

Universitat de Lleida

Document downloaded from:

<http://hdl.handle.net/10459.1/64971>

The final publication is available at:

<https://doi.org/10.1111/nph.13569>

Copyright

(c) Voltas et al., 2015

(c) New Phytologist Trust, 2015

1 **Intra-specific variation in the use of water sources by the circum-Mediterranean**
 2 **conifer *Pinus halepensis***

3

4 Jordi Voltas^{1*}; Devon Lucabaugh^{1†}; Maria Regina Chambel²; Juan Pedro Ferrio¹

5

6 ¹Department of Crop and Forest Sciences – AGROTECNIO Center, University of
 7 Lleida, Rovira Roure 191, Lleida E-25198, Catalunya, Spain

8 ²CIFOR-INIA, Ctra.de La Coruña km 7,5 E-28040, Madrid, Spain

9

10 *Corresponding author:

11 Jordi Voltas

12 Dept. of Crop and Forest Sciences-AGROTECNIO Center

13 Universitat de Lleida

14 Avda. Rovira Roure 191, E-25198 Lleida (Spain)

15 Tel: +34 973 702855

16 e-mail: jvoltas@pvcf.udl.es

17

18 [†]Present address: The Davey Tree Expert Company, 3025 Jones Mill Rd., Norcross, GA
 19 30071, USA

20

Total word count (excluding summary, references and legends):	6496	Nº of Figures:	4 (Fig. 2 and 4 in colour)
Summary:	196	Nº of Tables:	3
Introduction:	654	Nº of Supporting Information Files:	1
Materials and Methods:	2315		
Results:	1577		
Discussion:	1876		
Acknowledgments:	74		

21

22 **Summary**

23

- 24 • The relevance of inter-specific variation in the use of plant water sources has been
25 recognized in drought-prone environments. In contrast, the characterization of intra-
26 specific differences in water uptake patterns remains elusive, although preferential
27 access to particular soil layers may be an important adaptive response for species
28 along aridity gradients.
- 29 • Stable water isotopes were analysed in soil and xylem samples of 56 populations of
30 the drought-avoidant conifer *Pinus halepensis* grown in a common garden test.
- 31 • We show that most populations revert to deep soil layers as the main plant water
32 source during seasonal summer droughts. More specifically, we detected a clear
33 geographic differentiation among populations in water uptake patterns even under
34 relatively mild drought conditions (early autumn), with populations originating from
35 more arid regions taking up more water from deep soil layers. However, the
36 preferential access to deep soil water was largely independent of aboveground
37 growth.
- 38 • Our findings highlight the large plasticity and adaptive relevance of the differential
39 access to soil water pools among Aleppo pine populations. This study illustrates
40 how the selective pressure towards securing a source of water in dry environments
41 has interacted with demographic processes during post-glacial colonization to shape
42 species' adaptive genetic make-up.

43

44 **Key words:** adaptation, drought, Mediterranean climate, *Pinus halepensis*, stable
45 isotopes, water uptake, water-use strategies

46

47 **Introduction**

48 Access to deep water reservoirs is crucial for many tree species to withstand periods
49 of drought in seasonally dry climates (Valentini *et al.*, 1992; David *et al.*, 2007;
50 Eggemeyer *et al.*, 2009; Rossatto *et al.*, 2012). Taking advantage of stable isotopes as
51 natural tracers of water movement in the soil-water-atmosphere continuum, it has been
52 shown that the soil depth at which root water uptake occurs is highly dependent on
53 seasonal climate fluctuations (Snyder & Williams, 2000; Retzlaff *et al.*, 2001; Klein *et al.*
54 2014; Barbeta *et al.* 2015). Indeed, many woody shrubs and trees possess the ability
55 to switch active root absorption depth based on moisture availability in the soil (e.g.
56 *Banksia prionotes* [Dawson & Pate 1996]; *Prosopis velutina* [Snyder & Williams,
57 2000]; *Pistacia lentiscus* [Filella & Peñuelas 2003a]).

58 Stable water isotopes can also be used to determine taxonomic differences in the
59 source(s) of plant water within the soil profile. Although the relevance of inter-specific
60 variation in the use of different water sources has often been examined in dry
61 ecosystems (e.g. Dawson *et al.*, 1998; Barbour, 2007; West *et al.*, 2012; Comas *et al.*
62 2015), the characterization of genetic differences in water uptake patterns at the intra-
63 specific level remains unclear. So far, we are only aware of a single study examining
64 genetic differences in water uptake patterns through stable isotopes of water for
65 contrasting maize material (Zhang *et al.*, 2011). Nonetheless, preferential access to
66 particular soil layers may be an important effect of adaptive divergence for populations
67 of xeric species, as this trait may be linked to the array of evolutionary responses to
68 varying environmental conditions often observed within a species' distribution range
69 (Alberto *et al.*, 2013). Because the unprecedented intensity and duration of future
70 drought events are expected to impact negatively on the structure and function of forest
71 ecosystems (e.g. Park Williams *et al.* 2012), the assessment of intra-specific differences
72 in access to deep water pools may be relevant for understanding the short-term impact
73 of warming on contemporary populations and for tailoring mitigation strategies to
74 climate change.

75 The circum-Mediterranean conifer Aleppo pine (*Pinus halepensis* Mill.) is the most
76 widely distributed tree species in the Mediterranean basin, where it can be found under
77 a varied range of thermal and moisture conditions. The adaptive relevance of drought
78 stress in shaping the populations' genetic structure has been highlighted in previous
79 studies for traits such as biomass allocation (Chambel *et al.*, 2007; Climent *et al.*, 2008),
80 intrinsic water-use efficiency (Voltas *et al.*, 2008), wood anatomy (Esteban *et al.*, 2010)

81 and vulnerability to xylem embolism (Klein *et al.*, 2013). Altogether, these studies have
82 shown that populations thriving in dry environments exhibit different traits from their
83 counterparts originating from mesic areas.

84 We hypothesize that Aleppo pine has adapted to the recurrent drought episodes
85 typical of the Mediterranean basin by producing and maintaining a deep root system
86 that is dispersed into many layers of the soil profile. This adaptive characteristic may
87 allow trees to respond to precipitation pulses that eventually recharge the upper soil
88 layers in spring or autumn. Conversely, when the surface soil dries up, trees may revert
89 to deeper soil layers for water use. However, we also consider that such performance
90 may be fine-tuned to the environment owing to the disparity of selective pressures for
91 water use experienced by this species at the ecotypic level. Therefore, this study pursues
92 the following objectives: (i) to determine the main sources of water and their seasonal
93 variation for a widespread conifer (*Pinus halepensis*); (ii) to characterize differential
94 changes in the use of water sources over time at the intra-specific level (i.e. differential
95 phenotypic plasticity) for a highly representative set of seed sources covering most of
96 the present range of geographical distribution of this species; (iii) to clarify whether the
97 observed variability in phenotypic plasticity for plant water sources is of adaptive
98 relevance in relation to the diversity of life history strategies and environmental
99 conditions where this species can be found.

100

101 **Materials and Methods**

102 *Plant material*

103 Seed sources from 56 populations of Aleppo pine (*Pinus halepensis* Mill.) were selected
104 to represent most of the natural distribution range of the species, which spans the
105 Mediterranean basin (Fig. 1; Table S1). The populations originated from mainland
106 Spain, the Balearic Islands, France, Greece, Italy and Tunisia, and were grouped into 16
107 ecological regions based on geographic origin and ecotypic characteristics following
108 Climent *et al.* (2008). The seeds were collected at origin in 1995 from 20 to 30 open-
109 pollinated trees that were spaced at least 100 m apart. Bulk seed lots were prepared to
110 represent each of the different populations and a number of provenance trials were
111 established in Spain. For this purpose, one-year-old seedlings were produced using
112 standard container nursery practices in Spain during 1997. Then, the seedlings were
113 randomly allocated to six different experimental sites in April 1998.

114 For each seed source, climate variables at origin were obtained for the period 1960–
115 1990 from the Global Climatic Model WorldClim database (Hijmans *et al.*, 2005),
116 implemented in DIVA-GIS with a spatial resolution of 1 km². These variables were
117 chosen based on previous identification of climate drivers of ecotypic variation in
118 Mediterranean pines (Tapias *et al.*, 2004; Climent *et al.*, 2008; Voltas *et al.*, 2008) and
119 included, among others, annual mean temperature (T), temperature annual range (TAR),
120 annual precipitation (P) and annual summer precipitation (Ps).

121

122 *Study site*

123 One of the original six experimental sites located in Altura (39°49'29"N, 00°34'22"W,
124 640 m a.s.l.; Castellón province, eastern Spain) was used in this study (Fig. 1). We
125 selected this trial as representative of the average growing conditions in which the
126 species can be found in the Mediterranean (Gil *et al.*, 1996). It has a mean annual
127 precipitation of 652 mm, of which 19% falls in summer, a mean annual temperature of
128 13.8 °C and a mean annual potential evapotranspiration of 1,115 mm. These features,
129 together with a low soil water retention capacity, indicate that trees often suffer from
130 summer drought stress. The trial was installed on a reforestation site with a 5% slope
131 and a southeast aspect. Site preparation included tilling with a disk ripper before
132 planting. The soil is a calcic cambisol with a maximum depth of ~40 cm and a very
133 gravelly and cemented underlying horizon of CaCO₃ accumulation. The seedlings (16
134 per population) were planted systematically (2.5 × 2.5 m spacing) in four replicates
135 consisting of four-tree line plots following a latinized row-column design for a total of
136 896 test seedlings. This layout is suited for experiments that include a large number of
137 treatments, as it provides an effective control of field variation in two directions,
138 allowing adjustment for field trend using rows and columns along with an extra
139 blocking facility for contiguous replicates (Williams *et al.*, 2002).

140

141 *Data collection*

142 The field data were collected in 2010, a 10% drier (less rainy) than average year, on
143 three different days representing contrasting situations in terms of water shortage, in
144 accordance with the seasonal regime of precipitation typical of Western Mediterranean
145 (Fig. 1): 2 June (end of spring, wet), 28 July (peak summer, dry) and 27 September
146 (early autumn, drought recovery transition from dry to wet). These are referred to as D₁,
147 D₂ and D₃ respectively. On each day, xylem samples were obtained from 11:00 am to

148 1:00 pm solar time from rows of the experimental layout following a zigzag pattern.
 149 Samples were taken from healthy, east-facing branches of about 1.5 cm diameter in the
 150 top third of the crown using telescopic loppers. Shoot segments (about 5 cm length)
 151 were bark-peeled, placed immediately into glass vials and frozen in dry ice to prevent
 152 evaporation. Out of the total of four replicates, samples were taken from the first
 153 consecutive three, excluding the fourth replicate to allow the collection of samples
 154 under uniform environmental conditions. Furthermore, three trees were selected from
 155 every four-tree line plot in each of the three replicates based on phenotypic similarity
 156 (height and diameter at breast height – DBH). This was done to avoid the potentially
 157 large biasing effects of non-representative trees on the average isotopic records obtained
 158 per plot. Therefore, nine trees per population (i.e. three trees per plot) were monitored at
 159 each sampling date for a total of 504 sampled trees.

160 Soil samples were collected for the same days at two depth ranges (0–15 cm and 15–
 161 40 cm) using a straight tube probe thoroughly cleaned between consecutive samplings.
 162 From 7:00 am to 9:00 am solar time, samples were taken from soil pits dug in the
 163 middle of the row of four-tree line plots selected systematically in the field (spaced 25
 164 m within rows and 20 m within columns, following a staggered pattern). The aim of this
 165 systematic arrangement was to account for the spatial variation in the isotopic
 166 signatures of soil water, while keeping the number of soil samples to be taken during
 167 sampling days within reasonable limits. In particular, 42 samples were taken at each
 168 sampling day corresponding to two different soil layers, for a total of 126 soil samples.
 169 The soil extracted was placed quickly into glass vials and frozen in dry ice. All samples
 170 were kept frozen until processing and analysis. Growth data (height and diameter at
 171 1.30 m) were recorded for each tree in April 2010 (at age 14). Stem volume over bark
 172 (V_{ob}) was used as a surrogate for total standing biomass (Reinhardt *et al.*, 2006). V_{ob}
 173 was calculated by the following equation, assuming the stem to be conical (Climent *et*
 174 *al.*, 2008):

$$175$$

$$176 \quad V_{ob} = (\pi/12) \times D^2 \times H \quad (1)$$

$$177$$

178 where D is the diameter at 1.30 m and H is the total tree height.

179

180 *Isotopic analysis*

181 Xylem and soil water was extracted by cryogenic vacuum distillation (Otieno *et al.*,
182 2006). Prior to extraction, the xylem samples of the same plot were pooled together.
183 Therefore, 168 isotopic determinations (56 populations \times 3 replicates) were performed
184 per sampling date for xylem water. Sample tubes were placed in a heated silicone oil
185 bath (110-120°C), and connected with Ultra-Torr unions (Swagelok Company, Solon,
186 OH, USA) to a vacuum system (*ca.* 10^{-2} mbar) including U-shaped water traps in series
187 that were cooled with liquid N₂. The extraction time was 90 minutes for xylem and 120
188 minutes for soil samples. Captured water was then transferred into cap-crimp 2 ml vials,
189 and stored at 4°C until analysis.

190 The oxygen and hydrogen isotopic composition ($\delta^{18}\text{O}$ and $\delta^2\text{H}$ respectively) of water
191 was determined by isotope ratio infrared spectroscopy (IRIS) using a Picarro L2120-*i*
192 coupled to an A0211 high-precision vaporiser (Picarro Inc., Sunnyvale, CA, USA). The
193 estimated precision, based on the repeated analysis of four reference water samples, was
194 0.10‰ for $\delta^{18}\text{O}$ and 0.40‰ for $\delta^2\text{H}$. The isotopic compositions of this reference
195 material were 0.39‰, -5.34‰, -10.05‰ and -15.39‰ for $\delta^{18}\text{O}$, and -1.2‰, -43.3‰, -
196 72.9‰ and -114‰ for $\delta^2\text{H}$ (IAEA proficiency test 2011; Isotope Hydrology Section of
197 the International Atomic Energy Agency, M. Groening, pers. comm.).

198 Residual organic contaminants in the distilled water can interfere with the analysis of
199 plant and soil samples conducted with IRIS (West *et al.*, 2011; Martín-Gómez *et al.*,
200 2015). The presence of contaminants was checked through Picarro's ChemCorrect™
201 post-processing software. Approximately 50% of the xylem samples were flagged as
202 potentially contaminated by the Chemcorrect™ software, whereas only two soil
203 samples were flagged. Although the estimated concentration of contaminants for
204 flagged samples was very small (the maximum difference from pure water standards
205 was less than 7×10^{-5} Methanol units [equivalent to 0.01% MetOH] and less than 65
206 'Other' units [equivalent to 0.25% Ethanol]), we applied the post-processing correction
207 described in Martín-Gómez *et al.* (2015). Overall, there were only small differences
208 between corrected and non-corrected values, but the precision of population and soil
209 layer mean estimates was slightly improved using the corrected values. A small subset
210 including xylem and soil samples was also analysed by isotope-ratio mass spectrometry
211 for $\delta^{18}\text{O}$, showing a very good agreement with IRIS values even prior to correction
212 ($r^2=0.993$, RMSE=0.230, $n=10$).

213

214 *Statistical analysis*

215 The oxygen and hydrogen isotopic compositions of xylem water were subjected to
216 mixed model analysis of variance (ANOVA) for a latinized row-column design
217 independently for each sampling day. The fixed terms of the analysis were column,
218 replicate and population. The random terms were row within replicate, column within
219 replicate and intra-block error (Williams *et al.*, 2002). We also considered an alternative
220 model with *Vob* as a covariate to adjust for the possible size-dependent variation of
221 isotopic signatures. However, this effect was non-significant for both $\delta^{18}\text{O}$ and $\delta^2\text{H}$;
222 hence, any correction for an effect of tree size on isotopic records was deemed
223 unnecessary. We did not consider potential differences in competition intensity among
224 trees caused by mortality of adjacent trees as the survival rate at age 14 was very high
225 (95.1%).

226 The population effect in the ANOVA was further partitioned into two fixed terms
227 accounting for variation (1) among the 16 ecological regions and (2) among populations
228 within ecological region. For D_2 , only 54 populations were available for comparison
229 owing to problems encountered during the water extraction process, which led to highly
230 deviating isotopic values for populations 21 and 142. The stem volume for 2010 was
231 also subjected to mixed model ANOVA following the same partitioning of effects.
232 However, in this case an extra random term was included accounting for between-tree
233 variation. Tests of fixed effects were performed using Wald-type F -statistics and the
234 estimation of variance components through restricted maximum likelihood testing. Best
235 linear unbiased estimates (BLUES) of population and ecotypic effects were obtained for
236 $\delta^{18}\text{O}$ and $\delta^2\text{H}$ of xylem water and stem volume.

237 The oxygen and hydrogen isotopic compositions of soil water were also subjected to
238 mixed model ANOVA for a two factor (soil layer, sampling date) factorial following
239 the latinized row-column layout (fixed column, replicate, soil layer and sampling day
240 effects, a random row within replicate effect and a pooled intra-block error term). The
241 semi-variogram associated with this dataset did not reveal any obvious spatial pattern of
242 residual variation; hence, we did not apply any spatial adjustment to the original
243 isotopic soil data. BLUES were also obtained for the different combinations of soil layer
244 and sampling day for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ of soil water. Mixed model ANOVAs were
245 performed using the mixed procedure of SAS/STAT v. 9.2 (SAS Institute Inc., Cary,
246 NC).

247 The relative contributions of different sources to xylem water were estimated by
248 Bayesian mixing modelling using the SIAR (Stable Isotope Analysis in R) package
249 (Parnell *et al.*, 2010). Stable isotope mixing models are used extensively for studying
250 food webs, but can also be applied to the determination of plant water sources (Palacio
251 *et al.*, 2014; Barbeta *et al.*, 2015). We considered three different sources of soil water:
252 two soil depths (0–15 cm and 15–40 cm) and groundwater reservoirs remaining in
253 cracks and fissures within the calcic horizon, protected from evaporation. Hence,
254 BLUEs for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ of the upper and lower soil layers were used as input in the
255 mixing model for each sampling day. For groundwater, three alternative estimates were
256 tested: (1) the weighted average of monthly isotopic signatures of precipitation from
257 October to April (considered as the soil recharge period in the Mediterranean) over the
258 period 2000–2010 for Valencia, the nearest site (*ca.* 40 km) of the Spanish Network of
259 Isotopes in Precipitation (REVIP; Capilla *et al.* 2011); (2) the outcome of systematic
260 sampling of the aquifer system in the Júcar catchment, eastern Spain, covering the
261 period 1974–2006 (Instituto Geológico y Minero de España, 2007); (3) the average of
262 samples of water collected from three nearby fountains (natural springs). The estimates
263 were similar across methods: -6.2‰ and -39.4‰ (method 1), -6.5‰ and -35.6‰
264 (method 2), and -6.7‰ and -39.5‰ (method 3), for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ respectively. They
265 also yielded very similar relative contributions of plant water use at the population level
266 through the Bayesian mixing models. In particular, the mean correlation between
267 relative contributions of water sources for populations was 0.99 for upper soil, 0.99 for
268 lower soil and 0.98 for groundwater in D_2 . In D_3 , the correlations were 0.99 (upper soil),
269 0.96 (lower soil) and 0.86 (groundwater). We present here the results obtained using
270 records of spring water (method 3).

271 As target values ('consumers') in SIAR, we used the BLUEs of $\delta^{18}\text{O}$ and $\delta^2\text{H}$ of
272 xylem water at the population level. The trophic enrichment factor (TER) was set to 0,
273 assuming no fractionation during water uptake (Ehleringer & Dawson, 1992). We ran
274 500,000 iterations separately for the isotopic values of each population on each
275 sampling day, discarding the first 50,000 and thinning by a factor of 45, thereby
276 obtaining the most likely contribution (the mean of the posterior distribution of the
277 Markov chain Monte Carlo simulation) of each source for every population and
278 sampling day. This analysis evaluated differential patterns of active root zones both at
279 different temporal periods and among populations during the growing season.

280 Simple correlations were calculated to determine relationships among biophysical
 281 factors at origin (e.g. geographic coordinates, climate variables) and phenotypic traits
 282 (relative contributions of soil water sources and stem volume). We also analysed the
 283 relationships between carbon isotope composition ($\delta^{13}\text{C}$; surrogate for water-use
 284 efficiency, WUE) of wood holocellulose, as reported in Voltas *et al.* (2008), and the
 285 aforementioned phenotypic traits for a subset of 25 populations. Sampling day D₁ was
 286 not included in the correlation analysis as we did not detect significant population
 287 effects in the ANOVAs. We also used Euclidean geographical distances and ecological
 288 distances between each population and the trial site. In the latter case, we calibrated the
 289 climate data following Rutter & Fenster (2007) to calculate Gower's distance (GD):

290

$$291 \quad GD = \frac{1}{p} \sum_{i=1}^p \frac{|A_i - B_i|}{r_i} \quad (2)$$

292

293 where p is the number of environmental factors, A_i and B_i are the values of each
 294 environmental factor (T, TAR, P and Ps) at the two compared sites and r_i is the range
 295 of each environmental factor in the dataset. The differences among treatments and the
 296 correlation coefficients were considered statistically significant when $P < 0.05$.

297

298 **Results**

299 *Stable isotopes in xylem water*

300 The ANOVAs revealed significant differences among populations for D₂ (for $\delta^{18}\text{O}$ and
 301 $\delta^2\text{H}$) and D₃ (for $\delta^{18}\text{O}$) (Table 1). There was no significant differentiation among
 302 populations for D₁, regardless of isotope type. Grouping populations into ecological
 303 regions was effective at unveiling genetic variations of potential adaptive relevance, as
 304 the partitioning of population effects indicated that most variation related to changes at
 305 the ecotypic level. This was the case for both $\delta^{18}\text{O}$ and $\delta^2\text{H}$ on D₂ and D₃ (Table 1). For
 306 D₂, $\delta^{18}\text{O}$ of xylem water additionally displayed significant variation among populations
 307 within ecological regions.

308 For $\delta^{18}\text{O}$, populations ranged from -8.0‰ (population 184) to -5.4‰ (population
 309 103), and from -8.4‰ (population 156) to -5.5‰ (population 233) for D₂ and D₃
 310 respectively. For $\delta^2\text{H}$, populations varied from -56.3‰ (population 184) to -44.1‰
 311 (population 103), and from -54.7‰ (population 156) to -41.0‰ (population 92) for D₂
 312 and D₃ respectively. The corresponding range of values for ecological regions was as

313 follows: for $\delta^{18}\text{O}$, from -7.5‰ (11PY, Ibiza) to -6.0‰ (14IT, Italy) and from -7.2‰
314 (3ALC, Southern Plateau of Spain) to -5.9‰ (10ME, Menorca; 14IT, Italy) for D_2 and
315 D_3 respectively; for $\delta^2\text{H}$, from -51.2‰ (9MA, Majorca) to -46.0‰ (14IT, Italy) and
316 from -48.6‰ (9MA, Majorca) to -43.2‰ (14IT, Italy) in D_2 and D_3 respectively. The
317 relationships between D_2 and D_3 were significant across population means for both $\delta^{18}\text{O}$
318 ($r = 0.30$; $p = 0.030$) and $\delta^2\text{H}$ ($r = 0.32$; $p = 0.018$).

319

320 *Stable isotopes in soil water*

321 The ANOVAs showed significant differences in $\delta^{18}\text{O}$ and $\delta^2\text{H}$ for both soil layer and
322 sampling day effects (Table 2). The upper soil layer was consistently more enriched
323 with heavier isotopes (i.e. it exhibited higher isotopic compositions) than the lower soil
324 layer, regardless of isotope type (mean difference across sampling days = 3.13‰ and
325 14.62‰ for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ respectively). Also, D_3 showed higher overall values (-10.5‰
326 and -83.1‰ for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ respectively) than either D_1 (-14.0‰ and -107.9‰) or D_2
327 (-10.7 ‰ and -100.2 ‰) (Fig. 2). However, the absolute $\delta^{18}\text{O}$ difference between soil
328 layers was larger for D_2 when compared to either D_1 or D_3 . This caused a significant
329 soil layer by sampling day interaction (Table 2).

330

331 *Contribution of water sources to genotypic differences in water extraction patterns*

332 BLUEs of population means for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ were plotted independently for each
333 sampling day (Fig. 2). BLUEs of each soil layer were also included for the sake of
334 comparison, suggesting active rooting zones of populations. We also added the global
335 meteoric water line (GMWL) and the estimated values ($\delta^{18}\text{O}$ and $\delta^2\text{H}$) of groundwater
336 taken from records of spring water. For D_1 , when precipitation was abundant, the
337 isotopic xylem water values representing each of the 56 populations clustered around
338 the mean isotopic values of the top soil layer and fell along the isotopic soil line. For D_2 ,
339 the cluster of population means of isotopic xylem water values was located along the
340 GMWL and well apart from the line drawn from the isotopic soil values, although the
341 population means were closer to the lower soil layer $\delta^{18}\text{O}$ value than for D_1 . The data
342 collected on D_2 represent the response of Aleppo pines to the lowest precipitation
343 levels, highest temperatures and – probably – the least soil water availability
344 experienced in 2010 at the site (Fig. 1 inset). Finally, for D_3 the situation was in
345 between that observed for D_1 and D_2 , that is, the populations were again placed along
346 the GMWL, but closer to the isotopic soil line than for D_2 and around the lower soil

347 layer $\delta^{18}\text{O}$ and $\delta^2\text{H}$ values. The outstanding ecotypic structure of the isotopic signals can
 348 be observed in Fig. 2 (insets).

349 Although most population means were within the range of isotopic values of the
 350 three water sources used to characterize water uptake patterns, a few populations fell
 351 outside their limits (using 95% confidence intervals for means of water sources; mean \pm
 352 SE \times 1.96). This was noticeable for D₁ (populations 186, 202 and 233) and D₃
 353 (populations 82, 156, 184 and 203) (red dots; Fig. 2), hence suggesting an incomplete
 354 characterization of water sources. For D₁, the high isotopic values of this population
 355 subset pointed to a source of highly evaporated (superficial) water available during the
 356 rainy spring. For D₃, the low values attained by these atypical populations might
 357 indicate the existence of a deeper water source below the actual sampled soil. Deletion
 358 of these potential outliers did not change substantially the results of the ANOVA for D₁,
 359 but for D₃ the population and ecological region effects became only marginally
 360 significant for $\delta^{18}\text{O}$ ($p= 0.091$ and $p= 0.074$, respectively) and non-significant for $\delta^2\text{H}$
 361 ($p= 0.444$ and $p= 0.371$).

362 The relative contribution of each water source (upper and lower soil layers and
 363 groundwater) to tree water consumption averaged over populations is shown in Fig. 3
 364 for each sampling day. The mixing models revealed that trees were able to extract water
 365 simultaneously from the three water pools, but in varying relative amounts depending
 366 on the season. The transition from spring to summer (D₁ to D₂) is clearly noticeable as a
 367 large contribution of source water (61%) on D₁ came from the upper soil layer (0–
 368 15cm). In contrast, for D₂ and D₃ most of the water used originated from either
 369 groundwater reservoirs (44.8% on D₂) or the lower soil layer (52.5% on D₃).

370 The contribution of each soil water source for each sampling day (except D₁) and
 371 ecological region is depicted in a ternary plot (Fig. 4). For D₂, when significant
 372 genotypic differences were found for both $\delta^{18}\text{O}$ and $\delta^2\text{H}$, the contribution of the upper
 373 soil layer varied between 2.2% (191 [population]; 11PY, Ibiza [ecological region]) and
 374 43.6% (103; 5LI, East Spain) (mean= 14%; median= 10.8%). For the lower soil layer,
 375 the differences in contribution ranged from 10.4% (103; 5LI, East Spain) to 78.7%
 376 (184; 9MA, Majorca) (mean= 41.1%; median= 43%). The groundwater contribution
 377 varied between 17.6% (184; 9MA, Majorca) and 54.3% (31; 1CAT, Catalonia) (mean =
 378 44.8%; median=45.2%). For D₃, when significant population differences were found for
 379 $\delta^{18}\text{O}$, the contribution of the upper soil layer varied between 1.7% (156; 6BS, South
 380 Betic Mountains) and 50.1% (233; 14IT, Italy) (mean= 14.2%; median= 8.3%). For the

381 lower soil layer, the differences in contribution ranged between 15.9% (92; 4MS,
382 Iberian Range) and 95.2% (156; 6BS, South Betic Mountains) (mean= 52.5%; median=
383 51.4%). The groundwater contribution varied between 3.0% (156; 6BS, South Betic
384 Mountains) and 52.1% (101; 5LI, East Spain) (mean= 33.2%; median= 35.3%). For D₁,
385 genotypic changes in contributory soil water sources were not examined owing to lack
386 of significant population effects in stable isotopes.

387

388 *Stem volume*

389 The ANOVA for stem volume showed significant variation among populations (results
390 not shown). As for xylem water isotopes, grouping populations into ecological regions
391 proved to be effective in revealing potential adaptive variation, as the partitioning of
392 population effects (F -value= 4.85; $p < 0.0001$) indicated that changes at the ecotypic
393 level underlie most variation (F -value= 8.07; $p < 0.0001$). Yet, the variability in stem
394 volume among populations within ecological regions remained significant. Trees at the
395 trial site averaged 5.16 m (height), 8.74 cm (diameter at 1.30 m) and 12.80 dm³
396 (volume). BLUEs of population means for volume ranged from 4.89 dm³ (population
397 84) to 37.57 dm³ (population 212).

398

399 *Relationships between population traits and eco-geographical variables*

400 For sampling days D₂ and D₃, the relative contributions of soil water sources to xylem
401 water at the population level were correlated with a number of eco-geographical
402 variables characterizing the sites of origin (Table 3). Population differences in the
403 contribution of the upper soil layer were associated with the latitude (for D₂) and
404 longitude (for D₃) of origin, suggesting that populations originating from the northern
405 and eastern Mediterranean basin extracted relatively more water from shallow soil
406 layers than southern and western populations. Similarly, populations from regions
407 located far from the trial site, which is located in the western area of the species'
408 distribution, showed a more important contribution from shallow soil to the total
409 amount of xylem water.

410 Populations originating from wetter areas (higher P, higher precipitation of wettest
411 month) tended to extract less groundwater at peak summer (D₂). This was the most
412 relevant association between water uptake patterns and explicit climate factors (Table
413 3). Additionally, Gower's ecological distance to the trial was used as a means of
414 comparing the overall climatic characteristics at origin for each population with those of

415 the evaluation site. A larger Gower's distance was related to a higher mean annual
416 temperature ($r= 0.437$; $p<0.001$) and both lower summer precipitation ($r= -0.413$;
417 $p=0.002$) and a lower ratio of summer to annual precipitation ($r= -0.664$; $p<0.001$),
418 therefore denoting increasing aridity at origin. In this regard, populations originating
419 from more arid conditions tended to extract less water from shallow soil and more water
420 from deeper soil layers (Table 3). A reanalysis of the relationships between relative
421 contributions of water sources for D₃ and eco-geographical variables excluding the
422 atypical populations marked in Fig. 2 did not change substantially the aforementioned
423 associations (Table S2).

424 The differences in stem volume at the population level were also associated to eco-
425 geographical variables at origin (Table 3). Populations from the northern or eastern
426 Mediterranean basin tended to exhibit the largest volumes, as well as those populations
427 experiencing relatively low temperatures during summer. According to Gower's
428 distance, seed sources originating from highly-seasonal dry areas tended to grow less
429 than those from humid native sites. We also found a positive association between stem
430 volume and groundwater contribution in autumn.

431

432 **Discussion**

433 This study provides new insights into changes in the root activity of a widespread
434 circum-Mediterranean pine in response to seasonal fluctuations in water availability,
435 which reflects potentially adaptive differences among natural populations. To our
436 knowledge, this is the first work to address the crucial issue of intra-specific divergence
437 in water uptake patterns for a plant species typical of drought-prone environments such
438 as Aleppo pine.

439

440 *Nature and timing of genetic differences in stable isotopes for xylem water*

441 The trees experienced the lowest precipitation at the end of July (D₂), coupled with the
442 highest temperatures for the entire year of 2010 (Fig. 1). The genotypic differentiation
443 that is observed for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ during this central period of water shortage suggests
444 that adaptive variation in water uptake patterns exists among populations of Aleppo
445 pine. Indeed, most of the populations' variability in water isotopes could be attributed to
446 differences among ecological regions, underpinning the adaptive relevance of the
447 differential access to deep soil water pools. Our results therefore point to peak summer
448 as the most adequate period to highlight ecotypic variability in water uptake, which

449 parallels the results reported by Snyder & Williams (2000). In contrast, on D₁ (end of
450 spring) the trees experienced abundant precipitation prior to sampling, presumably
451 approaching saturation in the top soil layer and resulting in a lack of intraspecific
452 differentiation. On D₃ (beginning of autumn) water availability was probably
453 somewhere between D₁ and D₂, but the genotypic differences observed for $\delta^{18}\text{O}$ suggest
454 that Aleppo pine populations can still exhibit contrasting water uptake patterns during
455 recovery from a drought period. Overall, $\delta^{18}\text{O}$ and $\delta^2\text{H}$ trends in population means were
456 quite consistent across sampling dates (D₂ and D₃), but $\delta^{18}\text{O}$ was more effective in
457 discriminating among entries, as the statistical relevance of genetic effects was
458 consistently larger than for $\delta^2\text{H}$. This could be partly due to fractionation during water
459 uptake, which has been reported for hydrogen in woody xerophytes (Ellsworth &
460 Williams, 2007), but consistently discarded for oxygen (Wershaw *et al.*, 1966; Dawson
461 & Ehleringer, 1991; Barbour, 2007; Ellsworth & Williams, 2007).

462 Aleppo pine seems to be flexible utilizing different water pools whenever the
463 availability of source water is in abundance (Querejeta *et al.*, 2001). Previous studies
464 (Nambiar, 1990; Querejeta *et al.*, 2001; Klein *et al.* 2014) suggest that the ordinary soil
465 depth at which the majority of water is taken up in pines is 30–40 cm (approximate
466 maximum soil depth of our study), where nutrient levels are also at their highest
467 (Jackson *et al.*, 1996; Achat *et al.*, 2008). Based on our findings, Aleppo pine shows a
468 clear preference for extracting water from the upper soil layer when it is available (i.e.
469 D₁), irrespective of the geographic origin of the population. Conversely, during the
470 seasonal summer drought (i.e. when the soil surface dries up), Aleppo pine relies, to a
471 greater or lesser extent, on deep rooting as a consistent strategy for water uptake (Klein
472 *et al.* 2014). This was observed for D₂ in the cluster of population isotopic signatures
473 located principally on and around the GMWL and near the estimated groundwater
474 signal for the area. This suggests that root activity during drought events is also present
475 below the available soil (Eggemeyer *et al.*, 2009; Barbeta *et al.*, 2015), allowing Aleppo
476 pine populations to access moisture through the fissured calcic horizon.

477 Nonetheless, Aleppo pine populations may require a relevant contribution to
478 transpiration from groundwater, even during wet periods (Fig. 3). Oren & Sheriff (1995)
479 and Querejeta *et al.* (2001) have hypothesized that the deep roots of pines may
480 effectively supply a significant amount of water, but preferentially during the dry
481 season, as they are composed of long hydraulic pathways with relatively low
482 conductivity compared to shallow roots (Oren & Sheriff, 1995; Lintunen & Kalliokoski,

483 2010). The distribution of deep roots in pines is known to be sparse but important in
484 supplying the trees when the soil surface layers dry out (Nambiar & Sands, 1992; Achat
485 *et al.*, 2008). The development of a deep rooting system therefore seems essential for
486 Aleppo pine to survive the intense summer drought, increasing its competitive ability;
487 this points to the existence of a dimorphic root morphology for the species, including
488 superficial lateral roots and deeply penetrating sinker (tap) roots (Filella & Peñuelas,
489 2003b).

490

491 *Relative contribution of the different soil layers to the water used by populations*

492 Fig. 3 depicts a clear seasonal transition in which Aleppo pine switches water uptake to
493 deeper soil layers when the upper soil dries up in mid-summer. In the early growing
494 season (late spring, D₁), the upper soil (shallow) layer was found to be the main source
495 of water (contributing >50%) for most (≥70%) populations. In contrast, in peak summer
496 and early autumn, the upper soil layer contributed less than 25% as a water source for
497 the majority (≥75%) of populations. Thus, the mixing models predict that most genetic
498 entries would revert to groundwater as the main plant water source when shallower soil
499 layers approach desiccation. Here, our results suggest that the distribution of functional
500 fine roots in Aleppo pine varies significantly across populations and ecological regions
501 when tested under common garden conditions. This could be a consequence of seasonal
502 differences in the amount of soil water available at origin, which may have imprinted
503 the adaptive genetic make-up of this species for water uptake. Nevertheless, most
504 ecological regions showed a similar plastic reaction in their patterns of water use from
505 peak summer to early autumn (i.e. a relative decrease in groundwater uptake) (Fig. 4).
506 The main exception was the Ibiza ecotype (11), characterised by a high annual mean
507 temperature and low annual precipitation, which showed a similarly high contribution
508 of groundwater at both sampling times. Such contrasting ecohydrological performances
509 could be further investigated through a more thorough assessment of water use
510 strategies complementing the isotopic results (root distribution profiles, soil water
511 content dynamics at different soil depths, etc.; Raz-Yaseef *et al.* 2013; Klein *et al.*
512 2014).

513 Other studies involving inter-specific comparisons (e.g. Snyder & Williams, 2000)
514 produced larger ranges of variation in contributing sources than our study. Nonetheless,
515 the relative contributions from multiple water sources have been useful in discerning
516 variation in root activity triggered by seasonal changes in soil water availability. An

517 obvious limitation of the isotopic approach is its inability to characterize changes in the
518 absolute amount of soil water taken up by plants at varying spatiotemporal scales
519 (Snyder & Williams 2000). This shortcoming also applies to our study as xylem water
520 isotopes allow the quantification of possible differences in the relative contribution of
521 several sources to the water transpired by trees, but do not give clear answers to the
522 issue of how the absolute amount of water consumed may vary among populations or
523 among sampling times. In this regard, seasonal sap flow measurements and/or other
524 estimates of total tree water use would be valuable to overcome such limitation.

525

526 *Relationships between genetic patterns of water uptake and eco-geographical*
527 *characteristics at origin*

528 We detected a geographic structure in the water uptake patterns of Aleppo pine across
529 most of its current distribution range, with a tendency for northern and eastern
530 Mediterranean populations to rely more on shallow water during dry periods than their
531 southern and western counterparts. This demonstrates strong ecotypic differences in the
532 functional plasticity of root systems for this Mediterranean conifer, although the
533 easternmost (and some of the driest) populations of the species remain to be tested.
534 Geographic patterns of ecotypic variation in Aleppo pine have been reported for a
535 number of morphometric, anatomical and ecophysiological traits (e.g. Tognetti *et al.*,
536 1997; Chambel *et al.*, 2007; Climent *et al.*, 2008; Voltas *et al.*, 2008; Esteban *et al.*,
537 2010), pointing to a well-structured array of adaptive responses following the post-
538 glacial long-range colonization of the species to the western Mediterranean basin from
539 which genetic differentiation occurred (Gómez *et al.*, 2005; Grivet *et al.*, 2009). Voltas
540 *et al.* (2008) revealed that there is adaptive variation for improved water use efficiency
541 (WUE) following west to east and north to south gradients. Similarly, Climent *et al.*
542 (2008) reported that variation in allocation patterns to different plant organs also
543 follows comparable gradients within the distribution range of Aleppo pine. Hence,
544 mesic populations from the northern and eastern regions grow taller and allocate fewer
545 resources to reproduction (Climent *et al.*, 2008), are less water conservative (Voltas *et al.*
546 *et al.*, 2008) and concurrently tend to exploit more shallow water at the expense of water
547 taken up from deeper water sources (this work). Conversely, xeric populations
548 originating from the drier conditions of the southern and western regions of the
549 Mediterranean seem better able to exploit deep soil water in the dry season and possibly
550 allocate more resources to the development of a deeper tap root system, regarded as

551 advantageous strategies for withstanding dry periods (Canadell *et al.*, 1996). Since
552 drought events are becoming more frequent in the Mediterranean (IPCC 2014), Aleppo
553 pine trees from arid ecosystems may be shifting their optimal ecological niche (at least
554 in terms of water uptake), perhaps showing high survival rates and enhanced
555 performance in northern and eastern regions that are becoming progressively drier. This
556 mismatch between territory characteristics linked to fitness may be relevant for
557 delineating anticipatory measurements to mitigate climate change impacts on forest
558 functioning and composition (e.g. through assisted migration strategies).

559 The relationships between carbon isotope composition ($\delta^{13}\text{C}$, as reported in Voltas *et al.* [2008] for a subset of 25 populations) and the relative contributions of soil water
560 sources indicated a preferential access to lower soil water of high- $\delta^{13}\text{C}$ (i.e. high WUE)
561 populations in summer (D_2), whereas low- $\delta^{13}\text{C}$ origins extracted relatively more
562 groundwater in autumn (Table 3). Improved WUE and better access to deep soil water
563 (either lower soil or groundwater) are therefore relevant characteristics contributing to
564 the adaptive strategy of xeric populations of Aleppo pine in coping with drought.
565 Although slow growing populations exhibited higher $\delta^{13}\text{C}$ values (Table 3), the absence
566 of negative associations between stem volume and the relative contributions of deep
567 water sources (Table 3) suggests a lack of relevant trade-offs between aboveground
568 growth and water uptake patterns. Rather the opposite, a higher contribution of
569 groundwater was related to a larger stem volume in early autumn. This might imply that
570 intraspecific allocation patterns of dry matter to shoot production (Climent *et al.*, 2008)
571 are largely independent of the distribution of functional roots and the active rooting
572 depth, which are also population-dependent as suggested by xylem water isotopes.

574 In conclusion, this study demonstrates the large plasticity of the root system of
575 Aleppo pine, allowing this species to shift actively among water sources during the
576 year. But, more importantly, it points to the adaptive relevance of resource investment
577 in deep roots as a strategy to withstand dry periods in populations of Aleppo pine
578 originating from particularly harsh, drought-prone habitats. Probably, the relevance of
579 such a selective process towards securing a source of water in dry environments
580 strongly interacted with the imprint of demographic processes on the evolutionary
581 outcome for this species. Indeed, the ecotypic patterns observed in plant water sources
582 showed a good match with the westward expansion through the Mediterranean during
583 post-glacial colonization, which produced a marked loss in genetic diversity but also a

584 strong action of natural selection on populations facing new environmental challenges
585 (Grivet *et al.*, 2009; Grivet *et al.* 2011).

586

587 **Acknowledgements**

588 This work was funded by the Spanish project FENOPIN (AGL2012-40151-C03-03).
589 J.P.F. is supported by the Ramón y Cajal programme (RYC-2008-02050). An earlier
590 version of this work was presented by D.L. to obtain an MSc degree in the Erasmus
591 Mundus Programme MEDfOR. The authors acknowledge Mara Lucà and Pilar Sopena
592 for technical assistance and Javier Rodríguez and Marifé Díaz for providing isotope data
593 from the Red Española de Vigilancia de Isótopos en la Precipitación.

594

595 **References**

- 596 **Achat DL, Bakker MR, Trichet P. 2008.** Rooting patterns and fine root biomass of
 597 *Pinus pinaster* assessed by trench wall and core methods. *Journal of Forest Research*
 598 **13**: 165–175.
- 599 **Alberto FJ, Aitken SN, Alía R, González-Martínez SC, Hänninen H, Kremer A,**
 600 **Lefèvre F, Lenormand T, Yeaman S, Whetten R et al. 2013.** Potential for
 601 evolutionary responses to climate change – evidence from tree populations. *Global*
 602 *Change Biology* **19**: 1645–1661.
- 603 **Barbeta A, Mejía-Chang M, Ogaya R, Voltas J, Dawson TE, Peñuelas J. 2015.** The
 604 combined effects of a long-term experimental drought and an extreme drought on the
 605 use of plant-water sources in a Mediterranean forest. *Global Change Biology*. doi:
 606 10.1111/gcb.12785
- 607 **Barbour MM. 2007.** Stable oxygen isotope composition of plant tissue: a review.
 608 *Functional Plant Biology* **34**: 83–94.
- 609 **Busch DE, Ingraham NL, Smith SD. 1992.** Water uptake in woody riparian
 610 phreatophytes of the southwestern United States: a stable isotope study. *Ecological*
 611 *Applications* **2**: 450–459.
- 612 **Canadell J, Jackson RB, Ehleringer JB, Mooney H, Sala OE, Schulze E-D. 1996.**
 613 Maximum rooting depth of vegetation types at the global scale. *Oecologia* **108**: 583–
 614 595.
- 615 **Capilla JE, Rodríguez-Arevalo J, Castaño S, Díaz-Teijeiro M, Heredia J, Sánchez**
 616 **del Moral R. 2011.** Mapping Oxygen-18 in meteoric precipitation over Peninsular
 617 Spain using geostatistical tools. In AGU Fall Meeting Abstracts (Vol. 1, p. 04).
- 618 **Chambel MR, Climent J, Alía R. 2007.** Divergence among species and populations of
 619 Mediterranean pines biomass allocation of seedlings grown under two watering
 620 regimes. *Annals of Forest Science* **64**: 87–97.
- 621 **Climent J, Prada A, Calama R, Chambel R, Sánchez de Ron D, Alía R. 2008.** To
 622 grow or to seed: ecotypic variation in reproductive allocation and cone production by
 623 young female Aleppo pine (*Pinus halepensis*, *Pinaceae*). *American Journal of*
 624 *Botany* **95**: 833–842.

- 625 **Comas C, del Castillo J, Voltas J, Ferrio JP. 2015.** Point processes statistics of stable
 626 isotopes: analysing water uptake patterns in a mixed stand of Aleppo pine and Holm
 627 oak. *Forest Systems*. doi: 10.5424/fs/2015241-05846 .
- 628 **David TS, Henriques MO, Kurz-Besson C, Nunes J, Valente F, Vaz M, Pereira JS,**
 629 **Siegwolf R, Chaves MM, Gazarini LC *et al.* 2007.** Water-use strategies in two co-
 630 occurring Mediterranean evergreen oaks: surviving the summer drought. *Tree*
 631 *Physiology* **27**: 793–803.
- 632 **Dawson TE, Ehleringer JR. 1991.** Streamside trees that do not use stream water.
 633 *Nature* **350**: 335–337.
- 634 **Dawson TE, Pate JS. 1996.** Seasonal water uptake and movement in root systems of
 635 Australian phreatophytic plants of dimorphic root morphology: a stable isotope
 636 investigation. *Oecologia* **107**: 13–20.
- 637 **Dawson TE, Pausch RC, Parker HM. 1998.** The role of hydrogen and oxygen stable
 638 isotopes in understanding water movement along the soil–plant–atmospheric
 639 continuum. In: Griffiths H, ed. *Stable isotopes: integration of biological, ecological*
 640 *and geochemical processes*. Oxford, UK: Bios Scientific Publishers, 169–183.
- 641 **Eggemeyer KD, Awada T, Harvey FE, Wedin DA, Zhou X, Zanner CW. 2009.**
 642 Seasonal changes in depth of water uptake for encroaching trees *Juniperus*
 643 *virginiana* and *Pinus ponderosa* and two dominant C4 grasses in a semiarid
 644 grassland. *Tree Physiology* **29**: 157–169.
- 645 **Ehleringer JR, Dawson TE. 1992.** Water uptake by plants – perspectives from stable
 646 isotope composition. *Plant, Cell and Environment* **15**: 1073–1082.
- 647 **Ellsworth PZ, Williams DG. 2007.** Hydrogen isotope fractionation during water
 648 uptake by woody xerophytes. *Plant and Soil* **291**: 93–107.
- 649 **Esteban LG, Martín JA, de Palacios P, Fernández FG, López R. 2010.** Adaptive
 650 anatomy of *Pinus halepensis* trees from different Mediterranean environments in
 651 Spain. *Trees – Structure and Function* **24**: 19–30.
- 652 **Filella I, Peñuelas J. 2003a.** Partitioning of water and nitrogen in co-occurring
 653 Mediterranean woody shrub species of different evolutionary history. *Oecologia* **137**:
 654 51–61.
- 655 **Filella I, Peñuelas J. 2003b.** Indications of hydraulic lift by *Pinus halepensis* and its
 656 effects on the water relations of neighbour shrubs. *Biologia Plantarum* **47**: 209–214.

- 657 **Gil L, Díaz-Fernández PM, Jiménez-Sancho MP, Roldán M, Alía R, Agúndez D,**
658 **De Miguel J, Martín S, De Tuero M. 1996.** *Las regiones de procedencia de Pinus*
659 *halepensis Mill. en España.* Madrid, Spain: Ministerio de Medio Ambiente.
- 660 **Gómez A, Vendramin GG, González-Martínez SC, Alía R. 2005.** Genetic diversity
661 and differentiation of two Mediterranean pines (*Pinus halepensis* Mill. and *Pinus*
662 *pinaster* Ait.) along a latitudinal cline using chloroplast microsatellite markers.
663 *Diversity and Distributions* **11**: 257–263.
- 664 **Grivet D, Sebastiani F, González-Martínez SC, Vendramin GG. 2009.** Patterns of
665 polymorphism resulting from long-range colonization in the Mediterranean conifer
666 Aleppo pine. *New Phytologist* **184**: 1016–1028.
- 667 **Grivet D, Sebastiani F, Alía R, Bataillon T, Torre S, Zabal-Aguirre M, Vendramin**
668 **GG, González-Martínez SC. 2011.** Molecular footprints of local adaptation in two
669 Mediterranean conifers. *Molecular Biology and Evolution* **28**: 101–116.
- 670 **Hijmans RJ, Cameron SE, Parra JL, Jones PG, Jarvis A. 2005.** Very high resolution
671 interpolated climate surfaces for global land areas. *International Journal of Climate*
672 **25**: 1965–1978.
- 673 **Instituto Geológico y Minero de España. 2007.** Caracterización hidrogeológica de la
674 unidad hidrogeológica 08.47 (peñón-montgó-bernia). 2º fase. Technical Report.
675 [WWW document] URL
676 http://info.igme.es/SIDIMAGENES/130000/250/130250_0000008.pdf
- 677 **IPCC. 2014.** Pachauri RK, Meyer LA, eds. *Climate Change 2014: Synthesis Report.*
678 *Contribution of Working Groups I, II and III to the Fifth Assessment Report of the*
679 *Intergovernmental Panel on Climate Change.* IPCC, Geneva, Switzerland.
- 680 **Jackson RB, Canadell J, Ehleringer JR, Mooney HA, Sala OA, Schulze ED. 1996.**
681 A global analysis of root distributions for terrestrial biomes. *Oecologia* **108**: 389–
682 411.
- 683 **Klein T, Di Matteo G, Rotenberg E, Cohen S, Yakir D. 2013.** Differential
684 ecophysiological response of a major Mediterranean pine species across a climatic
685 gradient. *Tree Physiology* **33**: 26–36.
- 686 **Klein T, Rotenberg E, Cohen-Hilaleh E, Raz-Yaseef N, Tatarinov F, Preisler Y,**
687 **Ogée G, Cohen S, Yakir D. 2014.** Quantifying transpirable soil water and its
688 relations to tree water use dynamics in a water – limited pine forest. *Ecohydrology* **7**:
689 409–419.

- 690 **Lintunen A, Kallioikoski T. 2010.** The effect of tree architecture on conduit diameter
691 and frequency from small distal roots to branch tips in *Betula pendula*, *Picea abies*
692 and *Pinus sylvestris*. *Tree Physiology* **30**: 1433-1447.
- 693 **Martín-Gómez P, Barbeta A, Voltas J, Peñuelas J, Denis K, Palacio S, Dawson TE,**
694 **Ferrio JP. 2015.** Isotope ratio infrared spectroscopy: a reliable tool for the
695 investigation of plant-water sources? *New Phytologist*. doi: 10.1111/nph.13376.
- 696 **Nambiar EKS. 1990.** Interplay between nutrients, water, root growth and productivity
697 in young plantations. *Forest Ecology and Management* **30**: 213–232.
- 698 **Nambiar EKS, Sands R. 1992.** Effects of compaction and simulated root channels in
699 the subsoil on root development, water uptake and growth of radiata pine. *Tree*
700 *Physiology* **10**: 297–306.
- 701 **Oren R, Sheriff DW. 1995.** Water and nutrient acquisition by roots and canopies. In:
702 Smith WK, Hinckley TM, eds. *Resource physiology of conifers: acquisition,*
703 *allocation and utilization*. London, UK: Academic Press, 34–74.
- 704 **Otieno DO, Kurz-Besson C, Liu J, Schmidt MWT, Vale-Lobo do R, David TS,**
705 **Siegwolf R, Pereira JS, Tenhunen JD. 2006.** Seasonal variations in soil and plant
706 water status in a *Quercus suber* L. stand: roots as determinants of tree productivity
707 and survival in the Mediterranean-type ecosystem. *Plant and Soil* **283**: 119–135.
- 708 **Palacio S, Azorín J, Montserrat-Martí G, Ferrio JP. 2014.** The crystallization water
709 of gypsum rocks is a relevant water source for plants. *Nature Communications* **5**,
710 Art. no. 4660.
- 711 **Parnell AC, Inger R, Bearhop S, Jackson AL. 2010.** Source partitioning using stable
712 isotopes: coping with too much variation. *PloS One* **5**: e9672.
- 713 **Park Williams A, Allen CD, Macalady AK, Griffin D, Woodhouse CA, Meko DM,**
714 **Swetnam TW, Rauscher SA, Seager R, Grissino-Mayer HD et al. 2012.**
715 Temperature as a potent driver of regional forest drought stress and tree mortality.
716 *Nature Climate Change* **3**: 292-297.
- 717 **Querejeta JI, Roldán A, Albaladejo J, Castillo V. 2001.** Soil water availability
718 improved by site preparation in a *Pinus halepensis* afforestation under semiarid
719 climate. *Forest Ecology and Management* **149**: 115–128.
- 720 **Raz-Yaseef N, Koteen L, Baldocchi DD. 2012.** Coarse root distribution of a semi-arid
721 oak savanna estimated with ground penetrating radar. *Journal of Geophysical*
722 *Research: Biogeosciences* **118**: 1-13.

- 723 **Reinhardt E, Scott J, Gray K, Keane R. 2006.** Estimating canopy fuel characteristics
 724 in five conifer stands in the western United States using tree and stand
 725 measurements. *Canadian Journal of Forest Research* **36**: 2803–2814.
- 726 **Retzlaff WA, Blaisdell GK, Topa MA. 2001.** Seasonal changes in water source of four
 727 families of loblolly pine (*Pinus taeda* L.). *Trees – Structure and Function* **15**: 154–
 728 162.
- 729 **Rossatto DR, de Carvalho Ramos Silva L, Villalobos-Vega R, Sternberg LDSL,
 730 Franco AC. 2012.** Depth of water uptake in woody plants relates to groundwater
 731 level and vegetation structure along a topographic gradient in a neotropical savanna.
 732 *Environmental and Experimental Botany* **77**: 259–266.
- 733 **Rutter MT, Fenster CB. 2007.** Testing for adaptation to climate in *Arabidopsis*
 734 *thaliana*: a calibrated common garden approach. *Annals of Botany* **99**: 529–536.
- 735 **Snyder KA, Williams DG. 2000.** Water sources used by riparian trees varies among
 736 stream types on the San Pedro River, Arizona. *Agricultural and Forest Meteorology*
 737 **105**: 227–240.
- 738 **Tapias R, Climent J, Pardos JA, Gil L. 2004.** Life histories of Mediterranean pines.
 739 *Plant Ecology* **171**: 53–68.
- 740 **Tognetti R, Michelozzi M, Giovannelli A. 1997.** Geographical variation in water
 741 relations, hydraulic architecture and terpene composition of Aleppo pine seedlings
 742 from Italian provenances. *Tree Physiology* **17**: 241–250.
- 743 **Valentini R, Scarascia Mugnozza GE, Ehleringer JR. 1992.** Hydrogen and carbon
 744 isotope ratios of selected species of a Mediterranean macchia ecosystem. *Functional*
 745 *Ecology* **6**: 627–631.
- 746 **Voltas J, Chambel MR, Prada MA, Ferrio JP. 2008.** Climate-related variability in
 747 carbon and oxygen stable isotopes among populations of Aleppo pine grown in
 748 common-garden tests. *Trees – Structure and Function* **22**: 759–769.
- 749 **Wershaw RL, Friedman I, Heller SJ, Frank PA. 1966.** Hydrogen isotope
 750 fractionation of water passing through trees. In: Hobson GD, ed. *Advances in organic*
 751 *geochemistry*. Oxford, UK: Pergamon Press, 55–67.
- 752 **West AG, Goldsmith GR, Matimati I, Dawson TE. 2011.** Spectral analysis software
 753 improves confidence in plant and soil water stable isotope analyses performed by
 754 isotope ratio infrared spectroscopy (IRIS). *Rapid Communications in Mass*
 755 *Spectrometry* **25**: 2268–74.

- 756 **West AG, Dawson TE, February EC, Midgley GF, Bond WJ, Aston TL. 2012.**
757 Diverse functional responses to drought in a Mediterranean-type shrubland in South
758 Africa. *New Phytologist* **195**: 396–407.
- 759 **Williams ER, Matheson AC, Harwood CE. 2002.** *Experimental Design and Analysis*
760 *for Tree Improvement*. Collingwood, Australia: CSIRO.
- 761 **Zhang C, Zhang J, Zhao B, Zhu A, Zhang H, Huang P, Li X. 2011.** Coupling a two-
762 tip linear mixing model with a δD - $\delta^{18}O$ plot to determine water sources consumed by
763 maize during growth stages. *Field Crops Research* **123**: 196–205.

For Peer Review

764 **FIGURE LEGENDS**

765

766 **Fig. 1** Geographical layout of the natural distribution range of Aleppo pine,
 767 encompassing the 56 populations (black dots) and 16 ecological regions (abbreviation
 768 [codes in Table S1] and borderline) used in this study. The arrow points to the location
 769 of the trial site, accompanied by a climograph of 2010 (data from the meteorological
 770 station of Segorbe, 39°50'53''N, 00°29'05''W, 335 m a.s.l., *ca.* 10 km from the study
 771 site). The vertical lines in the climograph indicate field sampling dates: 2 June (D₁), 28
 772 July (D₂) and 27 September (D₃).

773

774 **Fig. 2** Best linear unbiased estimates (BLUEs) of xylem water isotopic values ($\delta^{18}\text{O}$ and
 775 $\delta^2\text{H}$) of populations (circles) and ecological regions (insets; codes in Table S1) together
 776 with isotopic values of upper (triangle up) and lower (triangle down) soil layers and
 777 groundwater (square) and their standard errors for three sampling days: (a) 2 June (D₁),
 778 (b) 28 July (D₂) and (c) 27 September (D₃). The global meteoric water line (dashed-line)
 779 and the isotopic soil line (solid line) are also included in the panels.

780

781 **Fig. 3** Relative mean contribution across populations (percentage \pm standard deviation)
 782 of soil water extracted by the trees on each of three sampling days (D₁: 2 June; D₂: 28
 783 July 28; D₃: 27 September) from three plant water sources obtained by SIAR Bayesian
 784 mixing models: upper soil layer at 0–15 cm, lower soil layer at 15–40 cm and
 785 groundwater (below 40 cm).

786

787 **Fig. 4** Ternary plot suggesting changes in active rooting depths of 16 ecological regions
 788 (ecotypes) of Aleppo pine for two sampling dates (D₂: 28 July [red numbers]; D₃: 27
 789 September [blue numbers]) as indicated by the relative contributions of three plant
 790 water sources: upper soil layer (0–15 cm), lower soil layer (15–40 cm) and groundwater
 791 (>40 cm). Code numbers of ecological regions are in Table S1.

792

793 Table 1. Mixed model analysis of variance for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ in xylem water for three
 794 sampling days (D₁, D₂ and D₃) and 56 populations of Aleppo pine grown in a common
 795 garden test. Only fixed effects of the model are shown in the table.

D ₁ (June 2 nd)			$\delta^{18}\text{O}$ (‰)		$\delta^2\text{H}$ (‰)	
Source of variation	Num <i>df</i>	Den <i>df</i>	<i>F</i> -value	Prob > <i>F</i>	<i>F</i> -value	Prob > <i>F</i>
Column	6	12	1.11	0.6583	0.47	0.8323
Block	2	12	1.49	0.1296	1.60	0.2020
Population (P)	55	42	0.85	0.6852	0.96	0.5513
Region (Reg)	15	42	0.62	0.8576	0.87	0.5970
P × Reg	40	42	0.94	0.5779	0.98	0.4972
D ₂ (July 28 th)			$\delta^{18}\text{O}$ (‰)		$\delta^2\text{H}$ (‰)	
Source of variation	Num <i>df</i>	Den <i>df</i>	<i>F</i> -value	Prob > <i>F</i>	<i>F</i> -value	Prob > <i>F</i>
Column	6	12	0.37	0.8981	1.06	0.3847
Block	2	12	2.77	0.0628	2.49	0.0832
Population (P)	53	57	1.95	<0.0001	1.39	0.0325
Region (Reg)	15	57	2.80	0.0002	1.89	0.0197
P × Reg	38	57	1.57	0.0139	1.14	0.2493
D ₃ (September 27 th)			$\delta^{18}\text{O}$ (‰)		$\delta^2\text{H}$ (‰)	
Source of variation	Num <i>df</i>	Den <i>df</i>	<i>F</i> -value	Prob > <i>F</i>	<i>F</i> -value	Prob > <i>F</i>
Column	6	12	0.89	0.5030	1.43	0.1975
Block	2	12	3.71	0.0246	1.76	0.1724
Population (P)	55	61	1.51	0.0086	1.32	0.0576
Region (Reg)	15	61	1.91	0.0176	1.62	0.0592
P × Reg	40	61	1.32	0.0841	1.14	0.2447

796 Table 2. Mixed model analysis of variance for soil water isotopic data ($\delta^{18}\text{O}$ and $\delta^2\text{H}$)
 797 from a common garden test of Aleppo pine. Only fixed effects of the model are shown
 798 in the table.

Source of variation	Num <i>df</i>	Den <i>df</i>	$\delta^{18}\text{O}$ (‰)		$\delta^2\text{H}$ (‰)	
			<i>F</i> -value	Prob > <i>F</i>	<i>F</i> -value	Prob > <i>F</i>
Column	6	99	2.70	0.0180	3.35	0.0048
Block	2	3	0.04	0.9606	0.61	0.3001
Soil layer (Layer)	1	99	76.64	<0.0001	57.05	<0.0001
Sampling day (Day)	2	99	9.95	0.0001	13.98	<0.0001
Layer \times Day	2	99	3.42	0.0368	0.85	0.4289

For Peer Review

799 Table 3. Correlation coefficients (and associated probabilities) for the contributions of
 800 plant water sources at the population level in peak summer (D₂) and early autumn (D₃)
 801 and a number of eco-geographical variables at origin, the stem volume (Vob) and the
 802 carbon isotope composition ($\delta^{13}\text{C}$) (extracted from Voltas *et al.*, 2008) of 56 populations
 803 of Aleppo pine. Correlations with eco-geographical variables do not include populations
 804 of uncertain origin (reforestations of the Spanish northern plateau and Amphipholia; see
 805 Table S1). Significant correlations at the 5 % level are marked in bold.

	Sampling D ₂			Sampling D ₃			Stem volume (Vob)
	Upper soil	Lower soil	Groundwater	Upper soil	Lower soil	Groundwater	
Latitude	0.293 (0.041)	-0.271 (0.059)	0.532 (0.716)	0.167 (0.241)	-0.209 (0.141)	0.134 (0.347)	0.297 (0.033)
Longitude	0.223 (0.123)	-0.184 (0.205)	-0.011 (0.938)	0.321 (0.021)	-0.275 (0.050)	0.053 (0.780)	0.266 (0.059)
Altitude	-0.128 (0.380)	0.087 (0.549)	0.047 (0.743)	-0.100 (0.483)	0.119 (0.431)	-0.059 (0.677)	-0.098 (0.494)
Annual Mean Temperature	-0.019 (0.892)	0.029 (0.838)	-0.030 (0.839)	0.092 (0.520)	-0.061 (0.668)	-0.013 (0.927)	-0.018 (0.900)
Max. Temperature of Warmest Month	-0.167 (0.251)	0.111 (0.446)	0.069 (0.636)	0.099 (0.488)	-0.017 (0.904)	-0.092 (0.518)	-0.295 (0.035)
Min. Temperature of Coldest Month	0.056 (0.699)	-0.005 (0.977)	-0.099 (0.499)	0.070 (0.624)	-0.040 (0.781)	-0.020 (0.884)	0.050 (0.727)
Annual Precipitation	0.131 (0.371)	0.012 (0.936)	-0.281 (0.051)	-0.074 (0.605)	0.077 (0.591)	-0.033 (0.813)	-0.074 (0.605)
Precipitation of Wettest Month	0.051 (0.728)	0.103 (0.486)	-0.333 (0.019)	-0.106 (0.461)	0.129 (0.365)	-0.080 (0.575)	-0.082 (0.567)
Precipitation of Driest Month	0.220 (0.129)	-0.263 (0.068)	0.176 (0.226)	0.103 (0.471)	-0.131 (0.359)	0.086 (0.548)	0.207 (0.145)
Geographical Distance	0.192 (0.186)	-0.164 (0.259)	-0.002 (0.984)	0.354 (0.011)	-0.290 (0.039)	0.037 (0.793)	0.226 (0.111)
Gower's Distance	-0.296 (0.039)	0.316 (0.027)	-0.152 (0.298)	0.054 (0.706)	0.050 (0.726)	-0.147 (0.304)	-0.311 (0.026)
Vob	0.204 (0.139)	-0.220 (0.109)	0.110 (0.427)	0.002 (0.986)	-0.179 (0.186)	0.281 (0.036)	-
$\delta^{13}\text{C}$	-0.514 (0.012)	0.558 (0.006)	-0.343 (0.108)	-0.108 (0.609)	0.382 (0.059)	-0.515 (0.008)	-0.499 (0.011)
n= 49 (51) for correlations between D ₂ (D ₃ , Vob) and eco-geographical variables							
n= 54 (56) for correlations between D ₂ (D ₃) and Vob							
n= 23 (25) for correlations between D ₂ (D ₃ , Vob) and $\delta^{13}\text{C}$							

806

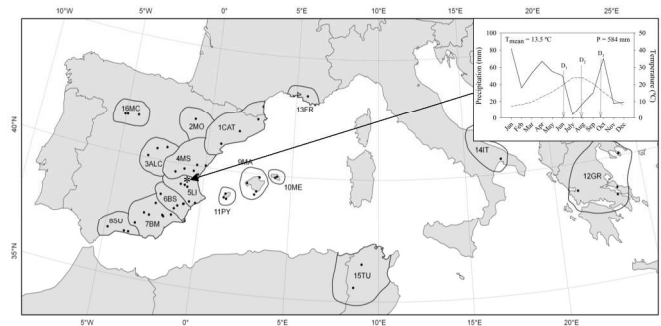


Fig. 1 Geographical layout of the natural distribution range of Aleppo pine, encompassing the 56 populations (black dots) and 16 ecological regions (abbreviation [codes in Table S1] and borderline) used in this study.

The arrow points to the location of the trial site, accompanied by a climograph of 2010 (data from the meteorological station of Segorbe, 39°50'53"N, 00°29'05"W, 335 m a.s.l., ca. 10 km from the study site).

The vertical lines in the climograph indicate field sampling dates: 2 June (D1), 28 July (D2) and 27 September (D3).

254x190mm (300 x 300 DPI)

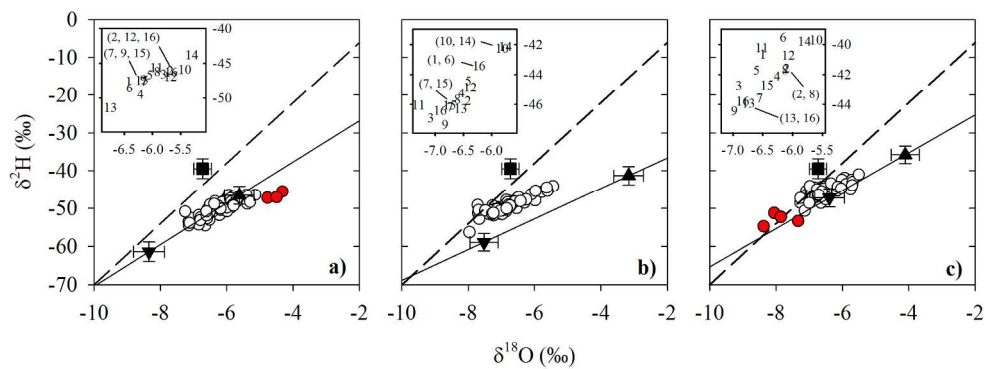


Fig. 2 Best linear unbiased estimates (BLUES) of xylem water isotopic values ($\delta^{18}\text{O}$ and $\delta^2\text{H}$) of populations (circles) and ecological regions (insets; codes in Table S1) together with isotopic values of upper (triangle up) and lower (triangle down) soil layers and groundwater (square) and their standard errors for three sampling days: (a) 2 June (D1), (b) 28 July (D2) and (c) 27 September (D3). The global meteoric water line (dashed-line) and the isotopic soil line (solid line) are also included in the panels.
226x114mm (300 x 300 DPI)

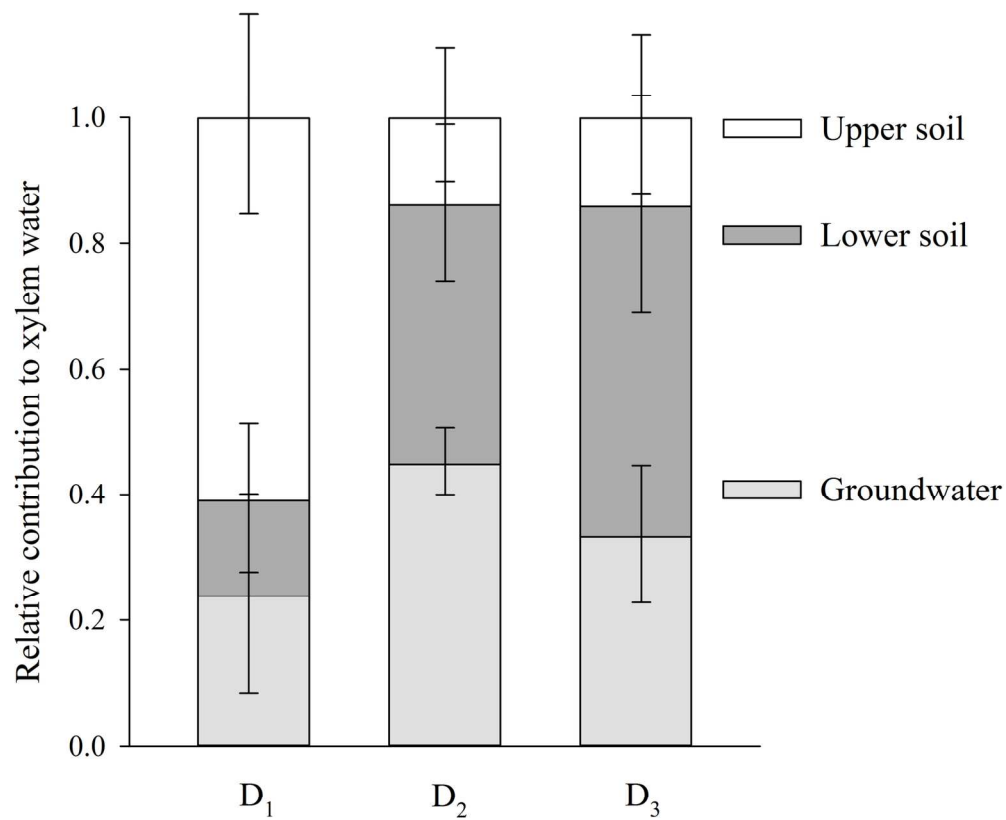


Fig. 3 Relative mean contribution across populations (percentage \pm standard deviation) of soil water extracted by the trees on each of three sampling days (D1: 2 June; D2: 28 July 28; D3: 27 September) from three plant water sources obtained by SIAR Bayesian mixing models: upper soil layer at 0–15 cm, lower soil layer at 15–40 cm and groundwater (below 40 cm).
156x172mm (300 x 300 DPI)

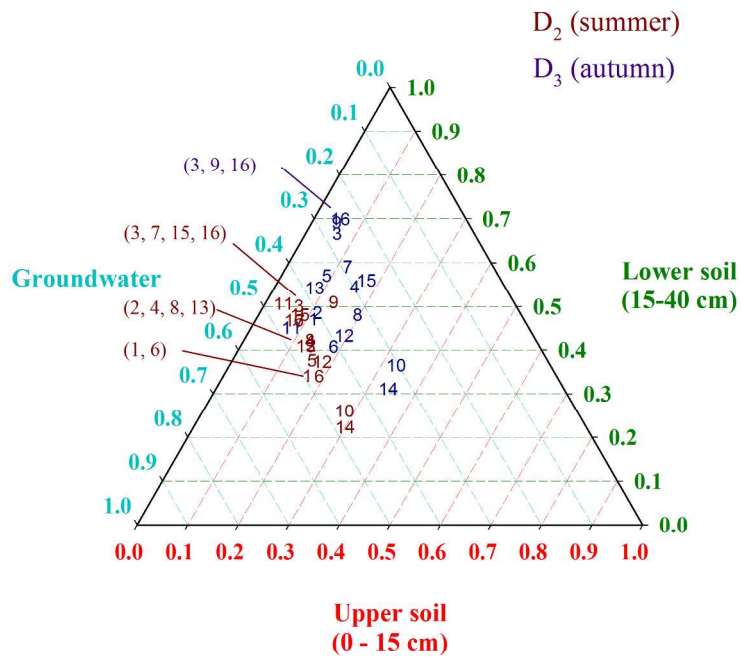


Fig. 4 Ternary plot suggesting changes in active rooting depths of 16 ecological regions (ecotypes) of Aleppo pine for two sampling dates (D₂: 28 July [red numbers]; D₃: 27 September [blue numbers]) as indicated by the relative contributions of three plant water sources: upper soil layer (0–15 cm), lower soil layer (15–40 cm) and groundwater (>40 cm). Code numbers of ecological regions are in Table S1.

209x296mm (300 x 300 DPI)