

Intraspecific variation in the use of water sources by the circum-Mediterranean conifer *Pinus halepensis*

Jordi Voltas¹, Devon Lucabaugh¹, Maria Regina Chambel² and Juan Pedro Ferrio¹

¹Department of Crop and Forest Sciences – AGROTECNIO Center, University of Lleida, Rovira Roure 191, Lleida E-25198, Spain; ²CIFOR-INIA, Ctra.de La Coruña km 7.5, E-28040 Madrid, Spain

Author for correspondence:

Jordi Voltas

Tel: +34 973 702855

Email: jvoltas@pvcf.udl.es

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Summary

- The relevance of interspecific variation in the use of plant water sources has been recognized in drought-prone environments. By contrast, the characterization of intraspecific differences in water uptake patterns remains elusive, although preferential access to particular soil layers may be an important adaptive response for species along aridity gradients.
- Stable water isotopes were analysed in soil and xylem samples of 56 populations of the drought-avoidant conifer *Pinus halepensis* grown in a common garden test.
- We found that most populations reverted to deep soil layers as the main plant water source during seasonal summer droughts. More specifically, we detected a clear geographical differentiation among populations in water uptake patterns even under relatively mild drought conditions (early autumn), with populations originating from more arid regions taking up more water from deep soil layers. However, the preferential access to deep soil water was largely independent of aboveground growth.
- Our findings highlight the high plasticity and adaptive relevance of the differential access to soil water pools among Aleppo pine populations. The observed ecotypic patterns point to the adaptive relevance of resource investment in deep roots as a strategy towards securing a source of water in dry environments for *P. halepensis*.

Introduction

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

Stable water isotopes can also be used to determine taxonomic differences in the source(s) of plant water within the soil profile. Although the relevance of interspecific variation in the use of different water sources has often been examined in dry ecosystems (Dawson *et al.*, 1998; Barbour, 2007; West *et al.*, 2012; Comas *et al.*, 2015), the characterization of genetic differences in water uptake patterns at the intraspecific level remains elusive. To date, we are only aware of a single study examining genetic differences in water uptake patterns, through the use of stable isotopes of water in genetically contrasting maize (*Zea mays*) material (Zhang

et al., 2011). Nonetheless, preferential access to particular soil layers may be an important effect of adaptive divergence for populations of xeric species, as this trait may be linked to the array of evolutionary responses to varying environmental conditions often observed within a species' distribution range (Alberto *et al.*, 2013). Because the unprecedented intensity and duration of future drought events are expected to impact negatively on the structure and function of forest ecosystems (Park Williams *et al.*, 2012), the assessment of intraspecific differences in access to deep water pools may be relevant for understanding the impact of warming on contemporary populations and for tailoring mitigation strategies to climate change.

The circum-Mediterranean conifer Aleppo pine (*Pinus halepensis*) is the most widely distributed tree species in the Mediterranean basin, where it can be found under a varied range of thermal and moisture conditions. The adaptive relevance of drought stress in shaping the populations' genetic structure has been highlighted in previous studies for traits such as biomass allocation (Chambel *et al.*, 2007; Climent *et al.*, 2008), intrinsic water use efficiency (WUE; Voltas *et al.*, 2008), wood anatomy (Esteban *et al.*, 2010) and vulnerability to xylem embolism (Klein *et al.*, 2013). These studies have shown that populations thriving in dry environments exhibit different traits from their counterparts originating from mesic areas.

We hypothesize that Aleppo pine has adapted to the recurrent drought episodes typical of the Mediterranean basin by

producing and maintaining a deep root system that is dispersed into many layers of the soil profile. This adaptive characteristic may allow trees to respond to precipitation pulses that eventually recharge the upper soil layers in spring or autumn. Conversely, when the surface soil dries up, trees may revert to deeper soil layers for water use. However, we also consider that such performance may be fine-tuned to the environment as a consequence of the disparity of selective pressures for water use experienced by this species at the ecotypic level. Therefore, this study pursued the following objectives: to determine the main sources of water and their seasonal variation for a widespread conifer (*P. halepensis*); to characterize differential changes in the use of water sources over time at the intraspecific level (i.e. differential phenotypic plasticity) for a highly representative set of seed sources covering most of the present range of geographical distribution of this species; to clarify whether the observed variability in phenotypic plasticity for plant water sources is of adaptive relevance in relation to the diversity of life history strategies and environmental conditions where this species can be found.

Materials and Methods

Plant material

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

For each seed source, climate variables at origin were obtained for the period 1960–1990 from the Global Climatic Model WorldClim database (Hijmans *et al.*, 2005), implemented in

DIVA-GIS with a spatial resolution of 1 km². These variables were chosen based on previous identification of climate drivers of ecotypic variation in Mediterranean pines (Tapias *et al.*, 2004; Climent *et al.*, 2008) and included annual mean temperature (T), maximum temperature of the warmest month (T_{\max}), minimum temperature of the coldest month (T_{\min}), temperature annual range ($T_{\max} - T_{\min}$; TAR), annual precipitation (P) and annual summer precipitation (P_s).

Study site

One of the original six experimental sites located in Altura (39°49'29"N, 00°34'22"W, 640 m above sea level; Castellón province, eastern Spain) was used in this study (Fig. 1). We selected this trial as representative of the average conditions in which the species can be found in the Mediterranean (Gil *et al.*, 1996). It has a mean annual precipitation of 652 mm, of which 19% falls in summer, a mean annual temperature of 13.8°C and a mean annual potential evapotranspiration of 1115 mm. These features, together with a low soil water retention capacity, indicate that trees often suffer from summer drought stress. The trial was installed on a reforestation site with a 5% slope and a southeast aspect. Site preparation included tilling with a disc ripper before planting. The soil is a calcic cambisol with a maximum depth of *c.* 40 cm and a very gravelly and cemented underlying horizon of CaCO₃ accumulation. The seedlings (16 per population) were planted systematically (2.5 × 2.5 m spacing) in four replicates consisting of four-tree line plots following a latinized row-column design for a total of 896 test seedlings. This layout is suited to experiments that include a large number of treatments, as it provides an effective control of field variation in two directions, allowing adjustment for field trend using rows and columns along with an extra blocking facility for contiguous replicates (Williams *et al.*, 2002).

Data collection

The field data were collected in 2010, a 10% drier (less rainy) than average year, on three different days representing contrasting situations in terms of water shortage, in accordance with the

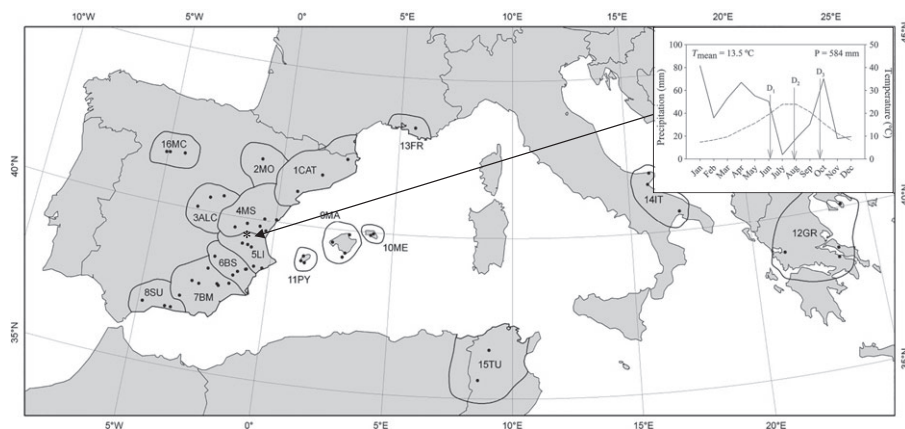


Fig. 1 Geographical layout of the natural distribution range of Aleppo pine (*Pinus halepensis*), encompassing the 56 populations (black dots) and 16 ecological regions (codes in Supporting Information Table S1) used in this study. The arrow points to the location of the trial site, accompanied by a climograph for 2010 (data from the meteorological station of Segorbe; 39°50'53"N, 00°29'05"W, 335 m above sea level, *c.* 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).

seasonal regime of precipitation typical of the western Mediterranean (Fig. 1): 2 June (end of spring, wet), 28 July (middle of summer, dry) and 27 September (early autumn, drought recovery transition from dry to wet). These are referred to as D₁, D₂ and D₃, respectively. On each day, xylem samples were obtained from 11:00 to 13:00 h solar time from rows of the experimental layout following a zigzag pattern. Samples were taken from healthy, east-facing branches of *c.* 1.5 cm diameter in the top third of the crown using telescopic loppers. Shoot segments (*c.* 5 cm length) were bark-peeled, placed immediately into glass vials and frozen in dry ice to prevent evaporation. Out of the total of four replicates, samples were taken from the first consecutive three to allow the collection of samples under uniform environmental conditions. Furthermore, three trees were selected from every four-tree line plot in each replicate based on phenotypic similarity (height and diameter at breast height (DBH)). This was done to avoid the potentially large biasing effects of nonrepresentative trees on the average isotopic records obtained per plot. Therefore, nine trees per population (i.e. three trees per plot) were monitored at each sampling date for a total of 504 sampled trees.

Soil samples were collected for the same days at two depth ranges (0–15 cm and 15–40 cm) using a straight tube probe thoroughly cleaned between consecutive samplings. From 07:00 to 09:00 h solar time, samples were taken from soil pits dug in the middle of the row of four-tree line plots selected systematically in the field (spaced 25 m within rows and 20 m within columns, following a staggered pattern). The aim of this systematic arrangement was to account for the spatial variation in the isotopic signatures of soil water, while keeping the number of soil samples to be taken during sampling days within reasonable limits. In particular, 42 samples were taken on each sampling day corresponding to two different soil layers, for a total of 126 soil samples. The soil extracted was placed quickly into glass vials and frozen in dry ice. All samples were kept frozen until processing and analysis. Growth data (height and diameter at 1.30 m) were recorded for each tree in April 2010 (at age 14 years). Stem volume over bark (Vob) was used as a surrogate for total standing biomass (Reinhardt *et al.*, 2006). Vob was calculated using the following equation, assuming the stem to be conical (Climent *et al.*, 2008):

$$\text{Vob} = (\Pi/12) \times D^2 \times H \quad \text{Eqn 1}$$

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

Isotopic analysis

Xylem and soil water was extracted by cryogenic vacuum distillation (Otieno *et al.*, 2006). Before extraction, the xylem samples of the same plot were pooled. Therefore, 168 isotopic determinations (56 populations \times three replicates) were performed per sampling date for xylem water. Sample tubes were placed in a heated silicone oil bath (110–120°C), and connected with Ultra-Torr unions (Swagelok Co., Solon, OH, USA) to a vacuum system (*c.* 10^{−2} mbar) including *U*-shaped water traps in series that were cooled with liquid N₂. The extraction time was 90 min for

xylem and 120 min for soil samples. Captured water was then transferred into cap-crimp 2-ml vials, and stored at 4°C until analysis.

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

Residual organic contaminants in the distilled water can interfere with the analysis of plant and soil samples conducted with IRIS (West *et al.*, 2011; Martín-Gómez *et al.*, 2015). The presence of contaminants was checked using Picarro Inc.'s CHEMCORRECT post-processing software. Approximately 50% of the xylem samples were flagged as potentially contaminated by the CHEMCORRECT software, whereas only two soil samples were flagged. Although the estimated concentration of contaminants for flagged samples was very small (the maximum difference from pure water standards was $< 7 \times 10^{-5}$ methanol units, equivalent to 0.01% MeOH, and < 65 'other' units, equivalent to 0.25% ethanol), we applied the post-processing correction described in Martín-Gómez *et al.* (2015). Overall, there were only small differences between corrected and noncorrected values, but the precision of population and soil layer mean estimates was slightly improved using the corrected values. A small subset including xylem and soil samples was also analysed by isotope-ratio mass spectrometry for $\delta^{18}\text{O}$, showing a very good agreement with IRIS values even before correction ($r^2 = 0.993$, RMSE = 0.230; $n = 10$).

Statistical analysis

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

The population effect in the ANOVA was further partitioned into two fixed terms accounting for variation among the 16 ecological regions, and among populations within ecological region. For D₂, only 54 populations were available for comparison because of problems encountered during the water extraction process, which led to highly deviating isotopic values for

populations 21 and 142. The stem volume for 2010 was also subjected to mixed model ANOVA following the same partitioning of effects. However, in this case an extra random term was included accounting for between-tree variation. Tests of fixed effects were performed using Wald-type F -statistics and the estimation of variance components through restricted maximum likelihood. Best linear unbiased estimates (BLUES) of population and ecotypic effects were obtained for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ of xylem water and stem volume.

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

The relative contributions of different sources to xylem water were estimated by Bayesian mixing modelling using the Stable Isotope Analysis in R (SIAR) package (Parnell *et al.*, 2010). Stable isotope mixing models are used extensively for studying food webs, but can also be applied to the determination of plant water sources (Palacio *et al.*, 2014; Barbeta *et al.*, 2015). We considered three different sources of soil water: two soil depths (0–15 cm and 15–40 cm) and groundwater reservoirs remaining in cracks and fissures within the calcic horizon, protected from evaporation. Hence, BLUES for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ of the upper and lower soil layers were used as input in the mixing model for each sampling day. For groundwater, three alternative estimates were tested: the weighted average of monthly isotopic signatures of precipitation from October to April (considered as the soil recharge period in the Mediterranean) over the period 2000–2010 for Valencia, the nearest site (*c.* 40 km) of the Spanish Network of Isotopes in Precipitation (REVIP; Capilla *et al.*, 2011); the outcome of systematic sampling of the aquifer system in the Júcar catchment, eastern Spain, covering the period 1974–2006 (Instituto Geológico y Minero de España, 2007); the average of samples of water collected from three nearby fountains (natural springs). The estimates were similar across methods: -6.2‰ and -39.4‰ (method 1), -6.5‰ and -35.6‰ (method 2), and -6.7‰ and -39.5‰ (method 3) for $\delta^{18}\text{O}$ and $\delta^2\text{H}$, respectively. They also yielded very similar relative contributions of plant water use at the population level through the Bayesian mixing models. In particular, the mean correlation between relative contributions of water sources for populations was 0.99 for upper soil, 0.99 for lower soil and 0.98 for groundwater on D₂. On D₃, the correlations were 0.99 (upper soil), 0.96 (lower soil) and 0.86 (groundwater). We present here the results obtained using records of spring water (method 3).

As target values ('consumers') in SIAR, we used the BLUES of $\delta^{18}\text{O}$ and $\delta^2\text{H}$ of xylem water at the population level. The

trophic enrichment factor (TER) was set to 0, assuming no fractionation during water uptake (Ehleringer & Dawson, 1992). We ran 500 000 iterations separately for the isotopic values of each population on each sampling day, discarding the first 50 000 and thinning by a factor of 45, thereby obtaining the most likely contribution (mean of the posterior distribution of the Markov chain Monte Carlo simulation) of each source for every population and sampling day. This analysis evaluated differential patterns of active root zones both at different temporal periods and among populations during the growing season.

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

$$\text{GD} = \frac{1}{p} \sum_{i=1}^p \frac{|A_i - B_i|}{r_i} \quad \text{Eqn 2}$$

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

Results

Stable isotopes in xylem water

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

For $\delta^{18}\text{O}$, populations ranged from -8.0‰ (population 184) to -5.4‰ (population 103), and from -8.4‰ (population 156) to -5.5‰ (population 233) for D₂ and D₃, respectively. For $\delta^2\text{H}$, populations varied from -56.3‰ (population 184) to -44.1‰ (population 103), and from -54.7‰ (population 156) to -41.0‰ (population 92) for D₂ and D₃, respectively. The

Table 1 Mixed model analysis of variance for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ in xylem water for three sampling days (D_1 , D_2 and D_3) and 56 populations of Aleppo pine (*Pinus halepensis*) grown in a common garden test

Source of variation	Numerator df	Denominator df	$\delta^{18}\text{O}$ (‰)		$\delta^2\text{H}$ (‰)	
			F-value	P > F	F-value	P > F
D_1 (2 June)						
Column	6	12	1.11	0.3522	0.47	0.8323
Block	2	12	1.49	0.2263	1.60	0.2020
Population (P)	55	42	0.85	0.7704	0.96	0.5513
Region (Reg)	15	42	0.62	0.8576	0.87	0.5970
P \times Reg	40	42	0.94	0.5779	0.98	0.4972
D_2 (28 July)						
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 \times Reg	38	57	1.57	0.0139	1.14	0.2493
D_3 (27 September)						
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 \times Reg	40	61	1.32	0.0841	1.14	0.2447

Only fixed effects of the model are shown in the table.

Table 2 Mixed model analysis of variance for soil water isotopic data ($\delta^{18}\text{O}$ and $\delta^2\text{H}$) from a common garden test of Aleppo pine (*Pinus halepensis*)

Source of variation	Numerator df	Denominator df	$\delta^{18}\text{O}$ (‰)		$\delta^2\text{H}$ (‰)	
			F-value	P > F	F-value	P > F
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

Only fixed effects of the model are shown in the table.

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

Stable isotopes in soil water

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

Contribution of water sources to genotypic differences in water extraction patterns

BLUEs of population means for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ were plotted for each sampling day (Fig. 2). BLUEs of each soil layer were also included for the sake of comparison, suggesting active rooting zones of populations. We also added the global meteoric water line (GMWL) and the estimated values ($\delta^{18}\text{O}$ and $\delta^2\text{H}$) of groundwater taken from records of spring water. For D_1 , when precipitation was abundant, the isotopic xylem water values representing each of the 56 populations clustered around the mean isotopic values of the top soil layer and fell along the isotopic soil line. For D_2 , the cluster of population means of isotopic xylem

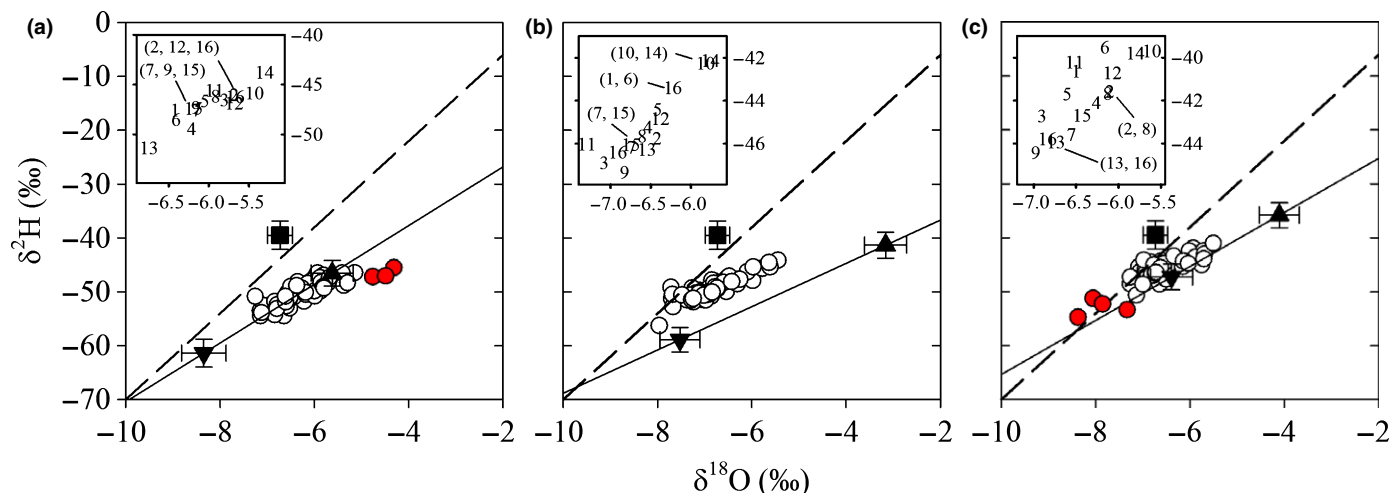


Fig. 2 Best linear unbiased estimates (BLUEs) of xylem water isotopic values ($\delta^{18}\text{O}$ and $\delta^2\text{H}$) of populations (circles) and ecological regions (insets; codes in Supporting Information Table S1) of Aleppo pine (*Pinus halepensis*) together with isotopic values of upper (upward-pointing triangle) and lower (downward-pointing triangle) soil layers and groundwater (square) and their SE for three sampling days: (a) 2 June (D_1), (b) 28 July (D_2) and (c) 27 September (D_3). The red circles indicate populations with mean values outside the range of isotopic values of the three water sources. The global meteoric water line (dashed line) and the isotopic soil line (solid line) are also included in the panels.

water values was located along the GMWL and well apart from the line drawn from the isotopic soil values, although the population means were closer to the lower soil layer $\delta^{18}\text{O}$ value than for D_1 . The data collected on D_2 represent the response of Aleppo pines to the lowest precipitation levels, highest temperatures and – probably – the least soil water availability experienced in 2010 at the site (Fig. 1, inset). Finally, for D_3 the situation was in between that observed for D_1 and D_2 ; that is, the populations were again placed along the GMWL, but closer to the isotopic soil line than for D_2 and around the lower soil layer $\delta^{18}\text{O}$ and $\delta^2\text{H}$ values. The outstanding ecotypic structure of the isotopic signals can be observed in Fig. 2 (insets).

Although most population means were within the range of isotopic values of the three water sources used to characterize water uptake patterns, a few populations fell outside their limits (using 95% confidence intervals for means of water sources; mean \pm SE \times 1.96). This was noticeable for D_1 (populations 186, 202 and 233) and D_3 (populations 82, 156, 184 and 203) (Fig. 2, red dots), hence suggesting an incomplete characterization of water sources. For D_1 , the high isotopic values of this population subset pointed to a source of highly evaporated (superficial) water available during the rainy spring. For D_3 , the low values attained by these atypical populations might indicate the existence of a deeper water source below the actual sampled soil. Deletion of these potential outliers did not change substantially the results of the ANOVA for D_1 , but for D_3 the population and ecological region effects became only marginally significant for $\delta^{18}\text{O}$ ($P=0.091$ and $P=0.074$, respectively) and nonsignificant for $\delta^2\text{H}$ ($P=0.444$ and $P=0.371$, respectively).

The relative contribution of each water source (upper and lower soil layers and groundwater) to tree water consumption averaged over populations is shown in Fig. 3 for each sampling day. The mixing models revealed that trees were able to extract water simultaneously from the three water pools, but in varying

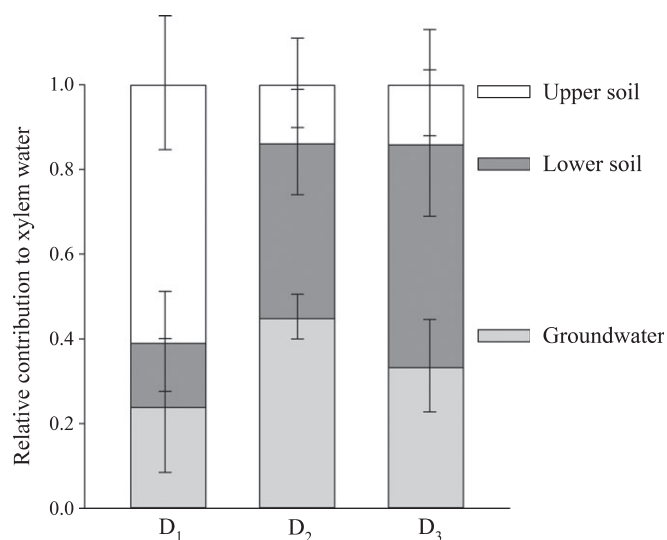


Fig. 3 Relative mean contribution across Aleppo pine (*Pinus halepensis*) populations (percentage \pm SD) of soil water extracted by the trees on each of three sampling days (D_1 , 2 June; D_2 , 28 July; D_3 , 27 September) from three plant water sources obtained by SIAR Bayesian mixing models: upper soil layer (0–15 cm), lower soil layer (15–40 cm) and groundwater (below 40 cm).

relative amounts depending on the season. The transition from spring to summer (D_1 – D_2) is clearly noticeable as a large contribution of source water (61%) on D_1 came from the upper soil layer (0–15 cm). By contrast, for D_2 and D_3 most of the water used originated from either groundwater reservoirs (44.8% on D_2) or the lower soil layer (52.5% on D_3).

The contribution of each soil water source for each sampling day (except D_1) and ecological region is depicted in a ternary plot (Fig. 4). For D_2 , when significant genotypic differences were found for both $\delta^{18}\text{O}$ and $\delta^2\text{H}$, the contribution of the upper soil layer varied between 2.2% (population 191; 11PY, Ibiza,

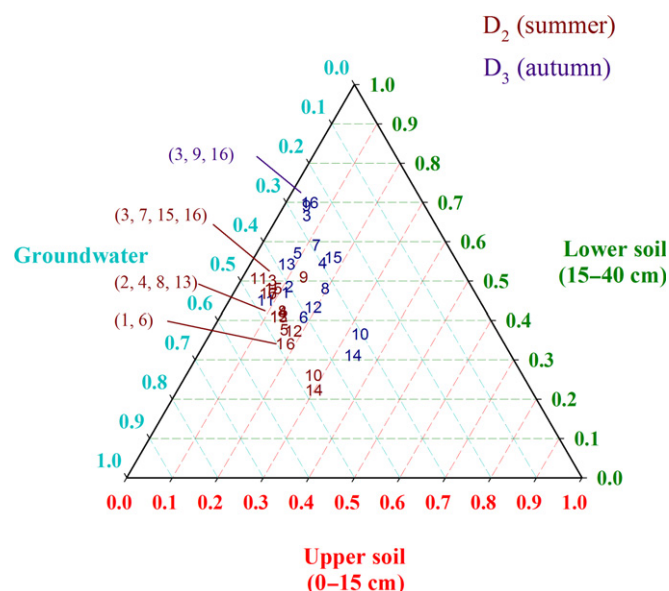


Fig. 4 Ternary plot suggesting changes in active rooting depths of 16 ecological regions (ecotypes) of Aleppo pine (*Pinus halepensis*) for two sampling dates (D_2 , 28 July, red numbers; D_3 , 27 September, blue numbers) as indicated by the relative contributions of three plant water sources: upper soil layer (0–15 cm), lower soil layer (15–40 cm) and groundwater (> 40 cm). Code numbers of ecological regions are given in Supporting Information Table S1.

ecological region) and 43.6% (103; 5LI, East Spain) (mean = 14%; median = 10.8%). For the lower soil layer, the differences in contribution ranged from 10.4% (103; 5LI, East

Spain) to 78.7% (184; 9MA, Majorca) (mean = 41.1%; median = 43%). The groundwater contribution varied between 17.6% (184; 9MA, Majorca) and 54.3% (31; 1CAT, Catalonia) (mean = 44.8%; median = 45.2%). For D_3 , when significant population differences were found for $\delta^{18}\text{O}$, the contribution of the upper soil layer varied between 1.7% (156; 6BS, South Betic Mountains) and 50.1% (233; 14IT, Italy) (mean = 14.2%; median = 8.3%). For the lower soil layer, the differences in contribution ranged between 15.9% (92; 4MS, Iberian Range) and 95.2% (156; 6BS, South Betic Mountains) (mean = 52.5%; median = 51.4%). The groundwater contribution varied between 3.0% (156; 6BS, South Betic Mountains) and 52.1% (101; 5LI, East Spain) (mean = 33.2%; median = 35.3%). For D_1 , genotypic changes in contributory soil water sources were not examined because of a lack of significant population effects in stable isotopes.

Stem volume

The ANOVA for stem volume showed significant variation among populations (results not shown). As for xylem water isotopes, grouping populations into ecological regions proved to be effective in revealing potential adaptive variation, as the partitioning of population effects (F -value = 4.85; $P < 0.0001$) indicated that changes at the ecotypic level underlie most variation (F -value = 8.07; $P < 0.0001$). Yet, the variability in stem volume among populations within ecological regions remained significant. Trees at the trial site averaged 5.16 m (height), 8.74 cm

Table 3 Correlation coefficients (and associated probabilities given in parentheses) for the contributions of plant water sources at the population level in the middle of summer (D_2) and early autumn (D_3) and a number of ecogeographical variables at origin, the stem volume (Vob) and the carbon isotope composition ($\delta^{13}\text{C}$) (extracted from Voltas *et al.*, 2008) of 56 populations of Aleppo pine (*Pinus halepensis*)

	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 ecogeographical 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 $\delta^{13}\text{C}$.

Correlations with ecogeographical variables do not include populations of uncertain origin (reforestations of the Spanish northern plateau and Amphipholia; see Supporting Information Table S1). Significant correlations at the 5% level are shown in bold.

(diameter at 1.30 m) and 12.80 dm³ (volume). BLUEs of population means for volume ranged from 4.89 dm³ (population 84) to 37.57 dm³ (population 212).

Relationships between population traits and ecogeographical variables

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

Populations originating from wetter areas (higher *P*, and higher precipitation of wettest month) tended to extract less groundwater in the middle of summer (D₂). This was the most relevant association between water uptake patterns and climate factors (Table 3). Additionally, Gower's ecological distance to the trial was used as a means of comparing the overall climatic characteristics at origin for each population with those of the evaluation site. A larger Gower's distance was related to a higher mean annual temperature ($r = 0.437$; $P < 0.001$) and both lower summer precipitation ($r = -0.413$; $P = 0.002$) and a lower ratio of summer to annual precipitation ($r = -0.664$; $P < 0.001$), therefore denoting increasing aridity at origin. In this regard, populations originating from more arid conditions tended to extract less water from shallow soil and more water from deeper soil layers (Table 3). A reanalysis of the relationships between relative contributions of water sources for D₃ and ecogeographical variables excluding the atypical populations marked in Fig. 2 did not change substantially the aforementioned associations (Table S2).

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

Discussion

This study provides new insights into changes in the root activity of a widespread circum-Mediterranean pine in response to seasonal fluctuations in water availability, which reflects potentially adaptive differences among natural populations. To our knowledge, this is the first work to address the crucial issue of intraspecific divergence in water uptake patterns for a plant

species typical of drought-prone environments such as Aleppo pine.

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

The trees experienced the lowest precipitation at the end of July (D₂), coupled with the highest temperatures for 2010 (Fig. 1). The genotypic differentiation that was observed for $\delta^{18}\text{O}$ and $\delta^2\text{H}$ during this central period of water shortage suggests that adaptive variation in water uptake patterns exists among populations of Aleppo pine. Indeed, most of the populations' variability in water isotopes could be attributed to differences among ecological regions, underpinning the adaptive relevance of the differential access to deep soil water pools. Our results therefore point to the middle of summer as the most suitable period to highlight ecotypic variability in water uptake, which parallels the results reported by Snyder & Williams (2000). By contrast, on D₁ (end of spring) the trees experienced abundant precipitation before sampling, presumably approaching saturation in the top soil layer and resulting in a lack of intraspecific differentiation. On D₃ (beginning of autumn), water availability was probably somewhere between those of D₁ and D₂, but the genotypic differences observed for $\delta^{18}\text{O}$ suggest that Aleppo pine populations can still exhibit contrasting water uptake patterns during recovery from a drought period. Overall, $\delta^{18}\text{O}$ and $\delta^2\text{H}$ trends in population means were quite consistent across sampling dates (D₂ and D₃), but $\delta^{18}\text{O}$ was more effective in discriminating among entries, as the statistical relevance of genetic effects was consistently larger than for $\delta^2\text{H}$. This could be partly attributable to fractionation during water uptake, which has been reported for hydrogen in woody xerophytes (Ellsworth & Williams, 2007), but consistently discarded for oxygen (Wershaw *et al.*, 1966; Dawson & Ehleringer, 1991; Barbour, 2007; Ellsworth & Williams, 2007).

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

Nonetheless, Aleppo pine populations may require a relevant contribution to transpiration from groundwater, even during wet periods (Fig. 3). Oren & Sheriff (1995) and Querejeta *et al.* (2001) have hypothesized that the deep roots of pines may effectively supply a significant amount of water, but preferentially during the dry season, as they are composed of long hydraulic pathways with relatively low conductivity compared with shallow roots (Oren & Sheriff, 1995; Lintunen & Kallioikoski, 2010). The distribution of deep roots in pines is known to be sparse but important in supplying the trees when the soil surface layers dry out (Nambiar & Sands, 1992; Achat *et al.*, 2008). The development of a deep rooting system therefore seems essential for Aleppo pine to survive the intense summer drought, increasing its competitive ability; this points to the existence of a dimorphic root morphology for the species, including superficial lateral roots and deeply penetrating sinker (tap) roots (Filella & Peñuelas, 2003b).

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

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

Other studies involving interspecific comparisons (Snyder & Williams, 2000) produced larger ranges of variation in contributing sources than our study. Nonetheless, the relative contributions from multiple water sources have been useful in discerning variation in root activity triggered by seasonal changes in soil water. An obvious limitation of the isotopic approach is its inability to characterize changes in the absolute amount of soil water

taken up by plants at varying spatiotemporal scales (Snyder & Williams, 2000). This shortcoming also applies to our study, as xylem water isotopes allow the quantification of possible differences in the relative contribution of several sources to the water transpired by trees, but do not give clear answers about how the absolute amount of water consumed may vary among populations or among sampling times. In this regard, seasonal sap flow measurements and/or other estimates of total tree water use would be valuable to overcome such limitations.

Relationships between genetic patterns of water uptake and ecogeographical characteristics at origin

We detected a geographical structure in the water uptake patterns of Aleppo pine across most of its current distribution range, with a tendency for northern and eastern Mediterranean populations to rely more on shallow water during dry periods than their southern and western counterparts. This demonstrates strong ecotypic differences in the functional plasticity of root systems for this Mediterranean conifer, although the easternmost (and some of the driest) populations of the species remain to be tested. Geographical patterns of ecotypic variation in Aleppo pine have been reported for a number of morphometric, anatomical and ecophysiological traits (Tognetti *et al.*, 1997; Chambel *et al.*, 2007; Climent *et al.*, 2008; Voltas *et al.*, 2008; Esteban *et al.*, 2010), pointing to a well-structured array of adaptive responses following the postglacial long-range colonization by the species of the western Mediterranean basin, after which genetic differentiation occurred (Gómez *et al.*, 2005; Grivet *et al.*, 2009). Voltas *et al.* (2008) revealed that there is adaptive variation for improved WUE following west to east and north to south gradients. Similarly, Climent *et al.* (2008) reported that variation in allocation patterns to different plant organs also follows comparable gradients within the distribution range of Aleppo pine. Hence, mesic populations from the northern and eastern regions grow taller and allocate fewer resources to reproduction (Climent *et al.*, 2008), are less water conservative (Voltas *et al.*, 2008) and concurrently tend to exploit more shallow water at the expense of water taken up from deeper water sources (this work). Conversely, xeric populations originating from the drier conditions of the southern and western regions of the Mediterranean seem better able to exploit deep soil water in the dry season and possibly allocate more resources to the development of a deeper tap root system, regarded as advantageous strategies for withstanding dry periods (Canadell *et al.*, 1996). As drought events are increasing in frequency and intensity in the Mediterranean (IPCC, 2014), Aleppo pine trees from arid ecosystems may show a gradual shift of their optimal ecological niche to the more mesic northern and eastern regions of its distribution range, which are expected to become progressively drier over the 21st Century. This mismatch between territory characteristics linked to fitness may be relevant for delineating anticipatory measures designed to mitigate climate change impacts on forest functioning and composition (e.g. through assisted migration strategies).

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 sources indicated a preferential access to lower soil water of high- $\delta^{13}\text{C}$ (i.e. high WUE) populations in summer (D_2), although low- $\delta^{13}\text{C}$ populations extracted relatively more groundwater in autumn (Table 3). Improved WUE and better access to lower soil water in the middle of summer are therefore relevant characteristics contributing to the adaptive strategy of xeric populations of Aleppo pine in coping with drought. Interestingly, this result differs from previous observations reporting positive associations between WUE and use of shallow soil water at the interspecific level (Flanagan *et al.*, 1992). Although slow-growing populations exhibited higher $\delta^{13}\text{C}$ values (Table 3), the absence of negative associations between stem volume and the relative contributions of deep water sources (Table 3) suggests a lack of relevant trade-offs between aboveground growth and water uptake patterns. Rather the opposite: a higher contribution of groundwater was related to a larger stem volume in early autumn. This might imply that intraspecific allocation patterns of dry matter to shoot production (Climent *et al.*, 2008) are largely independent of the distribution of functional roots and the active rooting depth, which are also population-dependent as suggested by xylem water isotopes.

In conclusion, this study demonstrates the high plasticity of the root system of Aleppo pine, allowing this species to shift actively among water sources during the year. But, more importantly, it points to the adaptive relevance of resource investment in deep roots as a strategy to withstand dry periods in populations of Aleppo pine originating from particularly harsh, drought-prone habitats. Probably, the relevance of such a selective process towards securing a source of water in dry environments strongly interacted with the imprint of demographic processes, hence determining the evolutionary outcome for this species. Indeed, the ecotypic patterns observed in plant water sources showed a good match with the westward expansion through the Mediterranean during postglacial colonization, which produced a marked loss in genetic diversity but also a strong action of natural selection on populations facing new environmental challenges (Grivet *et al.*, 2009, 2011).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Characteristics of the 56 populations of Aleppo pine used in this study

Table S2 Population-level correlations between contributions of plant water sources and ecogeographical variables at origin, stem volume and carbon isotope composition

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