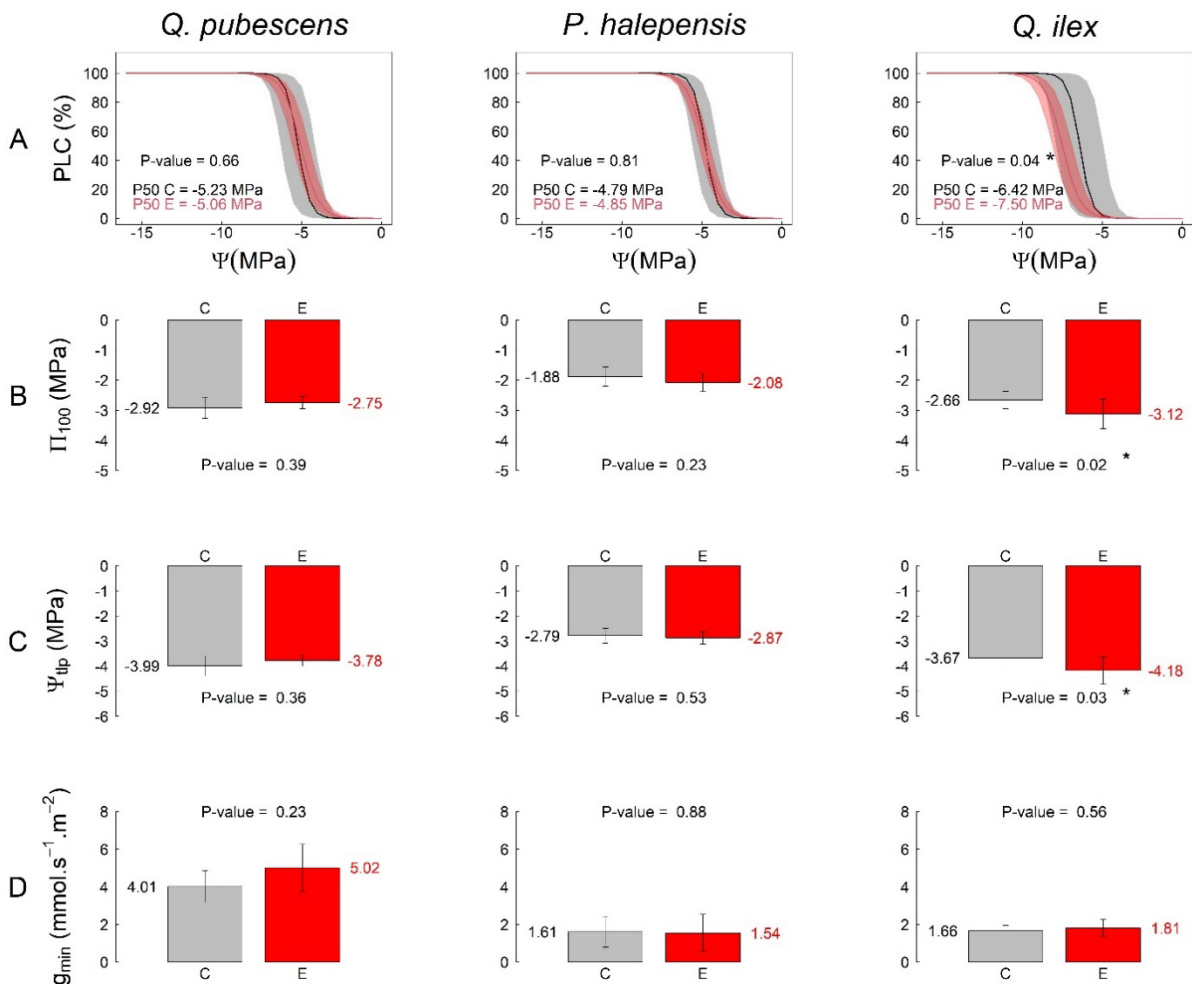
351 For *Q. ilex*,  $P_{50}$ ,  $\pi_{100}$  and  $\Psi_{tlp}$  were significantly more negative for trees growing in the rainfall  
 352 exclusion ( $P\_value = 0.04$  for  $P_{50}$ ;  $P\_value = 0.02$  for  $\pi_{100}$ ;  $P\_value = 0.03$  for  $\Psi_{tlp}$ ), whereas  $g_{min}$   
 353 remained unchanged ( $P\_value > 0.05$ ).

354

355

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357



358

Figure 3. Effects of rainfall exclusion on hydraulic traits for the three studied species. A. Xylem vulnerability curves to cavitation, represented by the percent loss of conductivity (PLC) as a function of water potential ( $\Psi$ ). Averages and standard deviations are respectively in bold lines and grey (control) or red (rainfall exclusion) areas. B. Osmotic water potential at full turgor ( $\pi_{100}$ ), C. water potential at turgor loss point ( $\Psi_{tip}$ ), D. minimum leaf conductance ( $g_{min}$ ) in the control (C, in grey) and rainfall exclusion (E, in red) treatments, for the three studied species. In the barplots, bars represent mean values; error bars represent standard deviation. P-values of treatment effect are presented; asterisks indicate a significant difference at P-value < 0.05.

359

360 Effect of rainfall exclusion on species hydraulic safety margins and xylem embolism.

361 For all species, we did not observe any statistical difference in HSM between rainfall exclusion and  
 362 control trees, based on either  $\Psi_{mid\ min}$  (Fig. 4A, P-value > 0.8 for each species) or  $\Psi_{tip}$  (Fig. 4B,  
 363 P-value > 0.9 for each species). Levels of native stem xylem embolism were also similar in both  
 364 treatments for *P. halepensis* or *Q. illex*, the only species analyzed (Fig. 5).

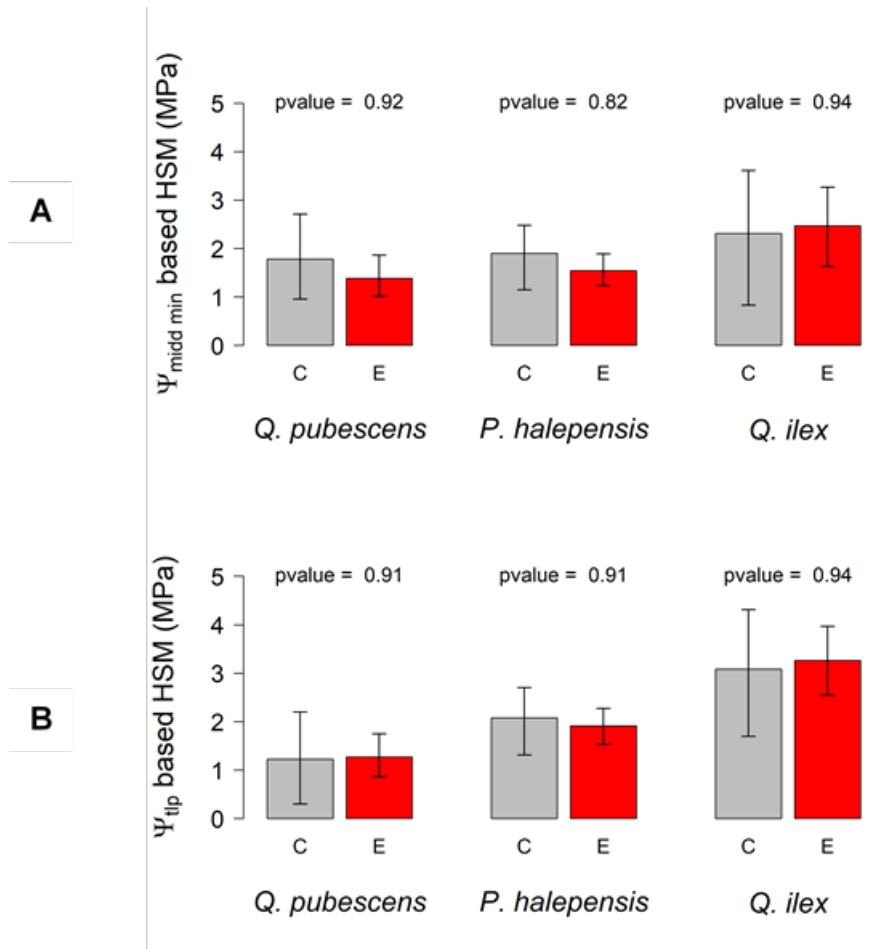
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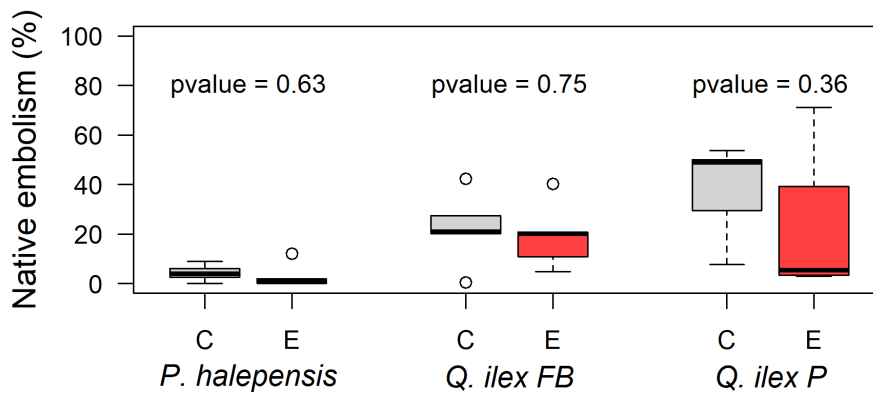


370

371 Figure 4. A. Hydraulic safety margins (HSM) in relation to species and treatment (C: control; E: rainfall  
372 exclusion). A. HSM defined as the difference between minimum midday water potentials ( $\Psi_{\text{midd min}}$ ) and water  
373 potential causing 50% loss of hydraulic conductivity (P50).  $\Psi_{\text{midd min}}$  correspond to the minimal values  
374 measured in 2015 for *Q. pubescens*, 2016 for *Q. ilex* in P and FB sites and 2018 for *P. halepensis*. B. HSM  
375 defined as the difference between turgor loss point ( $\Psi_{\text{tip}}$ ) and P50. Bars and errors bars represent respectively  
376 the median and the first/third quartiles (confidence interval) of the nonparametric bootstrap analysis.

377





378

379 Figure 5. Native stem xylem embolism (%) estimated from X-ray microtomography on 4 years-old branches  
 380 of *P. halepensis* and *Q. ilex* trees from the control (C) and rainfall exclusion (E) treatments at both Font-blanche  
 381 (FB) and Puéchabon (P) sites. The measurements were performed in March 2018. P\_value of Wilcoxon rank  
 382 sum tests comparing native stem xylem embolism between treatments are represented for both sites.

383

## 384 Discussion

385

386 In the current context of rapid global change, tree capacity to acclimate to increasing drought appears  
 387 crucial for the maintenance of forest structure, composition and function. Under drier conditions,  
 388 hydraulic failure risk should increase for trees if they cannot adjust key hydraulic traits involved in  
 389 the hydraulic safety. In the present study, we aimed to prospect the ability of three functionally  
 390 different species (different drought response strategies, clades, phenology, etc.) to modify these traits  
 391 under increasing drought conditions, and to assess the hydraulic risk ensued. Among the three studied  
 392 species, only *Q. ilex* exhibited significant plasticity in the hydraulic traits prospected, but all three  
 393 species maintained the hydraulic safety margins (HSM). In the following, we first discuss the effects  
 394 of the hydraulic adjustments of *Q. ilex* for its drought tolerance capacity. Then, we argue about the  
 395 likely causes leading to a lack of hydraulic adjustment under rainfall exclusion treatment for both *P.*  
 396 *halepensis* and *Q. pubescens*. Finally, we discuss the consequences of HSM homeostasis in response  
 397 to drier conditions across species for their hydraulic risk.

398

### 399 Homeostasis of HSM due to plasticity in *Q. ilex*

400

401 *Q. ilex* reached lower values of both  $\Psi_{pd\ min}$  and  $\Psi_{midd\ min}$  in the rainfall exclusion treatments  
 402 of both FB and P sites (Fig. 2, S6). Thus, the higher water stress experienced by this species in  
 403 response to increased drought could be the trigger for P50 and  $\Psi_{tlp}$  adjustments towards more negative  
 404 values (Fig. 3, S7).

405           Until now, studies focusing on long-term drought acclimation of *Q. ilex* had concluded that  
406 no plasticity of hydraulic vulnerability occurred for adult trees undergoing rainfall exclusion, in either  
407 branches (Limousin et al., 2010; Martin-StPaul et al., 2013) or in leaves (Limousin et al. 2022). Our  
408 results contradict those previous works. This could be explained by the facts that: 1) previous  
409 estimates of P50 in the xylem of branches were possibly biased by the open-vessel artifact (Martin-  
410 StPaul et al., 2014; Torres-Ruiz et al., 2014); 2) estimations of xylem vulnerability to cavitation in  
411 branches include several wood rings, which may hide xylem plasticity occurring after a specific  
412 aggravated drought; and 3) the rehydration kinetics method used to calculate leaf hydraulic  
413 conductance may not reflect only the xylem vulnerability to cavitation but also an extra-xylary  
414 component in the leaf mesophyll (Li et al., 2020; Limousin et al., 2022; Trifiló et al., 2016). The  
415 optical technique (Brodribb et al., 2016) used in this study for *Q. ilex* and *Q. pubescens* appears as a  
416 good solution to overcome these limitations. It allows the estimation of hydraulic vulnerability in the  
417 xylem only and in leaves, that are shorter lived, and therefore less affected than branches, by the  
418 potential biases due to regrowth and cavitation fatigue, or by methodological issues related to the  
419 flushing of native stem xylem embolism and vessels open at both ends (Gauthey et al., 2020).

420           In addition to the adjustment of xylem vulnerability to cavitation, our findings also highlighted  
421 that turgor-related traits also changed for this species under aggravated water stress conditions.  
422 Indeed, for *Q. ilex* trees growing in the rainfall exclusion treatments, lower  $\pi_{100}$  values were reported  
423 (Limousin et al., 2022), that translate into a significant reduction of  $\Psi_{tlp}$  (Bartlett et al., 2012). As  
424 stomatal closure is realised through the loss of stomata guard cells turgidity,  $\Psi_{tlp}$  could be used as a  
425 surrogate of the point of stomatal closure (Martin-StPaul et al., 2017). This suggests that *Q. ilex* trees  
426 from the rainfall exclusion treatments could close their stomata at lower water potentials than trees  
427 from the control treatments thus allowing them to maintain more photosynthetic activity or root  
428 growth under similar drought conditions. This adjustment may lead to an increased risk of xylem  
429 embolism if the vulnerability to cavitation, P50, is not also reduced. The apparent coordinated  
430 plasticities of  $\Psi_{tlp}$ , that is lowered by 0.5 MPa, and of P50 that is lowered by 1 MPa, could be beneficial  
431 to leaf gas exchange, without affecting the hydraulic safety margin (Fig. 4B). This maintenance of  
432 the HSM in spite of drier conditions seems confirmed by the similar levels of native stem xylem  
433 embolism observed in the *Q. ilex* trees growing inside the rainfall exclusion treatments and those  
434 growing under control conditions (Fig. 5). Limousin et al., (2022) showed that Fs increased  
435 significantly in the rainfall exclusion treatments, suggesting that lower  $\Psi_{tlp}$  do not result from internal  
436 cell water loss but rather from the accumulation of osmolytes. Hence, we can assume that, besides its  
437 action on stomatal control, osmotic adjustment in the exclusion treatments could also play a role in

438 limiting cell dehydration and hence drought-induced damages on leaves, which is crucial for this  
439 evergreen species with a leaf lifespan of two to three years (La Mantia et al., 2003).

440       Regarding the minimal leaf conductance ( $g_{\min}$ ), which is implied in water loss after stomatal  
441 closure (Duursma et al., 2019), we observed no plasticity in response to long-term increased drought  
442 for *Q. ilex* or any of the other species (Fig. 3). This suggests either that  $g_{\min}$  is not or little plastic, or  
443 that more precise methods, such as the drought box (Billon et al., 2020), should be used to detect  
444 plasticity in this trait.

445

446

447 Homeostasis of HSM despite lack of plasticity in *P. halepensis* and *Q. pubescens*

448

449 The most likely explanation for the lack of adjustments in hydraulic traits for *P. halepensis* and *Q.*  
450 *pubescens* is that these species experienced similar levels of extreme water stress under both control  
451 and rainfall exclusion treatments, and that that was no advantage in increasing HSM. This hypothesis  
452 appears plausible for *P. halepensis*, for which  $\Psi_{\text{pd min}}$  were similar between rainfall exclusion and  
453 control trees (Fig. 2). Such result is consistent with its behavior in response to drought. Indeed, *P.*  
454 *halepensis* is an isohydric species that strongly regulates its transpiration at the onset of drought,  
455 allowing it to limit soil water consumption and maintain both  $\Psi_{\text{pd}}$  and  $\Psi_{\text{midd}}$  (Figs. 2, 3) over a safe  
456 threshold of -3 MPa (Moreno et al., 2021). Recently, (Moreno et al., 2024) proposed that such  
457 isohydric behaviour is the results of two mechanisms : (i) its stomatal closure that happened at  
458 relatively high level of water stress and (ii) its ability to isolates itself from the ground and the  
459 atmosphere. Hence, its water use strategy seems sufficient in itself to limit hydraulic damages caused  
460 by rainfall reduction, without requiring xylem adjustments, at least under our experimental  
461 conditions. The fact that native stem xylem embolism were similar between rainfall exclusion and  
462 control treatments (Figs. 5, 6) further supports this idea.

463 For *Q. pubescens*, given its anisohydric behaviour like for *Q. ilex* (Damesin & Rambal, 1995; Poyatos  
464 et al., 2008), we expect trees from the rainfall exclusion to be exposed to more negative water  
465 potentials. In 2014, two years after the onset of the rainfall exclusion experiment, a significant  
466 difference between  $\Psi_{\text{pd}}$  of control and rainfall exclusion trees was observed. This result attests the  
467 effectiveness of the rainfall exclusion, which is in line with the significant reduction in soil moisture  
468 measured in the rainfall exclusion treatments (Genard-Zielinski et al., 2015, 2018). Nevertheless, for  
469 the following studied years,  $\Psi_{\text{pd min}}$  was similar between treatments. This result, which seems at first

470 sight contradictory, probably underpins other adjustments in unprospected traits. The figure S8 shows  
471 that at the onset of the experiment, the amount of leaf loss by the trees of the rainfall exclusion is 1.5  
472 higher than the one of the control treatment, suggesting highest leaf area index in the rainfall exclusion  
473 plot. Throughout the experiment, the gap between the litterfalls of the two treatments narrows to  
474 become similar in 2018. This result suggests that trees in the exclusion plot have reduced leaf  
475 production and therefore their overall leaf area, limiting overall transpiration and hence water  
476 potential decrease. Other adjustment could also occurred as, for example, an increase of rooting depth  
477 could be induced after aggravated water stress, as proposed by Martin-StPaul et al. (2013), enabling  
478 trees to access deeper soil water and thus limiting their drought exposure. Likewise, earlier leaf  
479 senescence could also occur in trees facing drier conditions (Wu et al., 2022). These additional  
480 adjustments occurring at the whole tree scale could be sufficient to dampen the rainfall exclusion  
481 effect on extreme tree water stress and prevent hydraulic damages (Fig. 5). Further investigations are  
482 however needed to identify the other traits implied in the homeostasis of hydraulic risk for this  
483 species.

484

485 The risk of maintaining the HSM for species vulnerability under future climatic  
486 conditions.

487

488 Although achieved through different means, our data report a maintenance of HSM under  
489 experimental 30 % rainfall exclusion (Fig. 4) for the three studied species. This suggest that no matter  
490 the drought response strategies adopting by these species, trees exposed to drier conditions adjusted  
491 functionally to preserve this trait. One might have expected that, in order to reduce hydraulic risk,  
492 trees could have adjusted to increase HSM values, through the development of a more cavitation  
493 resistant xylem. A reasonable explanation that could be advanced to explain this lack is the need for  
494 the plant to optimize fluxes relative to the hydraulic risk and that can be achieve through a myriad of  
495 strategies. The maintenance of HSM in response to plasticity that we reported in this study is in line  
496 with metanalysis from Choat et al. (2012), which reports a worldwide convergence of forest  
497 vulnerability to drought (estimated through tree HSM) at the interspecific level independently of the  
498 level of drought. The fact that trees seem to maintain HSM despite several years of increasing drought  
499 calls into question the natural capacity of forests to withstand accelerating climate change. To what  
500 extent HSM can be maintained in the future and at what cost, is still an open question. It remains to  
501 be known how much future extreme drought or heat waves may affect acclimated and non-acclimated  
502 trees. The next step is to use traits based mechanistic process-based models (Martin-StPaul et al.,

503 2017; Blackman et al., 2019, Ruffault et al. 2023), in order to characterize hydraulic risk in various  
504 conditions. In particular, they would enable to assess how the combination of several traits could  
505 translate into different levels of risk in a warmer, drier future.

506

507

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518

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