Consistent geographical gradient of water use efficiency evidences local adaptations to drought across the complete latitudinal distribution of *Quercus suber*

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ABSTRACT

Rationale: Increased aridity has led to drought-induced mortality or loss of health for many tree species. Of particular interest is to explore the response of the Mediterranean tree species cork oak (*Quercus suber*) to this declining phenomenon due to its severity and its large implications for the local economy and the provision of highly relevant ecosystem services.
Approach: To assess geographical variations in the response to water stress, we analyzed under controlled conditions the effects of four watering levels and the resistance to a terminal drought on seedlings collected from nine populations covering the complete latitudinal distribution of the species. We explored the response of a number of physiological traits and markers of oxidative stress potentially related with drought-resistance.
Findings: We found a highly plastic phenotypic response of most variables to water availability and a large influence of seedlings provenance in the drought-resistance strategies. Although the oxidative stress enzymes ruled out differential water stress throughout the distribution range, we found that seedlings from the southern limit are less vulnerable to drought than other populations. Southern seedlings adapted to xeric conditions displayed a larger sensitivity of stomata to changes in soil humidity and a higher water use efficiency. These physiological local adaptations coupled with larger acorn size in the southern populations, resulted in larger aboveground biomass and higher drought resistance at the southern distribution edge.
Conclusions: Our data suggest that this evergreen tree species relies on its physiological plasticity to develop adaptative features that allows it to overcome water shortage and that southern populations hold genetic diversity that could improve the species adaptation in the forecasted drought context with relevant implications for conservation programs.

Introduction

A broad knowledge of the ecological and geographical range of organisms is becoming increasingly important to promote effective planning and decision making for conservation and natural resource management. Such a task is especially pertinent in the ongoing climate change context due to the numerous and relevant harmful consequences of this phenomenon on Earth (Alberto et al., 2013; Keenan et al., 2011). Climate change is not only modifying the environments in which all living beings develop, but it is also projected to affect their global distribution patterns (Rubenstein et al., 2023). Changes in the species distribution range have the potential to reshape ecological communities, impact ecosystem services and functioning, disturb human health and well-being, and even have feedback effects on climate (Jing et al., 2022; Weiskopf et al., 2020). Therefore, disentangling how species are shifting as a function of climate change is key for meaningfully managing species and ecosystems. Whether abrupt changes in their geographical range will actually occur, however, depends on the extent to which these species are able to tolerate environmental modifications.

In most terrestrial biomes, trees are deemed to be keystone species as they support the survival of numerous multiple taxons and provide a broad spectrum of ecosystem services, with crucial feedbacks on biotic

and abiotic factors(Díaz et al., 2007). Thus, the way in which trees respond to changes in climate has the potential to largely affect the delicate balance ruling ecosystem functioning (Cramer et al., 2001). A major current challenge for the research community is to depict the framework of such selective pressure over time as accurately as possible in sessile species like trees, which have a limited migration capacity. Plant species have developed two main mechanisms to cope with environmental changes (Pérez-Ramos et al., 2013). On the one hand, local adaptations occur when a population evolves through a natural selection process responding to specific ecological conditions (Ducousso et al., 1996), being climate the most pressing factor causing population differentiation (Méndez-Cea et al., 2023; Saxe et al., 2001). On the other hand, plants can also deal with these new conditions through phenotypic plasticity, which is the range of phenotypes that a single genotype can express as a function of its environment (Chevin et al., 2013; Nicotra and Davidson, 2010; Reed and Frankham, 2001). Identifying responses to ecosystem variations will require a deep understanding of the environmental changes induced in the phenotype for a given plant species or population. Although phenotypic plasticity was used to be regarded as noise, we currently know that it is genetically controlled, heritable and of potential relevance to species' evolution (Bradshaw, 2006; Lande, 2009). Gaining an improved understanding of how plant species can take advantage of these two mechanisms will be crucial for predicting changes in species distributions, community composition and tree productivity under future climate change scenarios. Natural differences in functional traits throughout the species distribution range can be selectively fixed by local environmental conditions. Thus, site-specific characteristics can drive the selection of given functional trait values that benefit tree performance in such an environment, giving rise to local adaptations.

The Mediterranean biome has been widely acknowledged as a prominent hotspot of biodiversity (Myers et al., 2000), but also of vulnerability (Ochoa-Hueso et al., 2017). The Mediterranean basin is one of the European regions projected to be most impacted by climate change, with precipitation expected to decrease while temperature will rise causing an overall shortage in water availability as a result of increased evapotranspiration (Masson-Delmotte, 2021). Further, a higher frequency and severity of extreme droughts has been forecasted, especially during the summer season (Masson-Delmotte, 2021). These changes will imply strong consequences for Mediterranean forests and ecosystems (Martínez-Vilalta et al., 2012; Matías et al., 2019b; Vitale et al., 2012), as drought-related mortality has been found to be an emerging widespread event with critical implications for forest conservation (Hartmann et al., 2018). If these changes are as abrupt as projected, plant populations climatic boundaries may move faster than forest tree species can migrate (Higgins and Harte, 2006). Thus, the survival of forest tree species will depend in the short term on evolutionary change (Etterson and Shaw, 2001) through their phenotypic plasticity and their intra-specific genetic variation for adaptive traits (Marchin et al., 2008). For these reasons, great attention is being paid to disentangle the range dynamics of tree species, their forecasted patterns and adaptive functional traits in the Mediterranean region (Matías et al., 2019b; Morillas et al., 2023).

Cork oak (*Quercus suber* L.) is an iconic evergreen tree species typical of forest landscapes in the western coast of the Iberian Peninsula and at the north of Morocco. These forests not only hold a rich biodiversity and drive extensive and relevant ecological processes (e. g. carbon storage, water retention, nutrient cycling, soil conservation…), but they also have an important role in the local economy for many Mediterranean countries by the production of a renewable resource as cork (Ibáñez et al., 2014; Marras et al., 2014; Petroselli et al., 2013). Considering its significant implications for biodiversity, provision of ecosystem services and socio-economic interests, together with the alarming declining trends that this species is currently suffering (Catalão et al., 2022; Dorado et al., 2023; Matías et al., 2019a), it is of paramount importance to understand the potential scenarios that cork oak will face in the near future to address conservation and restoration programs (Beaumont et al., 2007; Keenan et al., 2011).

Despite cork oak is well adapted to water stress conditions through drought-adapted morphological traits (Morillas et al., 2023) and the activation of several physiological mechanisms to limit water evaporation (Lobo-do-Vale et al., 2023; Mediavilla et al., 2019), soil moisture seems to be the main constraining factor determining its geographical distribution. Previous research in common gardens proved that different cork oak populations across the distribution range display large variation in growth, morphological traits and survival (Gandour et al., 2007; Matías et al., 2019b; Morillas et al., 2023; Ramírez-Valiente et al., 2014, 2010). Adaptation to contrasting water availabilities has been proposed as the major mechanism responsible for the differences found in functional traits among populations (Matías et al., 2019b; Morillas et al., 2023). In Mediterranean environments, soil water availability is the most constraining factor determining plant species growth, reproduction, and survival (Gulías et al., 2002). Thus, tree fitness in these areas is largely affected by physiological traits controlling water loss such as the efficiency in the use of water (WUE) and the capacity of water transport through stomata (GSW), but also physiological traits involved in carbon uptake such as the maximum capacity to fix carbon (A_{max}). Besides, a combination of intracellular chemical reactions are also part of the plant's response to water stress (Porth et al., 2005). Given their important role, some of these traits have been suggested to have an adaptive value for cork oak under water stress (Matías et al., 2019b) and can be expected to be under selection in a water shortage context. However, the physiological and chemical responses of cork oaks to the forecasted increased drought are far from being fully understood.

The aim of this research was to explore the role of a number of physiological traits and markers of oxidative stress potentially involved in cork oak's drought resistance and their prevalence in natural populations located along the cork oak's latitudinal distribution. Namely, we quantified the functional responses of cork oak seedlings to the effect of increasingly severe levels of experimental drought and their resistance ability to a terminal drought. For this purpose, a controlled-conditions experiment was performed using acorns collected from nine populations of cork oak encompassing the complete latitudinal distribution of the species. The emerging seedlings were subjected to four different levels of water availability and following this phase, a terminal drought period was imposed. This approach allowed us to pinpoint the most vulnerable populations to water stress with implications for planning management strategies under a climate change context. We hypothesized that (i) Differences in water availability during the growing season will modulate the carbon uptake capacity and water use efficiency of cork oak seedlings, resulting in differential markers of oxidative stress. We also predicted that (ii) populations located in drier areas will display higher efficiency in the use of water during the growing period. Lastly, we projected that (iii) cork oak populations located near the southern limit of the species' distribution range will have developed physiological local adaptations to water shortage, making them less vulnerable to our experimental terminal drought compared to populations located in the central or northern parts of the distribution.

Materials and methods

Focal species and study sites

Cork oak (*Quercus suber* L.) is an emblematic evergreen tree species widely distributed in the Mediterranean biome, chosen as focal species for this study. To explore the role of local adaptations to drought conditions through the cork oak's distribution range, we selected nine populations along its latitudinal distribution gradient separated approximately 1° in latitude (Fig. 1, Table A.1). In November-December 2020 and following the natural ripening period of the species across its distribution range, we randomly selected five trees per population, with a minimum separation among them of 50 m, to account for the spatial
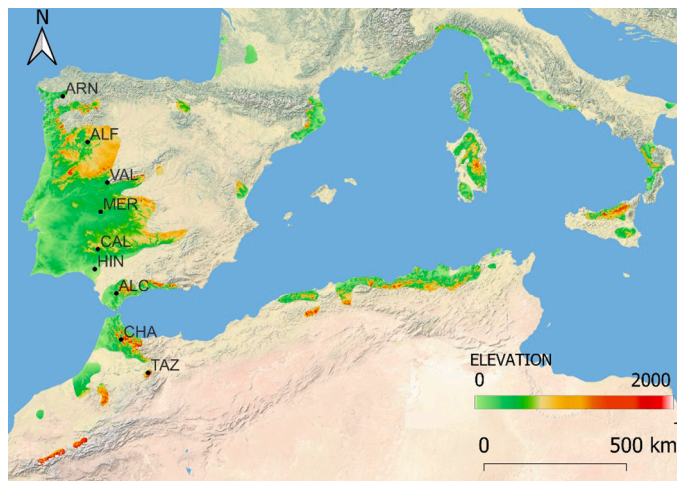
Fig. 1. Distribution map of *Quercus suber* showing its elevation gradient. The location of the source populations is indicated by black dots: ARN, ALF, VAL, MER, CAL, HIN, ALC, Cha and TAZ, sorted out from north to south, being ARN and TAZ the northern and southern most populations, respectively. Distribution data obtained from Caudullo et al. (2017).

variability of the sampling area. Fifty mature acorns per tree were collected, their viability was tested by the flotation method following Gribko (1995) and viable acorns were stored in wet vermiculite at 5 °C until sowing.

Experimental design

The experiment was performed at the greenhouse facilities of the University of Seville (CITIUS II), located at the city of Seville (Spain; 37°21′31″N 5°59′13″W) from January to September 2021. Temperature was stable throughout the experiment (25 °C in the day and 15 °C at night) and light conditions mimicked the forest understory (PAR 516.7 µmol $m^{-2} s^{-1}$ ± 50.13) with natural daily duration. The experiment covered the first spring and summer of life of tree seedlings, the most critical stage in the ontogeny of Mediterranean woody species (Mendoza et al., 2009). In January 2021, 360 tubular pots (15 cm in diameter and 20 cm height) were filled with a peat and river sand mixture (proportion 3:1 per volume) to allow a non-limiting nutrient availability and adequate drainage conditions. One individual acorn was sown per pot at 2 cm depth, 8 pots per mother tree, making a total of 40 pots per latitudinal population. To allow natural mycorrhization, all pots were watered with 300 ml of a soil microbial inoculum obtained from the maceration of 10 kg of soil collected under the canopy of natural *Q. suber* adult trees on 100 l of water for 48 h (Matías and Jump, 2014). This soil was collected from Doñana National Park (SW Spain), a central population to the latitudinal gradient of distribution, and was analyzed to assure the absence of the soil-borne pathogen Phytophthora *cinnamomi* (Sánchez et al., 2002). All pots were weekly irrigated at 100 % of soil water holding capacity (WHC, estimated as the amount of water (g) that the substrate held following water saturation) until 80 % of the seedlings emerged, which occurred after 13 weeks (April 2021).

After this initial experimental phase under optimal conditions, we simulated four different watering scenarios, each of them with 10 replicates: 1) 100 % of WHC, corresponding with a rainy growing season where soils remain wet most of the time, simulated through an irrigation until field capacity twice per week; 2) 50 % of WHC, simulating average moisture availability conditions in a cork oak forest during the spring season (Pérez-Ramos and Marañón, 2012); 3) 25 % of WHC, simulating a reduction of approximately 30 % over the previous scenario and according with the projections of the A1B scenario for the Mediterranean area at the end of the current century (Giorgi and Lionello, 2008); 4) 10 % of WHC, corresponding with an infrequently dry spring, an extreme

event that is expected to become more usual in the coming years (Mildrexler et al., 2018). These watering treatments were applied to experimental pots by adding a constant amount of water to all seedlings within the same watering level from 12 April to 1 July 2021, simulating the natural growing season. Following this differential treatment, watering was completely stopped simulating an extreme drought period and seedlings were subjected to a progressive drought until death (terminal drought hereafter) to determine seedling resistance to extreme drought conditions across provenances and watering levels. To determine the effectiveness of the watering treatments, soil moisture was measured twice a week using a ML3 Theta Probe soil moisture sensor (Delta-T Devices, Cambridge, UK) on the top 10 cm. To avoid the potential effect of small differences in environmental conditions, the position of every pot was randomly rotated monthly. Seedling survival was weekly monitored until all of them were dead.

Analyses of physiological traits, markers of oxidative stress and aboveground biomass in response to drought

Maximum leaf-level photosynthesis capacity (A_{max}), stomatal conductance (GSW) and ambient to leaf CO_2 difference (Ca) were measured on three to four individuals per provenance and watering treatment combination every fortnight before the onset of the extreme drought period (1st of July) and weekly along this period until the number of surviving seedlings prevented continuing with these measures. Gas exchange measurements were performed in randomly selected, fully expanded leaves by using a LI-6800 infrared gas-exchange analyzer system (Li-Cor Inc., Lincoln, NE, USA). These measurements took place between 10:00 h and 14:00 h using a multiphase flash fluorometer chamber fitted at 400 ppm reference CO_2 concentration, 1500 µmol $m^{-2} s^{-1}$ light-saturating photosynthetic photon flux density, block temperature at 25 °C to match ambient air temperature, and relative humidity on full scrub.

Plant stress at the end of the terminal drought was estimated through the analysis of the oxidative stress metabolite malondialdehyde (MDA), which is a useful marker of general lipid peroxidation. Also, the accumulation of the compatible organic solute proline was quantified. For doing this, we selected a random subset of three seedlings per provenance and watering treatment combination. The crude extracts for both metabolic determinations were obtained grinding 0.1 g of fresh leaves tissue with 2 ml of Ethanol 80 % (v/v). The homogenate was soaked at room temperature for 1 hour and finally centrifuged at 12,000 rpm for 5 min; the supernatant was used as a crude extract. The level of lipid peroxidation was determined by measuring MDA concentration with the thiobarbituric acid reactive substances method (Hodges et al., 1999). Proline was quantified as described by Pérez-López et al. (2023). Aliquots of 50 µL of ethanolic extract were added to 100 µL of reaction mix [ninhydrin 1% (w/v) in acetic acid 60% (v/v), ethanol 20% (v/v)] and heated at 95 °C for 20 min. After cooling, the absorbance of the reaction was measured at 520 nm. Proline concentration was determined from a standard curve. As proline levels were reported on fresh weight, the reduced plant relative water content responding to the experimental drought may have underestimated its concentration in the high watering treatments. For the analysis of carbon (C) and nitrogen (N) content in leaves and the relative abundance of ^{13}C ($\delta^{13}C$), 5 leaves of eight saplings per provenance and watering treatment combination were ground to powder following sapling death as result of the extreme drought period. Total carbon and nitrogen as well as the bulk isotopic composition were determined in these samples by dry combustion in a Flash 2000 elemental micro-analyzer coupled via ConFlo IV interface to a Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Carbon isotopic ratio is reported as part per thousand deviations (expressed as δ values) referred to appropriate IAEA standards:

$$\delta = \left[\frac{\text{R } sample - \text{R } standard}{\text{R } standard} \right] \times 1000 \qquad \text{(A1)}$$

where R is the $^{13}C/^{12}C$ ratio. The standard deviations of $\delta^{13}C$ were lower than $\pm\ 0.05$ ‰. The total C and N values were used for the computation of the leaf C:N ratio for each experimental treatment and provenance. These variables inform about water stress (proline, MDA), the efficiency in the use of water ($\delta^{13}C$), plant nutrition status (C:N), the maximum capacity to fix carbon (A_{max}), the capacity of water transport through stomata (GSW) and the ratio between intercellular CO_2 (Ci) and ambient CO_2 (Ca) concentration (Ci/Ca). The relevance of the latest variable lies in photosynthesis studies that proved that plants do not directly sense ambient CO_2 in the air (Ca) but rather use intercellular CO_2 (Ci) (Landsberg and Sands, 2011), which makes this index important for photosynthetic research. Following the terminal drought, dead seedlings were harvested and separated into stem, leaf and root fractions. We weighed the total aboveground biomass (g) corresponding to the sum of the shoot and leaf tissues.

Data and statistical analysis

Variations among provenances and water treatments during the experiment in soil moisture, photosynthetic rate (A_{max}), stomatal conductance (GSW) and the ratio of intercellular to ambient CO_2 (Ci/Ca) were tested using repeated-measures analyses of variance (rm-ANOVA) with provenance and watering treatment as between-subject factors and time as a within-subject factor. Data were checked for conformity with rm-ANOVA assumptions using Shapiro–Wilk normality test and Mauchly's test of sphericity. Normality tests of the residuals by time point indicated that they followed approximately a normal distribution. When sphericity could not be ensured, a Greenhouse–Geisser correction was applied. Pairwise comparisons were conducted by comparing main effects through post hoc tests using the Bonferroni correction. To investigate interactions among provenances, watering levels and time, data were divided into subsets according to different provenances and then were subjected to rm-ANOVA. We analysed the effect of geographical provenance and water availability during the growing period on seedling physiology by using linear models including provenance, watering treatments and their interaction as fixed factors to explore variations in proline, MDA, carbon isotopic discrimination levels, leaf C:N and aboveground biomass. The Akaike Information Criterion corrected for small sample sizes (AICc) was applied to choose the best model, i.e. lower AICc (Burnham and Anderson, 2004). The difference in AICc between the best model and the null model is provided by ΔAICc. R^2 indicates the proportion of variability in the outcome explained by the predictors (fixed effects). When data did not follow normality and/or homoscedasticity criteria, transformations were performed. When significant interactions between provenance and water treatments were detected, the post-hoc Tukey pairwise comparisons for each provenance were conducted to allow for more specific comparison. In the cases in which these interactions were not significant, the post-hoc Tukey pairwise comparisons were carried out for each variable with the whole dataset. All analyses were performed in R statistical software version 4.2.1 (R Development Core Team, 2022). All values are given as mean $\pm$ SE.

Results

Soil moisture

Watering levels applied during the experiment resulted in different values of soil moisture across treatments, progressively decreasing from 100 to 10 WHC levels during the whole treatment application (100 WHC: 26.2 ± 0.13 %; 50 WHC: 16.6 ± 0.09 %; 25 WHC: 9.9 ± 0.07 %; 10 WHC: 7.0 ± 0.08 %; Table 1). By contrast, we found no differences in soil moisture among pots containing seedlings from the different

Table 1

Results from repeated measurements ANOVA analyses (degree of freedom, F-statistics and P-value) explaining variations in soil moisture and physiological traits across provenances and watering treatments (WT) measured along time (Time). When significant interactions between provenance, water treatments and time were found, data were divided into subsets based on provenance and then were subjected to repeated measurement ANOVA analyses (see results in Fig. 4). Abbreviations: ARN, ALF, VAL, MER, CAL, HIN, ALC, CHA and TAZ are the populations where the acorns were collected. A_{max} means photosynthetic rate, GSW means stomatal conductance and Ci/Ca means the ratio of intercellular to ambient CO_2.

Variable	Factor	Df	F	P
Soil moisture	Provenance	7	1.7	0.0965
	WT	3	728.3	<0.0001
	Provenance x WT	21	0.7183	0.8135
A_{max}	Provenance	8	4.2370	0.001
	WT	3	42.27	<0.0001
	Time	5.165	110.93	<0.0001
	Time x Provenance	41.32	1.632	0.015
	Time x WT	15.49	5.244	<0.0001
	Provenance x WT	24.00	1.267	0.252
	Time x Provenance x WT	123.95	1.232	0.068
GSW	Provenance	8	2.848	0.014
	WT	3	54.73	<0.0001
	Time	5.342	50.26	<0.0001
	Time x Provenance	42.73	1.330	0.100
	Time x WT	16.03	5.853	<0.0001
	Provenance x WT	24.00	1.611	0.092
	Time x Provenance x WT	128.20	1.097	0.276
Ci/Ca	Provenance	8	2.976	0.011
	WT	3	62.32	<0.0001
	Time	4.014	128.03	<0.0001
	Time x Provenance	32.11	2.179	0.001
	Time x WT	12.04	6.451	<0.0001
	Provenance x WT	24.00	0.986	0.504
	Time x Provenance x WT	96.34	1.442	0.022

provenances (Table 1).

Geographical differences in the photosynthetic response to drought

Seedling photosynthetic performance strongly relied on the seed provenance and the water availability during the growing period (Table 1, Figs. 2, 3 and A.1a), as both factors were found to be significant for all the physiological variables monitored throughout the extreme drought phase of the experiment. Both sapling photosynthetic rate and stomatal conductance were lowest for the southern provenances and gradually increased in northern provenances, whereas the opposite trend was found for Ci/Ca concentration (Figs. 2a, 3 and A.1a). Rising soil water content from 10 to 100 % WHC resulted in progressively increased photosynthetic rate and stomatal conductance (Figs. 2b and A.1b) and decreased Ci/Ca concentration (Fig. 3). Regardless of the seedling provenance or water availability treatment, both photosynthetic rate and stomatal conductance dropped over time when subjecting seedlings to extreme drought (Figs. 2 and A.1). The contrary evolution was observed for Ci/Ca concentration, increasing over time in response to water limitation (Fig. 3). Although interactions between time and provenance and time and water treatment were detected frequently for these physiological variables, no significant interactions between provenance and water treatment were found for any of them (Table 1). The triple interaction between time, provenance and watering treatment was only detected for Ci/Ca concentration (Table 1), and the analysis of the independent subsets showed that water availability yielded differential Ci/Ca concentration values for all provenances excepting for two of them (Fig. 3c and 3h, see Tukey post hoc test at the figure caption).
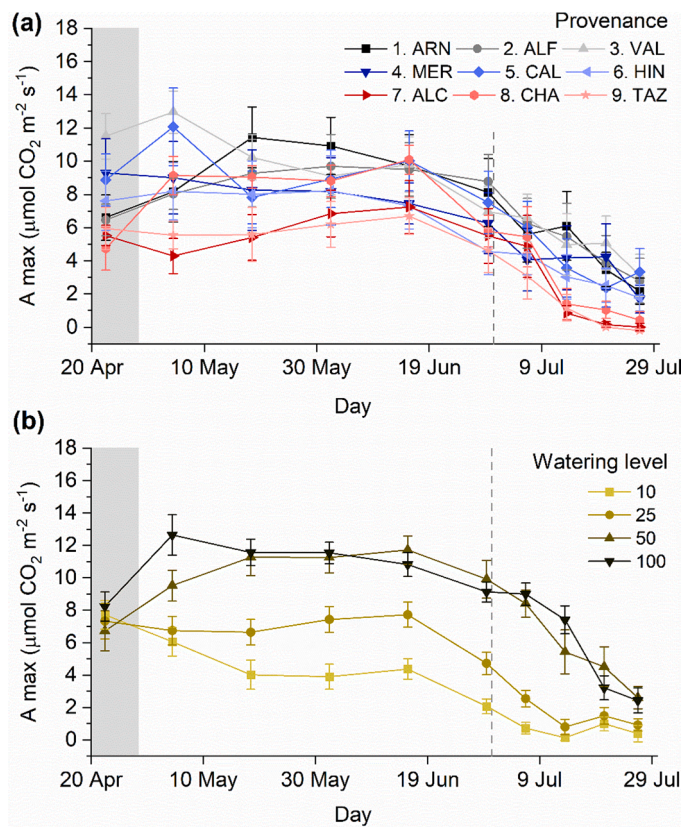
Fig. 2. Mean photosynthetic rate (A_{max}) of seedlings for all the provenances (a, watering treatments pooled) subjected to the different watering treatments (b, provenances pooled). The grey bar represents the emergence phase before the application of the watering treatment and dashed line indicates the start of the terminal drought period. Provenances displayed in panel a indicate where the acorns were collected, sorted out from north to south, being ARN and TAZ the northern and southern most populations, respectively. Water treatments displayed in panel b indicate saturated soil at 100 %, 50 %, 25 % and 10 % of water holding capacity, respectively. Predictors effects were calculated through repeated measurements ANOVA in Table 1. Differences among provenances for all watering treatments after Tukey post hoc test are denoted by different letters: ARN: a, ALF: ab, VAL: a, MER: abc, CAL: abc, HIN: abc, ALC: bc, CHA: abc, TAZ: c (a). Differences among watering treatments for all populations after Tukey post hoc test are denoted by different letters: 10: a, 25: b, 50: c, 100: c (b). Error bars represent ± SE.

Physiological responses and changes in markers of oxidative stress to water availability across the distribution range

Watering treatments applied during the experiment significantly affected all the analyzed functional traits considered in this study, except for leaf C:N ratio (Table 2, Table A.1, Table A.2 and Table A.3). Seed provenance was also found to be a key factor for most of these variables when responding to differential water availability, as it was included in the best model for all explored variables excepting for MDA (Table 2, A.3). We found a significant interaction between provenance and watering treatment for proline levels (Table 2). Thus, the independent analysis of each population revealed that watering availability determined proline concentration across provenances with the exception of population "ALC", which was unaffected by water conditions (Tables 2, A.1). Accordingly, the treatment 10 % WHC dramatically increased proline concentration for all populations excepting "ARN" and "ALC". Low watering availability consistently yielded increased proline, MDA and carbon isotopic discrimination levels (Fig. 4 and 5). Seedlings from northern provenance displayed more negative values of $\delta^{13}C$ compared to those with southern provenance (Fig. 5a). It is worth noting

that we did not find differences between the treatments 50 and 100 % WHC for any of the explored variables (Fig. 4 and 5). Plants with southern provenance developed higher aboveground biomass related to those with northern provenance, whereas high water availability consistently resulted in increased aboveground biomass for all provenances (Fig. 6).

Discussion

Functional responses to water availability

We investigated the physiological responses and changes in markers of oxidative stress of cork oak to both different water availability levels and to a terminal drought to explore the species' drought tolerance and its prevalence in natural populations. In increasingly arid Mediterranean forests, water is deemed the most constraining environmental factor for primary production (Austin et al., 2004). It was therefore expected that different levels of water availability during the cork oak seedling's early development would modulate its physiological performance determining the carbon uptake capacity and water use efficiency of the seedling and eventually resulting in differential markers of oxidative stress. Results of our study fully supported our first hypothesis. We found a significant effect of water availability for all explored physiological variables (Table 1). Cork oak seedlings displayed increasing levels of stomatal inhibition (Figure A. 1. b) and hence a reduction in carbon assimilation (Fig. 2b) when subjected to more restrictive water shortage (i.e., 25 % and 10 % of water holding capacity). The inclusion of the factor "water treatment" in the best linear model for markers of oxidative stress and $\delta^{13}C$ (Table 2) supports the large seedling plasticity to face with differential water availability. The highest values of proline and MDA, which levels depend on specific plant responses to water stress, were reported under the most restrictive water limitation treatment (Fig. 4a and b). Overproduction of these molecules has been previously identified as a biochemical process in plants responding to water stress. Proline is an amino acid that acts as an osmoprotectant to keep stable levels of water in the cytoplasm (Ahmed et al., 2009) and to prevent protein denaturation and cell membrane damage by promoting stability in the structure of enzymes and protecting their activity (Hessini et al., 2009). Previous work in cork oak observed a negative correlation between relative water content and proline accumulation in leaves, suggesting that proline amount is directly related to drought stress tolerance (Kwak et al., 2011). On the contrary, other authors provided evidence that proline is a good stress indicator but not always a good tolerance marker (Hnilickova et al., 2021). Intracellular proline also serves as a carbon and nitrogen reserve and, after water deficit, the proline catabolism may be essential for the recovery and restoration of cell homeostasis (Ashraf and Foolad, 2007; Kwak et al., 2011). The production of reactive oxygen species directly damages cell membrane phospholipids and increases lipid peroxidation (Zarafshar et al., 2014). Overall, the content of MDA is a measure of the degree of lipid peroxidation and is a useful marker of oxidative stress (Lima et al., 2002). Our results are supported by previous studies which have reported a similar increase in proline and MDA content in plants displaying specific responses to water stress (Liu et al., 2011), and in particular in other oak species (Cotrozzi et al., 2016; Jafarnia et al., 2018). Drought stress leads to an increase of ROS, which activates the antioxidant system to limit ROS accumulation. This system includes enzymatic and non-enzymatic components. Proline can act as an effective antioxidant molecule, protecting plants from ROS under water stress and reducing the MDA content and its deleterious effects (Szabados & Savouré, 2010; Hnilickova et al., 2021). It is important to note that the populations with the lowest levels of proline (ARN and ALC) showed high MDA content. This result suggests that proline plays a key role in reducing the deleterious consequences of ROS derivatives caused by water stress in both genotypes.

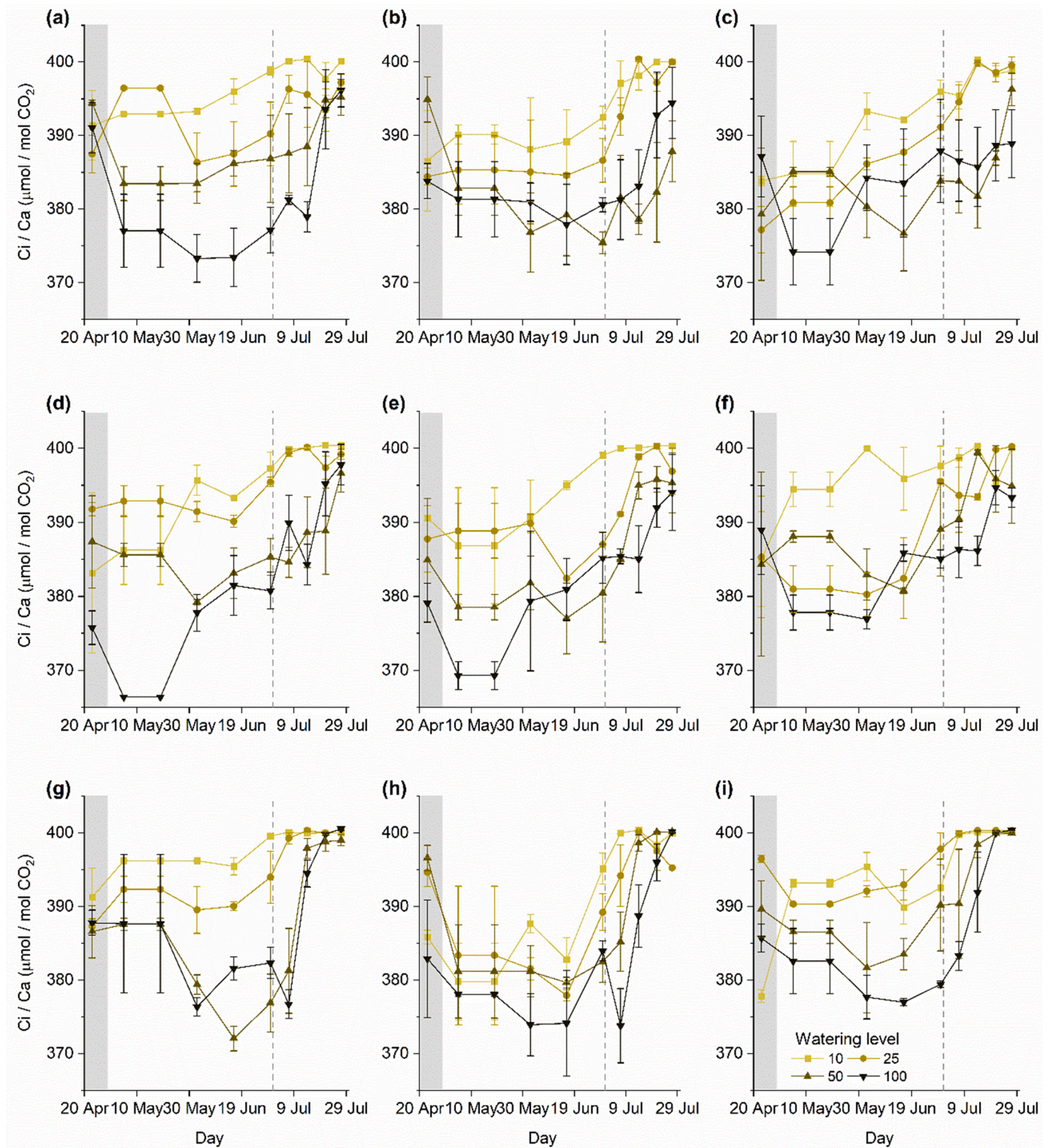The same pattern was found in the carbon isotopic discrimination

Fig. 3. Mean ratio of intercellular to ambient CO_2 (Ci/Ca) of seedlings subjected to the different watering treatments for the different provenances where the acorns were collected sorted out from north to south, being (a) and (i) the northern and southern most populations, respectively. The grey bar represents the emergence phase before the application of the watering treatment and dashed line indicates the start of the terminal drought period. Water treatments indicate saturated soil at 100 %, 50 %, 25 % and 10 % of water holding capacity, respectively. Predictors effects were calculated through repeated measurements ANOVA in Table 1. Differences among watering treatments for each population after Tukey post hoc test are denoted by different letters: 10: a, 25: a, 50: ab, 100: b (a), 10: a, 25: ab, 50: bc, 100: c (b), 10: a, 25: a, 50: a, 100: a (c), 10: a, 25: a, 50: b, 100: b (d), 10: a, 25: ab, 50: bc, 100: c (e), 10: a, 25: b, 50: b, 100: b (f), 10: a, 25: ab, 50: bc, 100: c (g), 10: a, 25: a, 50: a, 100: a (h), 10: a, 25: a, 50: ab, 100: b (i). Error bars represent ± SE.

Table 2

Results from linear models using Akaike Information Criterion corrected for small sample size (AICc) for the assessed changes in markers of oxidative stress and physiological and morphological traits. For each variable, the initial model accounted for the population where the acorn was collected (P), the water treatment applied (WT), and their interaction (PxWT). When significant interactions between provenance and water treatments were found (i.e. proline), the post-hoc Tukey pairwise comparisons for each population were used. The best model for each variable is the one with the lowest AICc. Abbreviations: ALC, ALF, ARN, CAL, CHA, HIN, MER, TAZ and VAL are the populations where the acorns were collected. MDA means malondialdheyde, $\delta^{13}C$ means carbon isotopic discrimination and C:N means leaf carbon to nitrogen ratio.

Response variable	Population	Best model	N	Δ AICc	R^2
Proline		$P + WT + PxWT$	108	145.51	0.86
	ALC	intercept	12	11.92	0
	ALF	WT	12	16.38	0.90
	ARN	WT	12	1.25	0.64
	CAL	WT	12	2.86	0.68
	CHA	WT	12	16.78	0.9
	HIN	WT	12	20.1	0.92
	MER	WT	12	10.94	0.84
	TAZ	WT	12	14.26	0.88
	VAL	WT	12	18.3	0.91
MDA		WT	109	96.7	0.55
$\delta^{13}C$		$P + WT$	288	133.64	0.4
C:N		P	288	165.09	0.45
Aboveground biomass		$P + WT$	392	200.18	0.42

analyses. The less negative $\delta^{13}C$ values reported for seedlings subjected to the lowest water availability (Fig. 5a) indicate a higher capacity to fix carbon per unit of water loss (i.e., a higher water use efficiency; Farquhar et al., 1989). This result implies that cork oak seedlings are able to modulate their water use efficiency according to climatic conditions, buffering the effects of the projected aridity to some extent and keep fixing carbon for a longer period, and highlights the natural drought tolerance of this species. As leaf nitrogen content positively determines the biochemical efficiency of carbon assimilation, the C:N ratio may have been related to the assimilation rate achieved for a given stomatal conductance. However, the C:N ratio showed no trend with water availability in our study (Fig. 5b). In line with this result, previous research did not find a correlation between N mass and rainfall for other plant species (Wright et al., 2005) or for cork oaks (Gouveia and Freitas, 2009). Even though the watering treatment applied to the seedlings produced a homogeneous response through all the explored variables, it

is interesting to note that differences between saturated soil at 100% and 50% of water holding capacity were not found for any physiological traits or markers of oxidative stress (Figs. 2, 3, 4, 5, 6 and A.1). This finding supports the notion that cork oaks are highly plastic plant species deeply adapted to water-limited environments. According to our results, a dramatic reduction in water availability (25% of soil water holding capacity) is required to significantly affect the physiology or chemical functioning of this drought-tolerant plant species. As expected, aboveground growth of cork oak seedlings from all provenances significantly declined when exposed to severe water limitations compared with non-stressed seedlings (Fig. 6). The reduction in plant growth is a frequent response to water shortage as a result of restrained photosynthesis (Ghanbary et al., 2017).

Differences among provenances in drought-resistance strategies

Cork oak seedlings exhibited a contrasting response to water availability across provenances, which suggested differential mechanisms to cope with water shortage throughout the cork oak's latitudinal distribution. It is worth noting that, although "provenance" was included in the best model for proline (Table 2), we found no geographical trend (i. e., no differences between northern and southern populations). Accordingly, the factor "provenance" was not accounted for in the best model for MDA (Table 2). Therefore, our results point out that cork oak populations coming from the whole species' distribution range suffered the same level of water stress when subjecting for a common watering level, regardless of their provenance. Despite this fact, seedlings from different provenance presented differential local adaptations to water shortage. Seedlings from northern provenance, historically less constrained by water, displayed lower stomatal limitations (Figure A. 1 a) and higher levels of photosynthetic rates (Fig. 2a) allowing them to fix carbon for a longer period. However, seedlings from the southern edge, adapted to frequent and severe drought, presented higher water use efficiency (Fig. 5a) which was ultimately translated into larger biomass (Fig. 6). These results support our hypothesis proposing the presence of differential physiological local adaptations to water shortage throughout the species' distribution range.

Physiological traits values were not consistent throughout the cork oak's distribution range, showing increased performance in northern populations compared to southern ones in terms of gas exchange parameters (including photosynthetic rate and stomatal conductance, Figs. 2a and A. 1 a). However, a common pattern emerged among provenances for these variables, as evidenced for a significant decline in
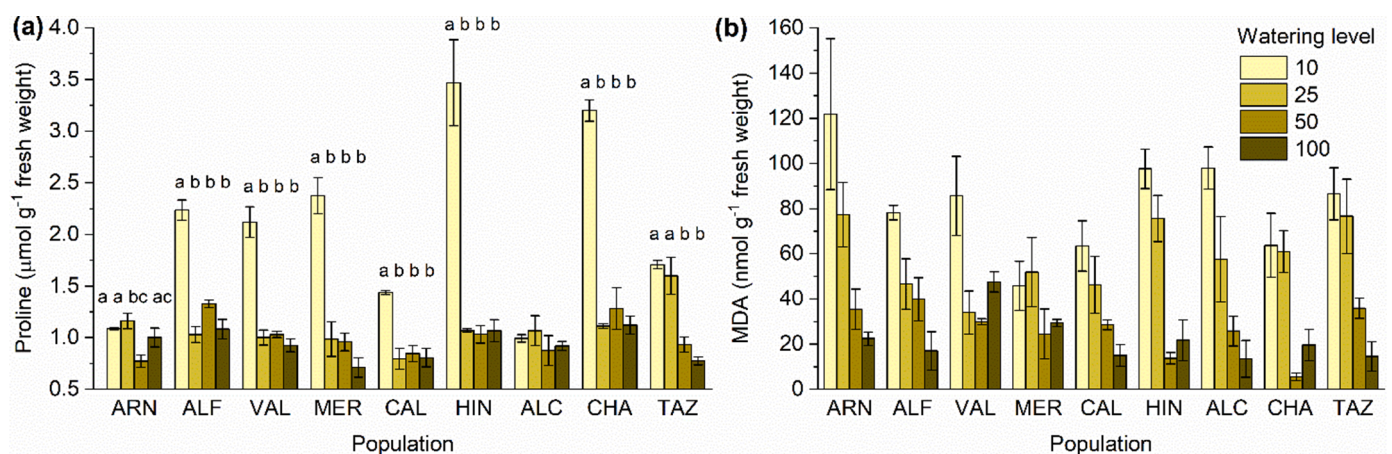

Fig. 4. Responses of proline (a) and malondialdehyde (MDA, b) to terminal drought for each one of the watering treatments and provenances. 100, 50, 25 and 10 means saturated soil at 100 %, 50 %, 25 % and 10 % of water holding capacity, respectively. The x-axis represents the populations where the acorns were collected, sorted out from north to south, being ARN and TAZ the northern and southern most populations, respectively. Predictors effects were calculated using equations for best models reported in Table 2 and parameters values in Table A.2, A.3 and A.4. Differences among watering treatments for all populations after Tukey post hoc test are denoted by different letters: see panel (a), 10: a, 25: b, 50: c, 100: c (b). Error bars represent ± SE.
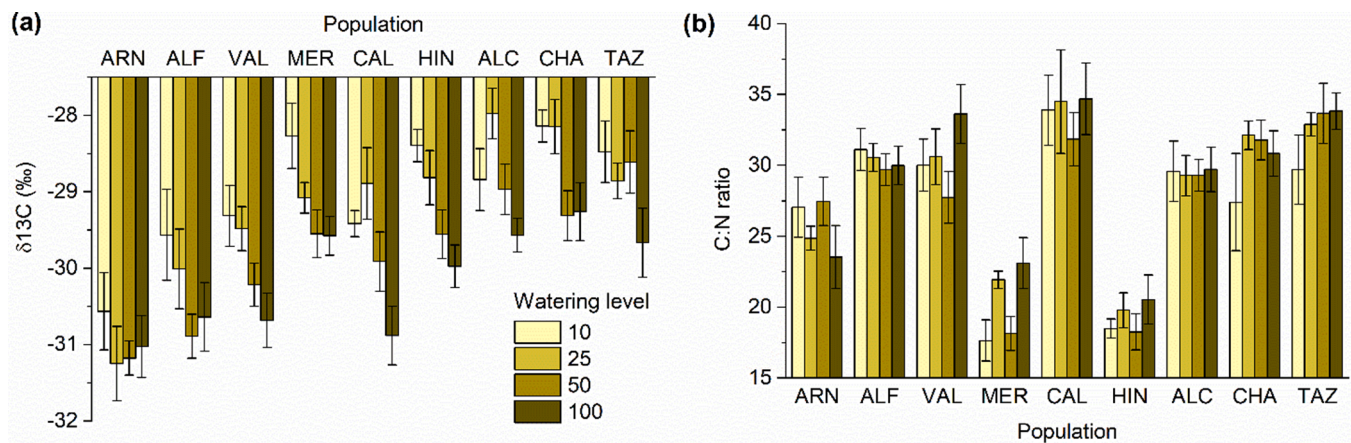
Fig. 5. Responses of carbon isotopic discrimination (δ^{13}C, a) and leaf carbon to nitrogen ratio (C:N, b) to terminal drought for each one of the watering treatments and provenances. 100, 50, 25 and 10 means saturated soil at 100 %, 50 %, 25 % and 10 % of water holding capacity, respectively. The x-axis represents the populations where the acorns were collected, sorted out from north to south, being ARN and TAZ the northern and southern most populations, respectively. Predictors effects were calculated using equations for best models reported in Table 2 and parameters values in Table A.4. Differences among watering treatments for all populations after Tukey post hoc test are denoted by different letters: 10: a, 25: a, 50: b, 100: b (a) no differences were found (b). Error bars represent $\pm$ SE.
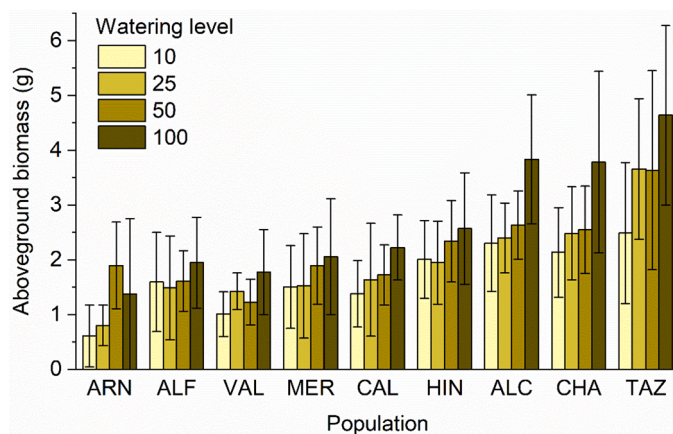

Fig. 6. Responses of aboveground biomass to terminal drought for each one of the watering treatments. 100, 50, 25 and 10 means saturated soil at 100 %, 50 %, 25 % and 10 % of water holding capacity, respectively. The x-axis represents the populations where the acorns were collected, sorted out from north to south, being ARN and TAZ the northern and southern most populations, respectively. Predictors effects were calculated using equations for best models reported in Table 2 and parameters values in Table A.4. Differences among watering treatments for all provenances after Tukey post hoc test are denoted by different letters: 10: a, 25: ab, 50: b, 100: c. Error bars represent $\pm$ SE.

all populations when responding to the terminal drought (Figs. 2a and A.1 a), whereas Ci/Ca concentration consistently increased regardless the provenance (Fig. 3). The gradual decrease of stomatal conductance and photosynthetic rate with increasing water deficit is a characteristic response of plants in general and oak species in particular (Cotrozzi et al., 2016; Ghanbary et al., 2017), and one of the main physiological strategies to cope with drought. Imposed drought effects were most severe for the southernmost populations (ALC, CHA and TAZ), with reductions of the stomatal conductance and photosynthetic capacity of oak seedlings reaching negative values (Figs. 2a and A. 1 a). Interestingly, this diminished physiological performance of southern populations was noted from the emergence phase, even before the application of the watering treatments. Thus, southern seedlings showed a conservative strategy for stomata control in response to changes in soil water availability in order to reduce transpiration under water stress. Stomatal conductance is a main driver of hydraulic changes that determine the plant response to environmental stress and is tightly

correlated with carbon fixation (Flexas et al., 2014). A close relationship between photosynthetic rate and stomatal conductance as observed in this study, was reported by other studies in *Quercus* species (Damesin and Rambal, 1995; Peñuelas et al., 1998) and in cork oak (Faria et al., 1998). The reduced photosynthetic rate responding to water stress could be due to the decrease in stomatal conductance, but also to mesophilic changes such as changes in the intercellular CO_2 concentration (Cotrozzi et al., 2016), finally leading to a reduction in photochemical efficiency of photosystem II. The response of photosynthesis to water shortage in *Quercus* species growing under natural conditions has been extensively reported (Damesin and Rambal, 1995; Faria et al., 1998; Salleo and Gullo, 1990). Negative photosynthetic values during the terminal drought period in the southernmost populations imply that leaf respiration predominates over photosynthesis (Gallé et al., 2007) and there is a net carbon loss, which would eventually lead to carbon starvation and plant death (McDowell et al., 2008). Conversely, we found that seedlings from northern provenance maintained positive carbon assimilation when subjected to the same terminal drought, being able to keep the carbon gain for a longer period regardless of the water shortage (Figs. 2 and A. 1). This result directly relates to the pattern found in our previous report, which described how cork oak seedlings from the northern distribution range had a higher survival rate compared to those from the south (Morillas et al., 2023).

Given the paramount importance of water availability for plant performance, we expected that cork oak southern populations influenced by drier climate would display higher efficiency in the use of water compared to northern populations. In agreement with this hypothesis, we found a clear trend towards less negative δ^{13}C values in the southern populations (Fig. 5) supported by the inclusion of the "provenance" factor in the best linear model for this variable (Table 2). This result supports a marked geographical increase in the water use efficiency with decreasing latitude across the species' distribution range. We further found that lower water availability yielded less negative δ^{13}C values (Fig. 5). The fact that this pattern was consistent across populations implies that seedlings from the whole distribution range can modulate their water use efficiency according to environmental conditions. Cork oak has been previously acknowledged as a highly plastic drought-resistant species, which can display a conservative water use (Besson et al., 2014; Grant et al., 2010). The significant increase of δ^{13}C values in cork oak leaves towards the most xeric end of our gradient (Fig. 5) suggests that this species can largely adjust the gaseous exchange at the leaf level according to local water availability. Thus, southern populations of cork oaks may maintain carbon assimilation by

minimizing water loss through fine-tuned stomatal control (Otieno et al., 2007) and by extracting water from deep soil reserves (David et al., 2007; Kurz-Besson et al., 2006), resulting in higher water use efficiency. These findings concur with our previous report in which we found that the same cork oak southern populations analyzed in this study showed a clear trend toward maximizing traits related with belowground growth (i.e., root surface area, root average diameter and root volume) to increase deep water uptake (Morillas et al., 2023). Our findings are in line with previous research relating increased water use efficiency values to local aridity for the studied species (Gouveia and Freitas, 2009) and other tree species (Damesin et al., 1997; Moore et al., 1999; Pérez-Ramos et al., 2013; Sun et al., 2003), and imply that not all cork oak populations are equally vulnerable to water stress.

In this study, we found that cork oak seedlings originating from the southern distribution range displayed larger aboveground biomass than those from the northern distribution area (Fig. 6). These results are in agreement with our previous report in which we identified the larger acorn size found in the southern populations as a determining trait influencing cork oak seedling growth in this same study area (Morillas et al., 2023). The relevance of seed size for seedling growth has been previously acknowledged by large empirical evidence (Baraloto et al., 2005; Pérez-Ramos et al., 2010; Ramírez-Valiente et al., 2009). Another factor potentially contributing to the differential aboveground biomass developed across provenances is the contrasting water use efficiency displayed by cork oak populations distributed throughout the species distribution range. The positive relationship between water use efficiency and aboveground biomass reported in this study would allow seedlings from southern provenance to fix a larger amount of carbon per unit of water transpired, which would eventually result in larger biomass compared to seedlings from northern provenance. Although most of studies exploring the relationship between water use efficiency and aboveground growth or biomass in water-limited environments report a positive relationship (e.g., Dudley, 1996b, 1996a; Ludwig et al., 2004), some studies yielded neutral (Ludwig et al., 2004) or even negative relationships (Donovan et al., 2007).

Conclusions

The results of this research support the presence of a marked geographical gradient of physiological adaptations to water stress across the complete latitudinal distribution of cork oak. Despite that the analysis of the stress indicator metabolites (proline and MDA) ruled out the differential water stress level among northern and southern provenances, seedlings from the southern limit seem to be less vulnerable to experimental drought compared to populations located in the central or northern parts of the distribution. Southern seedlings exhibited local adaptations to water shortage such as a higher sensitivity of stomata to changes in soil humidity, adjusting a better stomatal control of transpiration in response to drought, which allows for a fine-tuned physiological performance. Accordingly, seedlings from the more xeric provenance at the southern edge displayed the highest water use efficiency, increasing thus their resistance to drought. These physiological adaptations of southern-range seedlings to local conditions, along with other morphological traits related to drought resistance (i.e., larger acorn size or root investment, Morillas et al., 2023), resulted in larger aboveground biomass and higher drought resistance at the southern distribution limit (Matías et al., 2019b). Beyond these geographical differences, we also found evidence for phenotypic plasticity in terms of physiological traits in the response of cork oak seedlings to different levels of water availability. Thus, the plasticity of the different cork oak ecotypes, and in particular physiological traits, may be used to buffer the response of tree populations under a global change scenario, where extreme drought events are expected to increase in severity and frequency. Our results suggest that despite the potential impact of increasing aridity on the northern most populations, the southern populations may hold genetic diversity that would influence the potential of

the species to adapt to novel conditions. The intraspecific genetic diversity harbored by southern populations may provide an important adaptive advantage in response to the forecasted increased drought in the Mediterranean Basin. Thus, this ecotype plays an important role in terms of biodiversity conservation and features a high potential for restoration programs in more xeric environments. Notably, these areas are worthy of deeper scientific research and public awareness for preserving cork oak populations from extinction.

CRediT authorship contribution statement

Lourdes Morillas: Writing – original draft, Visualization, Software, Formal analysis, Data curation. **María José Leiva:** Writing – review & editing, Investigation. **Jacinto Gandullo:** Writing – review & editing, Investigation. **Ignacio M. Pérez-Ramos:** Writing – review & editing, Investigation. **Jesús Cambrollé:** Writing – review & editing, Investigation. **Luis Matías:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data is available in an open repository https://doi.org/10.12795/11441/156332.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100432.

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