

# Short range shifts in plant physiological responses to induced water stress: Experimental evidence of intraspecific trait variability differentiating neighbouring Mediterranean plant populations

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## ARTICLE INFO

### Keywords:

Aridity  
Drought  
Environmental variability  
Local homogeneity  
Intraspecific variability  
Plant functional traits

## ABSTRACT

Mediterranean plants are facing increased periods of drought, potentially jeopardizing their survival. Despite the known influence of intraspecific variability on plants' responses to the environment, most studies focus on differences between species and overlook short distances. Following the observation of three nearby populations of *Quercus ilex* and *Cistus salvifolius* inheriting different sets of drought-related functional traits in common garden, we tested if these populations could perform differently under water stress. We observed drought responses on plantlets from both species, measuring key physiological traits such as gas exchanges and spectrometric indexes. Our results show similar responses from populations of *C. salvifolius* while *Q. ilex* had high intraspecific variability, with increased drought-resistance in plants hailing from dry provenances, reflected by improved water use efficiency, photosynthetic capacity, and reduced metabolic response to stress. These observations support the idea that, despite the short distances, neighbouring plant populations can adapt differently to drought, changing the way they withstand climate and its changes.

## Abbreviations

ARI1	anthocyanin reflectance index 1
ARI2	anthocyanin reflectance index 2
BTV	between species trait variability
CE	apparent carboxylation efficiency
Ci	intercellular CO <sub>2</sub> concentration
CR1	carotenoid reflectance index 1
CR2	carotenoid reflectance index 2
E	transpiration rate
ETR	electron transport rate
Fv/Fm	maximal quantum yield of PSII photochemistry
gs	stomatal conductance
ITV	intraspecific trait variability
NDVI	normalized difference vegetation index
PFT	plant functional trait
P <sub>N</sub>	net photosynthetic rate
PRI	photochemical reflectance index
RWC	relative water content
WUE	water-use efficiency (= PN/E)
IWUE	intrinsic water-use efficiency (= PN/g <sub>s</sub> )

ΦPSII effective quantum yield of PSII photochemistry

## Introduction

In the last decades, drought events have been consistently increasing in several parts of the world, with their occurrence projected to increase both in frequency and intensity (IPCC 2023). Plants mainly experience drought through water stress, which is known to widely affect plant physiology and photosynthetic capacity: water stress can induce decreases in water potential and stomatal opening, leading to reduced photosynthetic capacity and subsequently increased photooxidation, due to the accumulation of reactive oxygen species (ROS) (Osakabe et al., 2014).

Given their sessile nature, plants need to adopt several adaptive strategies to deal with water stress, aimed at either avoiding, tolerating, or completely escaping drought: they can withstand aridity by carefully balancing their metabolism to maintain homeostasis, shift towards a different balance that allows for reduced water availability within their tissues or change their life cycle to completely avoid dry periods (Basu et al. 2016). These strategies are not mutually exclusive, but their adoption requires acquiring suitable plant functional traits (PFTs) that

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<https://doi.org/10.1016/j.stress.2024.100556>

Received 18 April 2024; Received in revised form 7 July 2024; Accepted 29 July 2024

Available online 2 August 2024

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will inevitably lead to some trade-offs (Violle et al. 2007; Wright et al. 2005).

The connection between PFTs and adaptive strategies is so strong that trait distributions have been effectively used, along with environmental niches (Thuiller et al. 2005), to forecast future species distributions (Heilmeier 2019). Earlier attempts in this field were mostly focused on between-species variability (BTW), until the scientific community began to address the importance of intraspecific variability (ITV), i.e. the intrinsic variability observed within each species, in shaping plants' ability to respond to climate change (Albert et al. 2011; Matesanz and Ramírez-Valiente 2019; Siefert et al. 2015).

Despite the rising attention towards ITV, this factor is still largely neglected in favour of studies focused on comparing differences between species (Moran et al. 2016; Siefert et al. 2015) and, even when considered, its source is often ignored (Lajoie and Vellend 2018; Matesanz and Ramírez-Valiente 2019). ITV is indeed driven by two main factors: genetic adaptation and phenotypic plasticity (Ghalambor et al. 2007). The former introduces heritable differences that allow specific populations to increase their fitness to their own environment while the latter allows each individual of a species to respond to their own perceived environment with an array of plastic responses that can vary within populations, individuals and even within the individual itself (Albert et al. 2011). Logically, plasticity-driven ITV will not shift a population ability to withstand drought, as the perceived variation will only be due to the conditions in which the population is being observed. Conversely, adaptation-driven ITV introduces population-specific traits that will be inherited by subsequent generations, potentially introducing a differentiating factor that may change populations' response capabilities.

A common habit in ecology studies focused at looking into plant ITV is to include distant populations as potential *ecotypes*. An ecotype is a population which can be distinguished "by a composite of variation in many traits and allele frequencies across loci over space" (Lowry 2012), i.e. which shows several heritable properties that allow the group to thrive in a specific environment. This variation requires isolation, and in natural conditions this is often ensured with distance. For example, pollen from the *Quercus* genus can travel up to 65 Km from its source (Maya-Manzano et al. 2016), generally prompting for the use of longer distances for this kind of studies. However, while geographical distances can introduce major environmental variation while minimizing genetic fluxes between populations, this should not lead to the conclusion that distance is necessary for the emergence of ecotypes: as a matter of fact, climate heterogeneity plays a far more important role in shaping natural selection (Hufford and Mazer 2003), and local climate heterogeneity can be quite common (Ford et al. 2013; Garcia et al. 2022; Opedal et al. 2015). Nevertheless, neighbouring and proximate populations are still often assumed to be homogeneous, by simply arguing with the role of locality due to the short distances involved (Iozia and Varone 2023), despite the multiple reported examples of local ITV reported from literature (Costa-Pereira et al. 2018; Linhart and Grant 1996). While ITV at short distances is often disregarded, it has been recently observed that several species from central Italy, placed on the extremes of a local aridity gradient, show potentially adaptive divergences between close-by provenances (with a mean distance between sites of 64.4 Km; Iozia et al. 2023), with multiple PFTs that can be observed diverging even after being grown in a common garden (Iozia and Varone 2023).

In particular, the two Mediterranean species *Quercus ilex* and *Cistus salviifolius* both adopted significantly different PFTs distributions between provenances when grown in a common garden (Iozia and Varone 2023), despite *C. salviifolius* appearing far less plastic than *Q. ilex* when observed in situ from a multi-dimensional trait perspective (Iozia et al. 2023). Similar conclusions could be obtained by scaling up observations: *Q. ilex* is a species known to show a very high degree of ITV with multiple ecotypes across the Mediterranean (Peguero-Pina et al. 2014) which are renowned for their different capabilities to withstand drought (Martin-StPaul et al. 2011; Navarro-Cerrillo et al. 2018), while *C. salviifolius* shows a complex pattern of variation that appears to not be

linked to climate adaptation (Farley and McNeilly 2000). However, a main limitation of these reports was that they never investigated the physiological ties between drought and adaptation: drought resistance was simply assessed from the nature of the observed differences in PFTs.

This leads to an interesting question to resolve: *do local divergences in PFTs shape each populations' capabilities to withstand drought?* The implications of this question are quite thrilling: are the divergences observed on PFTs reflected by variations in physiology? Is it possible that populations distant a few tens of Km from each other might respond differently to climate change? Should we consider them neighbouring ecotypes? The choice of working with two different species also allows us to evaluate whether phenotypic changes between populations could be similar across them.

A feasible way to answer these questions is to monitor plant physiology, a task which can be accomplished in multiple ways: gas-exchanges can be directly measured to keep track of net photosynthetic rate ( $P_N$ ), stomatal conductance ( $g_s$ ), and multiple indexes related to water use; moreover, the effective ( $\Phi_{PSII}$ ) and maximal quantum yield ( $F_v/F_m$ ) of PSII, along with other spectral indexes of photochemical, anthocyanin and carotenoid leaf internal status can be measured via spectrometry to monitor plant metabolism. The advantage of coupling multiple approaches is that this can overcome the limitations of each single approach: gas exchanges can provide punctual information about the photosynthetic status of the plant in the range of seconds, reflectance indexes provide insights into the status of the photosystems in the scale of hours and photochemical reflectance is bound to the accumulation of metabolites, thus responding in the scale of days.

All these traits are affected by drought in some way: as hydric stress increases plants initially react by reducing stomatal opening, reflected by  $g_s$ , (Osakabe et al. 2014), which slows down water losses at the expense of losing access to the atmospheric  $CO_2$ . This ultimately affects  $P_N$ , which lowers, and may even alter water use efficiency (Ullah et al. 2019). Quantum yield may also vary in response to drought, as it can be observed for *Q. ilex*, with declines both in  $\Phi_{PSII}$  and  $F_v/F_m$  (Chaves et al. 2002). Moreover, with stress ROS increase and plants need to react by deploying defensive antioxidant metabolites such as anthocyanins (Xu and Rothstein 2018) and carotenoids (Havaux 2014), in order to limit oxidative damage.

Considering our populations, we would expect a case of adaptation to be directly reflected in higher  $P_N$  rates from dry provenances under hydric stress, as a consequence of a more efficient water use, lower physiological damage (monitorable with carotenoid and anthocyanin content and NDVI) and possibly higher  $g_s$ . These metabolically expensive adaptations would allow plants from drought-subjected sites to survive aridity better than their wet-sites neighbours, especially for species that rely on resistance such as *Q. ilex* (Laureano et al. 2008).

To find the answer, we performed an experiment: subjecting the same plants of *Q. ilex* and *C. salviifolius* that were grown in a common garden by Iozia and Varone (2023) to an induced water stress, we observed how plants from neighbouring populations that show adaptively divergent PFTs respond to typical drought conditions, accurately measuring their gas exchanges and physiological responses. The choice to work on two different species allowed us to further refine our investigation, understanding the complexity of drought responses and how co-adapted species might respond to this stress factor.

## Materials and methods

### Study species

The species involved in this study are *Quercus ilex* L. (holm oak) and *Cistus salviifolius* L. (sage-leaved rock rose), two species likely evolved in the Mediterranean climate (Blondel and Aronson 1999; Correia and Catarino 1994), which are often found co-occurring in central Italy. Despite the common habitat, characterized by wet winters and warm, dry summers (Zunzunegui et al. 2011), these two species are deeply

different.

*C. salvifolius* is a semi-deciduous plant (Harley et al. 1987), capable of avoiding drought by shedding its leaves every four to eight months to adopt thicker, more resistant leaves during the dry period (Grant et al. 2014). Conversely, *Q. ilex*'s functional type is evergreen sclerophyllous (Martín-Sánchez et al. 2022), meaning its main strategy is to withstand drought with the adoption of thicker, more physiologically expensive leaves and deeper roots that optimize water inputs and outputs.

Moreover, while *Q. ilex* is often found as a tree, dominating mixed forests at the climax stage (Poissonet et al. 1978), *C. salvifolius* is a shrub which prefers glades, exposed to the typical direct sunlight of pioneer stages (Grant et al. 2014). This species is among the first to colonize clearings opened by wildfires, due to an adaptation which induces germination after its seeds are exposed to very high heat (Trabaud and Oustric 1989).

#### Plant material and original provenances characterization

One-year old plantlets were obtained from the experimental garden of Sapienza University of Rome (42 m asl, 41°54'8.07" N, 12°31'2.65" E), after being grown in 8 litres plastic pots with a ratio of 4 to 1 of soil and expanded clay respectively (Ondoño et al. 2015), as described by Iozia and Varone (2023).

Both species were obtained from seeds collected in three common sites with a mean distance from each other of 64.4 Km, chosen along the extremes of the *Latium* region's aridity gradient. Provenances were characterized by Iozia and coworkers (2023) with the Chelsea Climate model (Karger et al. 2017) and validated with the nearest meteorological stations (ARSIAL 2022), coupled with edaphic analyses the results of which are hereby reported.

Provenance Site A, *Castel Fusano*, is located at 41°43'23.6" N, 12°19'55.7" E, its mean elevation is 3 m.a.s.l. and it's subjected to an intense summer drought regime. Its mean total annual rainfall is 847.7 mm, and annual mean temperatures oscillate between a minimum of 9.9 °C and a maximum of 22.3 °C, with a mean of 16.0 °C. Coupled with a high sand content (~90 % of sand, ~5 % silt, ~5 % clay), this site can be considered among the driest sites of the region.

Provenance Site B, *La Farnesiana*, is located at 42°11'38.9" N, 11°52'33.1" E, its mean elevation is 150 m.a.s.l. and its summer drought regime is also fairly intense. With mean total annual rainfall of 938.9 mm, mean annual temperatures oscillating between 9.5 °C (minimum) and 21.4 °C (maximum) with a mean of 15.1 °C, and mostly sandy soils (~70 % sand, ~20 % silt, ~10 % clay), this site can be considered as an intermediate site, relatively dry to the rest of the region.

Provenance Site C, *Tivoli*, is located at 41°57'51.5" N, 12°48'54.9" E, its mean elevation is 430 m.a.s.l. and its summer drought regime is almost non-existent. Mean total annual rainfall amount to 1306.1 mm, and mean annual temperatures oscillate between a minimum of 8.1 °C and a maximum of 22.1 °C, with a mean of 13.9 °C. Soils from this site are also relatively siltier (~60 % sand, ~25 % silt, ~15 % clay). This site is therefore among the wettest sites of the region.

#### Experimental design

All measures were conducted in the experimental garden of Sapienza University of Rome. In July 2023, eight one-year-old plantlets were randomly chosen for each species and for each provenance, and randomly arranged inside a sun-exposed greenhouse that was kept with opened windows to allow for air circulation. Moreover, inside the greenhouse the position of plants was switched daily to avoid micro-climatic bias.

On the 3rd of July, after an adaptation period of four weeks during which plants were regularly watered to field capacity (measured with a HydroSense II Handheld Soil Moisture Sensor, Campbell Scientific, Logan, UT, USA), we randomly selected five individuals from each species and from each provenance to conduct the induced water stress

experiment. We opted for this sample size because it is considered the minimum necessary to maintain representativeness (Pérez-Harguindeguy et al. 2016) while still allowing us to quickly perform our sampling under comparable environmental conditions.

The remaining three plants per site per species were used as a control group. Water stress was induced by withholding water throughout the experiment. Leaf gas exchange measurements and leaf spectral measurements were carried out on each plant every two to three days, while the hydric status of plants was monitored with the leaf relative water content (RWC). To ensure each group was kept in similar edaphic conditions, we also monitored soil moisture by measuring volumetric water content (VWC,%) with a handheld soil moisture sensor (HydroSense II, Campbell Scientific, Logan, UT, USA) on each pot at dawn ( $p > 0.05$ , ANOVA; data not shown). The water stress experiment continued until the mean  $g_s$  from stressed plants would reach  $0.05 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$  or below, as this threshold is commonly considered indicative of a severe water stress condition among all  $C_3$  plants (Medrano et al. 2002).

The end of the experiment was reached after 16 solar days, when the last group of individuals reached the critical threshold. During the entire experiment, climate was monitored every half hour, with two dataloggers (HOBO UX100-011A Temp/RH, 2.5 %; Onset, MA, USA) placed inside the greenhouse, while air temperature and relative humidity were also measured with a handheld thermo-hygrometer (CP 11, Rotronic, CH) before and after each measuring session.

Throughout the experimental period, from the 3rd to the 19th of July, daily mean temperature (between dawn and sunset) inside the greenhouse was  $31.95 \pm 9.59 \text{ °C}$  and mean relative air humidity was  $60.77 \pm 21.71\%$ . During measuring times, which lasted from approximately 9am to 11am, mean air temperature was  $31.13 \pm 4.21 \text{ °C}$  and mean air humidity was  $50.30 \pm 11.23\%$ . These temperatures are similar to the temperatures naturally experienced by plants during summer at midday at their original locations (ARSIAL 2022; Karger et al. 2017). Temperatures at the time of measurement and relative humidity were also employed to determine leaf vapour pressure deficit (VPD, kPa), following the equations presented by Jiao and coworkers (2019).

#### Leaf water status

Relative water content (RWC,%) was measured on fully expanded upper leaves every two days, concurrently to the other measurements. RWC was calculated on single leaves from each plant using the leaf fresh mass (FM, g) measured right after leaf collection, the leaf water-saturated mass (SM, g) measured after a complete rehydration at 5 °C in the darkness for 24 h, and the leaf dry mass (DM, g) measured after drying at 90 °C with an oven for 72 h to ensure complete dehydration. To calculate RWC, we used the formula  $\text{RWC} = (\text{FM} - \text{DM}) / (\text{SM} - \text{DM}) \times 100$ , following Pepe et al. (2022).

#### Gas exchanges

Gas exchanges and photosynthetically active radiation (PAR,  $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ ) were measured with an infrared gas exchange analyser (ADC LCPro+, ADC, Hoddesdon, UK), using a  $6.5 \text{ cm}^2$  leaf chamber exposed to natural environmental conditions. To minimize diurnal photoinhibition (Werner et al. 2002) and reduce bias, measurements were all carried out in light-saturating conditions ( $\text{PAR} > 1000 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ ) from 8am to 10am, employing single fully expanded upper leaves of comparable age and light exposure from each plant. Original measurements included transpiration rate ( $E$ ,  $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), sub-stomatal  $\text{CO}_2$  concentration ( $C_i$ , ppm), stomatal conductance ( $g_s$ ,  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), and net photosynthetic rate ( $P_N$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ); from which we calculated the instantaneous water use efficiency (WUE,  $\mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$ ), the intrinsic water use efficiency (IWUE,  $\mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}$ ), and the apparent carboxylation efficiency (CE,  $\text{mol}^{-1}$ ) using the following formulas:  $\text{WUE} = P_N / E$ ,  $\text{IWUE} = P_N / g_s$ ,  $\text{CE} = P_N / C_i$ .

### Leaf chlorophyll fluorescence

The effective quantum yield ( $\Phi\text{PSII}$ ) and the maximal quantum yield ( $F_v/F_m$ ) of PSII were measured with a handheld PAM fluorometer (FluorPen FP 110, Photon Systems Instruments, Drásov, CR) on the same leaves used to carry out the gas exchange measurements, on each measuring day, right after gas-exchanges measurements between 10am to 10:30am.  $\Phi\text{PSII}$  was calculated on light-adapted leaves as  $\Phi\text{PSII} = (F_m - F_s)/F_m'$ , with  $F_m'$  being the maximal fluorescence yield under a light-saturating pulse of  $8'000 \mu\text{mol (photons)} m^{-2} s^{-1}$  and  $F_s$  the steady-state fluorescence yield of leaves illuminated at  $1'600 \mu\text{mol (photons)} m^{-2} s^{-1}$ , according to Genty et al. (1989). Since  $\Phi\text{PSII}$  is equivalent to  $F_v/F_m$  in dark-adapted samples,  $F_v/F_m$  was simply obtained by using the same instrument on leaves that were adapted to dark conditions for at least 25 min.  $\Phi\text{PSII}$  was also used to calculate the electron transport rate (ETR,  $\mu\text{mol (electrons)} m^{-2} s^{-1}$ ), using the equation  $\text{ETR} = \Phi\text{PSII} \times \text{PAR} \times 0.5 \times 0.84$  (Krall and Edwards 1992).

### Leaf reflectance indexes

Reflectance spectra were obtained from the same single leaves used in gas-exchanges and leaf chlorophyll fluorescence from each plant on each measuring day, using a radiometrically-calibrated handheld spectroradiometer (LM 510-V/UVIS, Photon Systems Instruments, Drásov, CR) with a wavelength range of 340 to 790 nm. Measurements were carried out right after leaf chlorophyll fluorescence measurements, between 10:30am and 11am. After calibrating the instrument with a standard calibration plate (Photon Systems Instruments, Drásov, CR), leaves would be directly placed in the leaf compartment of the instrument with the adaxial side of the leaf directed towards the sensor. Specific wavelengths obtained from the reflectance spectra were then used to calculate the normalized difference vegetation index (NDVI) as  $\text{NDVI} = (780 \text{ nm} - 630 \text{ nm}) / (780 \text{ nm} + 630 \text{ nm})$ , the photochemical reflectance index (PRI) as  $\text{PRI} = (531 \text{ nm} - 570 \text{ nm}) / (531 \text{ nm} + 570 \text{ nm})$ , the carotenoid reflectance indexes (CR1 and CR2) as  $\text{CR1} = (1 / 510 \text{ nm}) - (1 / 550 \text{ nm})$  and  $\text{CR2} = (1 / 510 \text{ nm}) - (1 / 700 \text{ nm})$ , and the anthocyanin reflectance indexes (ARI1 and ARI2) as  $\text{ARI1} = (1 / 550 \text{ nm}) - (1 / 700 \text{ nm})$  and  $\text{ARI2} = 790 \text{ nm} \times [(1 / 550 \text{ nm}) - (1 / 700 \text{ nm})]$ . NDVI can be considered as an index of greenness, commonly used to delineate vegetative stress (Huang et al. 2021), while PRI can be reasonably seen as a plant health index given its correlation with chlorophyll fluorescence parameters and de-epoxidation state of the xanthophyll cycle (Peguero-Pina et al. 2008).

### Statistical analysis

For this study, we initially measured a wide range of PFTs that we subsequently refined into a smaller subset of variables. Relationships between all the considered physiological traits (RWC,  $\Phi\text{PSII}$ ,  $F_v/F_m$ , ETR, NDVI, PRI, CR1, CR2, ARI1, ARI2, Ci, E, gs,  $P_N$ , WUE, IWUE, and CE) were first explored by carrying out Pearson's linear correlation analysis, applying a False Discovery Rate to the results to reduce the occurrence of Type 1 error (Fig. 1S). The resulting correlation matrices were then used to identify highly correlated PFTs that could be safely discarded from further analysis given their proven functional redundancy.

To answer our main question, two-way PERMANOVA was then performed on each species separately, using Mahalanobis distances to take into account the covariance structure observed in the data, set to 10'000 permutations. Tests were run using provenance and time since last hydration as independent factors. The first factor served to determine whether we could find general differences between groups hailing from each site, while the second factor could be considered as a response to water stress intensity.

Each individual variable was then further analysed by performing two-way ANOVAs, using the same factors previously used for the

PERMANOVA (site and time since last hydration), followed by post-hoc Tukey's test. This served to assess the presence of significant variation between sites for each trait, while the sampling day was used to determine the effects of water stress. Two-way ANOVA on RWC was also used to effectively determine water stress itself.

The magnitude of the water stress response was further assessed using the Hedges' G effect size index (Sullivan and Feinn 2012), a statistical tool designed to quantify the amount of variation between groups, allowing to overcome the limitations of simply relying on p values to determine significance. This index was chosen because it provides a bias correction to the more commonly used Cohen's D for small sample sizes. For this last batch of tests, Effect size was calculated as the difference in trait means between control values (represented by values measured at Day 0) and measurements from the last day of the experiment for each species divided by the pooled standard deviation. Our results were then compared to the categories suggested by Cohen (1988), which assess relative size values between 0.2 and 0.5 as small, values between 0.5 and 0.8 as medium, and values over 0.8 as large.

For all analysis, the significance level was fixed at  $p \leq 0.05$ . Two-way PERMANOVA and two-way ANOVA were carried out using the PAST statistical software (Hammer 2001), while Pearson's linear correlation analysis was performed using the function *corr.test* from the *corrplot* 0.92 package (Wei et al. 2017) for the software R 4.0.3 (R Core Team 2022). The effect size was computed using the *effectsize* 0.8.2 package (Ben-Shachar et al. 2021). Data were hereby shown as mean  $\pm$  standard deviation ( $n = 5$  plants).

## Results

### Leaf water status

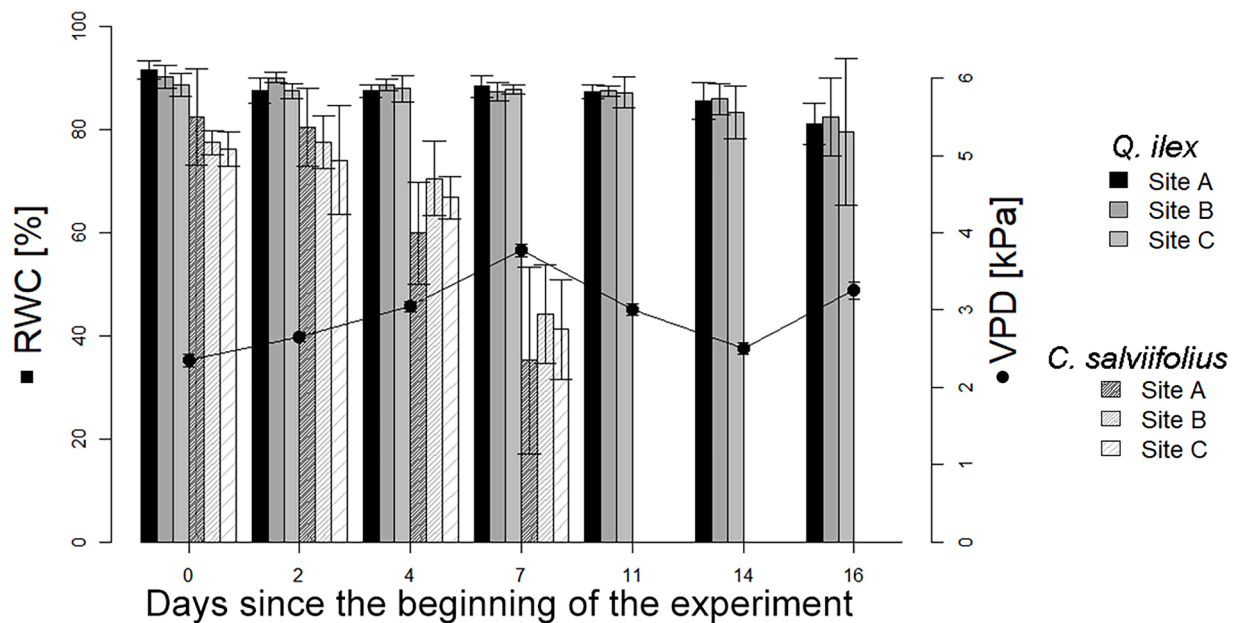
The water stress experiment lasted a total of 16 solar days, as the limit threshold was reached on the 10th of July by *C. salvifolius*, and on the 19th of July by *Q. ilex*. During the whole study period, VPD did not vary significantly between populations ( $p = 0.873$ , ANOVA), and it fluctuated between  $2.35 \pm 0.08$  kPa and  $3.77 \pm 0.08$  kPa, corresponding to conditions typically experienced by these species during summer (Oliet and Jacobs 2007; Prévosto et al. 2020) and reaching a peak on Day 7 (Fig. 1).

As expected, two-way ANOVA on RWC (Table 1) showed a significant decrease for both species ( $p < 0.001$ ) during the experiment, reflecting an increase in water stress (Fig. 1). Post-hoc Tukey test reveals that *C. salvifolius*' RWC began to fall on the 7th of July, after 4 days of being withheld water, while *Q. ilex* could maintain its leaf water status stable up to 17th of July, corresponding to 14 solar days since the beginning of the experiment.

The same two-way ANOVA revealed that plants of both species from each provenance maintained similar levels of RWC within their leaves ( $p = 0.33$ ) when considering sites as a factor, associated with a not significant day  $\times$  site interaction factor ( $p = 0.35$ ). This observation allows us to consider time as a good proxy for water stress in the following analyses, given RWC steadily decreases over time (Fig. 1) yet there are no significant differences between the way RWC decreases over time for all populations (Table 1).

### Variable selection

Pearson's linear correlation analysis carried out on all physiological traits revealed a strong and significant relationship between most of the considered traits for both species (Fig. 1S). In similar PAR conditions, ETR and  $\Phi\text{PSII}$  are connected by a linear relationship which is reflected by the high correlation found between these traits among all groups of both species. Therefore, we decided to remove ETR from the subsequent analysis. Given both CR1 and CR2 increase linearly with carotenoids content, these two variables were also found to be very highly correlated (Fig. 1S). A similar relationship exists between ARI1 and ARI2, as both



**Fig. 1.** Relative Water Content (RWC) of *Q. ilex* and *C. salvifolius*, and Vapour Pressure Deficit (VPD) during the experimental period. Measurements carried out on Day 0 correspond to control values. Data are shown as daily mean  $\pm$  standard deviation ( $n = 5$ ). Bars represent Relative Water Content, while dots represent Vapour Pressure Deficit as mean values at sampling time. Groups correspond to provenances for each species: Site A = Castel Fusano, Site B = La Farnesiana, Site C = Tivoli. Full bars represent *Q. ilex*, while dashed bars represent *C. salvifolius*.

**Table 1**

F values from two-way ANOVA carried out to test the effect of provenance, water stress (proxied by time since last hydration), and their interaction (provenance  $\times$  water stress) on several traits from *Q. ilex* and *C. salvifolius*. RWC = relative water content,  $\Phi$ PSII = quantum yield of PSII, Fv/Fm = maximal quantum yield of PSII, NDVI = normalized difference vegetation index, PRI = photochemical reflectance index, CR1 = carotenoid reflectance index 1, ARI1 = anthocyanin reflectance index 1, gs = stomatal conductance,  $P_N$  = net photosynthetic rate, IWUE = intrinsic water use efficiency, CE = apparent carboxylation efficiency.

Species	Trait	Provenance	Water Stress	Provenance * Water Stress	
<i>Q. ilex</i>	RWC	$F = 1.1$	$F = 7.5^{**}$	$F = 0.2$	
	$\Phi$ PSII	$F = 0.5$	$F = 4.4^{**}$	$F = 0.3$	
	Fv/Fm	$F = 0.4$	$F = 8.9^{**}$	$F = 0.9$	
	NDVI	$F = 12.3^{**}$	$F = 4.3^{**}$	$F = 0.9$	
	PRI	$F = 0.3$	$F = 10.4^{**}$	$F = 0.7$	
	CR1	$F = 16.1^{**}$	$F = 40.2^{**}$	$F = 6.2^{**}$	
	ARI1	$F = 5.9^{**}$	$F = 30.9^{**}$	$F = 2.4^{**}$	
	gs	$F = 3.2^*$	$F = 49.1^{**}$	$F = 0.9$	
	$P_N$	$F = 10.0^{**}$	$F = 36.8^{**}$	$F = 0.8$	
	IWUE	$F = 4.6^*$	$F = 6.8^{**}$	$F = 1.2$	
	CE	$F = 5.2^{**}$	$F = 16.9^{**}$	$F = 1.5$	
	<i>C. salvifolius</i>	RWC	$F = 0.6$	$F = 55.1^{**}$	$F = 1.1$
		$\Phi$ PSII	$F = 1.8$	$F = 40.1^{**}$	$F = 0.4$
		Fv/Fm	$F = 5.1^{**}$	$F = 125.1^{**}$	$F = 1.7$
NDVI		$F = 0.4$	$F = 4.1^*$	$F = 0.6$	
PRI		$F = 3.0$	$F = 31.4^{**}$	$F = 0.8$	
CR1		$F = 0.0$	$F = 43.7^{**}$	$F = 1.7$	
ARI1		$F = 1.6$	$F = 19.1^{**}$	$F = 1.2$	
gs		$F = 1.4$	$F = 21.0^{**}$	$F = 1.5$	
$P_N$		$F = 3.0$	$F = 61.9^{**}$	$F = 1.8$	
IWUE		$F = 0.2$	$F = 13.6^{**}$	$F = 1.7$	
CE		$F = 0.3$	$F = 20.4^{**}$	$F = 1.3$	

\*\*  $p \leq 0.001$ .

\*  $p \leq 0.05$ .

increased with Anthocyanin content (Fig. 1S), thus we opted to remove CR2 and ARI2 from further analyses to avoid redundancy.

Pearson's linear correlation analysis also supported the removal of

variables Ci, E and WUE from the dataset, given gas exchanges were measured under the same VPD across species and origins. This rendered E and gs functionally redundant, similarly to Ci and IWUE, as Pearson's correlation analysis demonstrated (Fig. 1S). Under similar VPD, WUE and IWUE are also linearly correlated, supporting the removal of one of these variables (WUE) as well.

*Multivariate analysis*

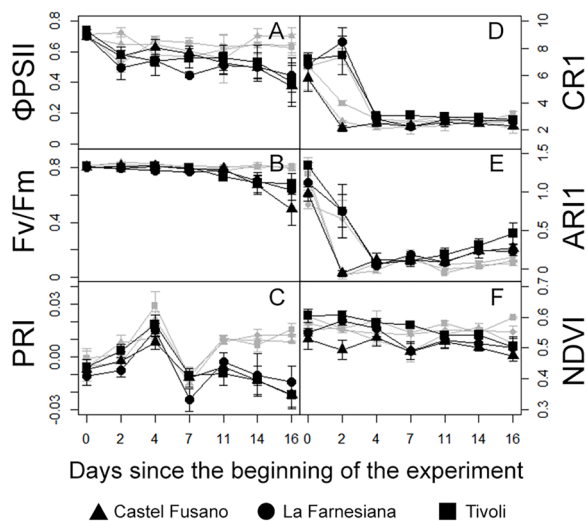
Pearson's correlation analysis highlighted how some of the remaining variables (RWC,  $\Phi$ PSII, Fv/Fm, NDVI, PRI, CR1, ARI1, gs,  $P_N$ , IWUE, and CE) still shared some level of correlation between groups (Fig. 1S), and this was taken into account in the following multivariate analysis: to account for correlations between traits, we opted to use Mahalanobis distance to compute two-way PERMANOVAs (Table 2). PERMANOVA highlighted a strong and significant effect of water stress (proxied by time since last hydration) to the overall trait variation for both species ( $p < 0.001$ ), while also highlighting a significant effect of provenance ( $p < 0.001$ ) for *Q. ilex* but not for *C. salvifolius* ( $p = 0.10$ ). For this test we opted to use time as a proxy for water stress intensity, following similar studies on *Q. ilex* (Guardia et al. 2012), and the first day of measurements as control values following Varone and Gratani (2015). This choice was allowed by the consideration that control plants that were kept under daily irrigation had maintained constant values and showed no significant variation in the considered parameters for the entire duration of the experiment (Figs. 2, 3, 4, and 5;  $p > 0.05$ , ANOVA).

**Table 2**

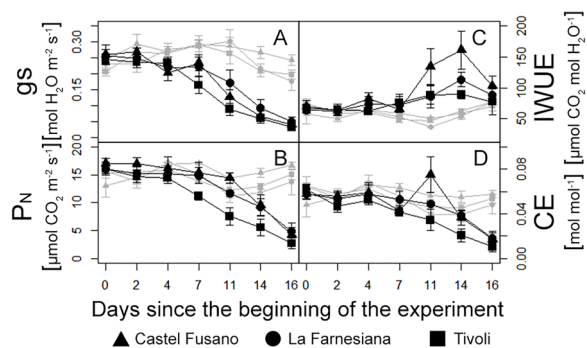
F values from two-way PERMANOVA carried out to test the effect of provenance, water stress (proxied by time since last hydration), and their interaction (provenance  $\times$  water stress) on *Q. ilex* and *C. salvifolius*.

Species	Provenance	Water Stress	Provenance * Water Stress
<i>Q. ilex</i>	$F = 3.0^{**}$	$F = 5.2^{**}$	$F = 1.0$
<i>C. salvifolius</i>	$F = 1.1$	$F = 4.7^{**}$	$F = 0.9$

\*\*  $p \leq 0.0001$ .



**Fig. 2.** (A) Quantum yield of PSII ( $\Phi$ PSII), (B) maximal quantum yield of PSII (Fv/Fm), (C) photochemical reflectance index (PRI), (D) carotenoid reflectance index 1 (CR1), (E) anthocyanin reflectance index 1 (ARI1), and (F) normalized difference vegetation index (NDVI) of *Q. ilex* during the study period. Measurements in light grey correspond to control values. Mean  $\pm$  standard error is shown as points and error bars ( $n = 5$ ; control  $n = 3$ ). Measurements from each population are represented by triangles for Castel Fusano, circles for La Farnesiana, and squares for Tivoli, respectively.



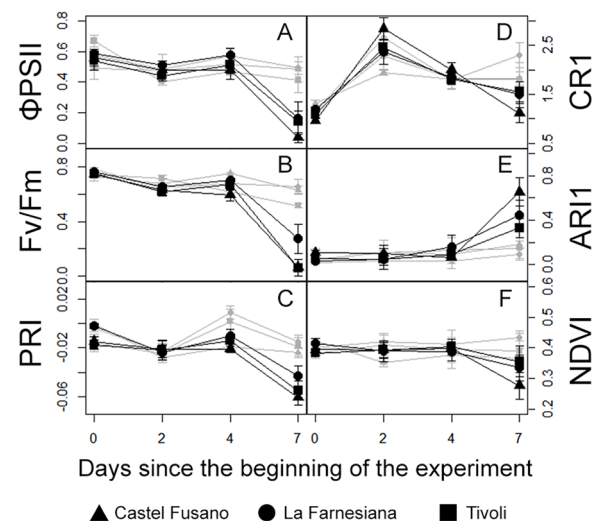
**Fig. 3.** (A) stomatal conductance (gs), (B) net photosynthetic rate ( $P_N$ ), (C) intrinsic water use efficiency (IWUE), and (D) apparent carboxylation efficiency (CE), of *Q. ilex* during the study period. Measurements in light grey correspond to control values. Mean  $\pm$  standard error is shown as points and error bars ( $n = 5$ ; control  $n = 3$ ). Measurements from each population are represented by triangles for Castel Fusano, circles for La Farnesiana, and squares for Tivoli, respectively.

### Single trait analysis

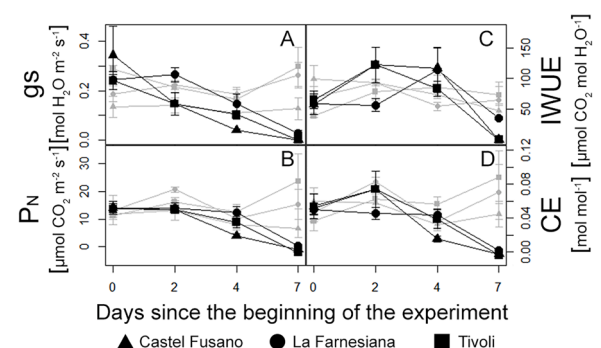
All considered traits significantly changed during the experiment (Table 1), as both species suffered a severe water stress, highlighted by the decrease in RWC, which went from  $78.74 \pm 5.00$  % on Day 0 to  $40.20 \pm 12.49$  % on Day 7 for *C. salviifolius* and from  $90.16 \pm 2.08$  % on Day 0 to  $81.03 \pm 8.58$  % on Day 16 for *Q. ilex* (Fig. 1). The physiology of both species was significantly affected, as they suffered a significant decline in RWC,  $P_N$ , gs, NDVI,  $\Phi$ PSII, and Fv/Fm. Declines were appreciably faster and more accentuated for *C. salviifolius*, as declines started appearing on Day 4 for this species (Figs. 4 and 5), while traits from *Q. ilex* mostly began to decrease on Day 11 (Figs. 2 and 3).

Considering ITV, the only trait that was found to vary significantly between sites for *C. salviifolius* was Fv/Fm ( $p < 0.01$ ): plants from site B appeared to suffer less damage to PSII, associated with mildly higher but not significant decreases in PRI ( $p = 0.06$ ) and A ( $p = 0.06$ ) (Table 1).

Overall, *C. salviifolius* was characterized by damage to PSII that



**Fig. 4.** (A) Quantum yield of PSII ( $\Phi$ PSII), (B) maximal quantum yield of PSII (Fv/Fm), (C) photochemical reflectance index (PRI), (D) carotenoid reflectance index 1 (CR1), (E) anthocyanin reflectance index 1 (ARI1), and (F) normalized difference vegetation index (NDVI) of *C. salviifolius* during the study period. Measurements in light grey correspond to control values. Mean  $\pm$  standard error is shown as points and error bars ( $n = 5$ ; control  $n = 3$ ). Measurements from each population are represented by triangles for Castel Fusano, circles for La Farnesiana, and squares for Tivoli, respectively.



**Fig. 5.** (A) stomatal conductance (gs), (B) net photosynthetic rate ( $P_N$ ), (C) intrinsic water use efficiency (IWUE), and (D) apparent carboxylation efficiency (CE), of *C. salviifolius* during the study period. Measurements in light grey correspond to control values. Mean  $\pm$  standard error is shown as points and error bars ( $n = 5$ ; control  $n = 3$ ). Measurements from each population are represented by triangles for Castel Fusano, circles for La Farnesiana, and squares for Tivoli, respectively.

began to appear since Day 2 (Fv/Fm: -14.67%), coupled with an increase of carotenoids followed by a drop (Day 2 CR1: +136.11%; Day 7 CR1: -45.49%) and gradual increases in anthocyanins that peaked on Day 7 (ARI1: +585.71%), when NDVI significantly lowered (NDVI: -20%) (Fig. 4).  $P_N$  and gs started to significantly decrease on the Day 4 ( $P_N$ : -40%; gs: -64%). CE and IWUE increased under the mild stress of Day 2 before declining, highlighting the severe physiological damage due to the water stress (Fig. 5).

Conversely, *Q. ilex* was found to significantly vary between sites, both in metabolic traits such as carotenoids and anthocyanin contents ( $p$  for CR1 and ARI1  $< 0.01$ ); punctual gas-exchanges traits such as stomatal conductance and net photosynthetic rates ( $p$  for gs and  $P_N < 0.05$ ), photosynthetic efficiency traits ( $p$  for IWUE and CE  $p < 0.05$ ); and greenness traits such as NDVI ( $p < 0.01$ ) (Table 1). The main differences were between sites A and C: plants from the dry site Castel Fusano immediately showed a decrease in carotenoids from Day 2, also

observable in its controls, when plants from the other provenances showed a peak increase followed by a drop on Day 4 that was most prominent in the wet site Tivoli (Fig. 2). Meanwhile, all three Sites showed similar trends in anthocyanins levels, with a drop on Day 2 (more severe on plants from Site A and controls) and a gradual increase from Day 7 (Day 4 for Castel Fusano) which reached higher levels from the population hailing from Tivoli, the wet site. This delay was underlined by a significant effect of the interaction between water stress (proxied by time) and Provenance on CR1 and ARI1 ( $p < 0.01$ ).

This population was also the first to respond to the water stress with decreased photosynthetic rates, as  $g_s$  and  $P_N$  began to lower on Day 7 (with a drop from  $P_N$ :  $16.07 \pm 1.73 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  and  $g_s$ :  $0.24 \pm 0.03 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$  on Day 0 to  $P_N$ :  $11.19 \pm 3.61 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  and  $g_s$ :  $0.164 \pm 0.06 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$  on Day 7) while plants from drier sites were able to maintain gas exchanges stable up to Day 11. This is reflected by photosynthetic efficiency traits such as IWUE and CE, which were significantly lower in plants hailing from Site C and significantly higher in plants hailing from site A, with site B in between but more closely resembling site C for IWUE and Site A for CE (Fig. 3). Interestingly, NDVI was initially higher for plants hailing from site C, than plants from the dry and intermediate sites, but while those groups remained stable, plants from Tivoli suffered a higher decrease in NDVI.

Our results highlight both fast and slow responses as hydric stress increases: ROS-responsive metabolic traits such as CR1 and ARI1 were quicker to respond, showing a rapid decrease in anthocyanins as soon as Day 2 (ARI1: -57.89%), also observable in the control plants, that were then followed by an increase from Day 4 to Day 16 (ARI1: +342.86%), and two different behaviours with carotenoids between sites, with an immediate decrease for plants hailing from site A (CR1: -56.21%), and a quick increase by plants hailing from sites B and C, less pronounced for the latter (Site B CR1: +24.78%, Site C CR1: +4.17%), similarly observable in control groups (Fig. 3). Similar fluctuations were also appreciable on PRI. Interestingly, this trait fluctuated during the experiment, with a temporary decrease that coincided with the highest VPD recorded, and a recovery that was significantly higher in control groups. Changes in these traits were then followed by reduced  $P_N$  and  $g_s$  on Day 11 ( $P_N$ : -31.65%;  $g_s$ : -48.00%), and only from the Day 14  $\Phi\text{PSII}$ , Fv/Fm, and CE began to lower. NDVI was found to be significantly lower only on the last day of the experiment ( $p < 0.01$ ). IWUE was found to improve under moderate stress, gradually increasing between Day 7 to Day 14 and then lowering again on the Day 16, when all populations of *Q. ilex* reached severely stressed conditions (Fig. 3).

Effect sizes for *C. salviifolius* were mostly higher in traits related to damage from water stress and reduction in photosynthetic rates and

efficiency (RWC, Fv/Fm, NDVI,  $P_N$ , and IWUE) while *Q. ilex* was found to show a higher effect size in traits related to stress response (namely CR1, ARI1,  $g_s$ ,  $P_N$  and CE) (Fig. 6). Most traits were found to suffer a large treatment effect size (Hedges'  $G > 0.8$ ), with just NDVI and IWUE suffering small to medium effects.

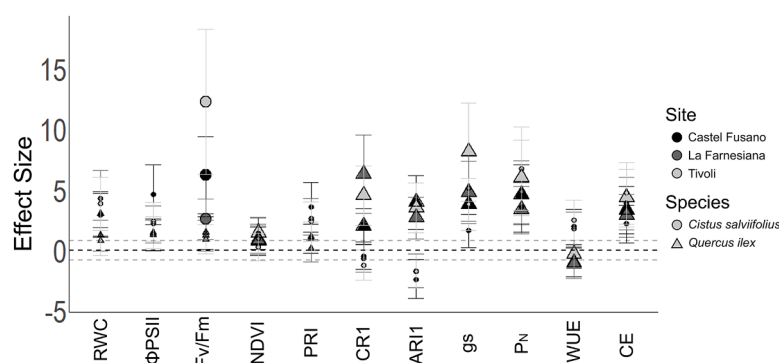
Considering ITV, the effect on *C. salviifolius*  $\Phi\text{PSII}$  was higher for Sites A and C (Hedges'  $G = 6.23$  and  $12.30$ , respectively), given plants from La Farnesiana were able to suffer a lower stress on PSII (Hedges'  $G = 2.61$ ). *Q. ilex*, on the other hand, showed a more variable response between provenances, with a higher effect of water stress on carotenoids related traits for sites B and C, a higher effect on ARI1 for site A, and the highest effect on gas exchange rates ( $P_N$  and  $g_s$ ) on plants from site C (Fig. 5). Effect size on CE was also higher on plants hailing from Tivoli. Finally, IWUE was found to show a small effect size on the population from site C (Hedges'  $G = -0.29$ ) and a large effect size on plants hailing from sites A and B (Hedges'  $G = -1.00$  and  $-1.08$ , respectively).

## Discussion

We began this investigation seeking to find out whether local divergences in previously observed plant morphology (Iozia and Varone 2023) could shape populations' capabilities to withstand drought. Our experiment allowed us to respond to this question affirmatively, as our multivariate analysis highlighted a significant effect of water stress and provenance for *Q. ilex*. Conversely, the Mediterranean shrub *C. salviifolius* showed a different behaviour, responding to the water stress experiment with an overall deterioration of physiological status that did not present significant differences between the three provenances overall.

For this conclusion, we resorted to using time since the last event of hydration as a proxy for water stress. This choice was supported by two observations: I) RWC significantly and consistently decreased over time in both species (Fig. 1) and II) there was no significant difference in RWC reductions between populations (Table 1). The first point ensures that time represents a gradual decrease in water availability while the second point ensures that these stress levels are directly comparable between populations.

The different behaviour observed between the two species is quite interesting, as both were previously found to show significantly different morphological traits between seedlings of these populations when grown in a common garden: specifically, *Q. ilex* seedlings from dry sites were found to be generally shorter, while *C. salviifolius* seedlings from the same sites had thicker, denser leaves (Iozia and Varone 2023). Perhaps more interestingly, a multivariate analysis on traits measured



**Fig. 6.** Effect size of the water stress over trait expression for *Q. ilex* and *C. salviifolius*. The comparisons were made between the first and the final respective days of the experiment: between Day 0 and Day 16 for *Q. ilex* and between Day 0 and Day 7 for *C. salviifolius*. Bigger symbols correspond to traits that show significant ( $p < 0.05$ , ANOVA) ITV. RWC = relative water content,  $\Phi\text{PSII}$  = quantum yield of PSII, Fv/Fm = maximal quantum yield of PSII, NDVI = normalized difference vegetation index, PRI = photochemical reflectance index, CR1 = carotenoid reflectance index 1, ARI1 = anthocyanin reflectance index 1,  $g_s$  = stomatal conductance,  $P_N$  = net photosynthetic rate, IWUE = intrinsic water use efficiency, CE = apparent carboxylation efficiency. Dots and error bars are Hedges'  $g$  and 95% confidence intervals. Circles represent measurements from *C. salviifolius*, triangles represent measurements from *Q. ilex*. Black symbols represent values from Site A (Castel Fusano), dark grey symbols represent values from Site B (La Farnesiana) and light grey symbols represent values from Site C (Tivoli).

on the same populations of these species in natural conditions highlighted significant variations between groups for *Q. ilex* (that were found to be shorter and have thicker leaves in the dry site) but not for *C. salviifolius* (which differences in plant height and leaf density wouldn't be detectable in a multivariate space) (Iozia et al. 2023). A possible explanation for this behaviour lies in the fact that multiple PFTs are often needed to adopt different survival strategies, and differences in single traits may not be representative of a shift in strategy, while combinations of traits may be more suitable to describe niche differences (Kraft et al. 2015). However, the high level of covariation we observed between our traits suggests the amount of traits needed to determine population's drought resistances may not be high. Responses over time highlighted a mix of drought resistance and drought avoidance for both species, as the first days of treatment were characterized by fast responses by plants' metabolism and the last days by optimizations in gas exchanges and water use efficiency.

The first sign of stress for a plant is the production of ROS, which play a crucial role in sensing stress and signalling the activation of the stress-response network that activates plants' defence mechanisms (Mittler et al. 2022). These Reactive Species are highly oxidizing, thus plants need to counterbalance their production with secondary metabolites such as carotenoids and anthocyanins, that will protect the plant from oxidation (Havaux 2014; Xu and Rothstein 2018). The sudden productions and drops of these compounds we could observe with spectrometry (CRI, and ARI1) as soon as the 5th of July reflect this need, as plants perceive the stressful environment and begin to activate their defences.

As water stress increased, we could appreciate both species changing the way they regulated gas exchanges, optimizing their water use efficiency (IWUE) with their stomatal control to the expense of  $P_N$ . Coupled with the reductions in gs that we observed in *Q. ilex* but not in *C. salviifolius*, these observations highlight how this last species appears to be less capable to control water losses through this mechanism. Damage to PSII and reductions in plant greenness (assessed by NDVI) tended to follow once the stress increased, along with a decrease in PRI in respect to control values.

It's important to remember that gas exchanges regulation is typically the first line of defence of a plant, followed by ROS increase as stress takes over (Osakabe et al. 2014), and the activation of stress response metabolism mediated by hormone-mediated pathways such as ABA (Zhang et al. 2006). Our observations are not in direct contradiction with this fact: the initial decrease in anthocyanins and carotenoids, linked to an increase in ROS (Havaux 2014; Xu and Rothstein 2018), was also observable in the control groups, suggesting the possibility of an external factor that possibly influenced all groups at the beginning of the experiment. Conversely, increases in anthocyanins observable since Day 7 mostly affected stressed plants, highlighting an increased need to control ROS that is also reflected in higher damage to the plant represented by a lower PRI.

Apart for general behaviours, our results present a significant amount of ITV for *Q. ilex*, strongly implying that this variation may be affecting its resistance to drought. The first difference we could appreciate is the lack of the production peak of carotenoids for plants hailing from provenance A. Secondary metabolism of this population seems less affected by drought, especially over the first days of the experiment. This is reflected by a lower effect size as well. Plants from Sites B and C, conversely, seem more affected by this stress as later anthocyanins levels suggest.

Gas exchanges were also found to reflect different drought resistance for these populations of *Q. ilex*, as plants from the wet site (Tivoli) were strongly affected by the induced water stress with an early drop in gas exchanges, and consequently to  $P_N$ . This was reflected by the lower photosynthetic efficiency (represented by IWUE and CE), highlighting how plants from the other sites were much more efficient under water stress and less affected on their photosynthetic capacity, performing similarly to each other ( $p = 0.40$ ). Tivoli was also the provenance from

which *Q. ilex* leaves greenness, represented by the NDVI, was most impacted while damage to the whole plant (represented by PRI) and PSII was found to affect populations similarly. Such a result could possibly derive from further differences in alternative drought coping mechanisms capable of stabilizing leaf RWC, such as differences in the accumulation of osmoprotectant osmolytes (Singh et al. 2015), which may merit further investigation in future research.

Overall, these observations are compatible with our prediction that plants hailing from dry sites of the region would perform better under drought conditions and confirm that local variances found within the range of tens of Km can indeed naturally select for heritable traits that will be affected differently by drought and climate change. This ecotypic behaviour is very important, as it implies some species response to drought may be much difficult to accurately predict.

Now, to answer the question "can we consider these populations ecotypes?" we need to make a consideration: while our results highlight the presence of specific groups that thrive in different climates due to a composite of morphological, metabolic and physiological traits, we can't forget important limitations to our study, such as the role of epigenetics on shaping PFTs (Moran et al. 2016). Common garden experiments need to be maintained for several generations before this influence can be excluded, thus genetic analysis would be needed to overcome this limitation. However, the observed improvements to drought resistance reflected by the adoption of drought-related morphological traits that were previously observed on these populations both in situ and under common growing conditions (Iozia et al. 2023; Iozia and Varone 2023) constitute a strong clue that we may be observing different ecotypes of *Q. ilex* from proximate localities. Another limit to our observations may rise from the effect of stress intensity: by completely withholding water, plants quickly experienced exposure to a stressful environment. In the case of *Cistus salviifolius*, this rapid decrease was enough to reach critical thresholds in just a week, possibly hiding potential ITV that could be highlighted with a different temporal resolution. To bypass this limitation, a different experimental design with controlled reductions of water availability may be advisable.

Despite our limits, we can still attempt to determine why we observed ITV in drought resistance between populations of *Q. ilex* but not in *C. salviifolius*. The first explanation may be given by functional types: since *Q. ilex* is mostly found with an arboreal habit, its necessity to adapt to different environments may be higher than the drought-semideciduous bush *C. salviifolius*. Moreover, given *C. salviifolius* is a pioneer species that tends to avoid drought by shedding its leaves and adopting a fast life cycle that takes advantage of temporary clearings (Grant et al. 2014), while *Q. ilex* is a slow growing plant that needs to withstand the environment for several years to complete its life cycle (Martín-Sánchez et al. 2022), it makes sense that *Q. ilex* would be more apt to adapt to different environments. This species is known to be quite competitive in its natural environment, resorting to its plasticity and resilience (Poissonet et al. 1978) to adapt to different environments (Martín-Sánchez et al. 2022). *Q. ilex* is in fact widely renowned for its tendency to develop ecotypes (Peguero-Pina et al. 2014), which are usually linked to different drought resistance (Martin-StPaul et al. 2011; Navarro-Cerrillo et al. 2018). Conversely, *C. salviifolius*, is prone to follow its optimal niche in the environment, quickly colonizing open areas that may reflect lower microclimatic variation between sites and its ITV patterns do not appear to follow drought or climate adaptation (Castro et al. 2023; Farley and McNeilly 2000).

In conclusion, our results support the idea that ITV can shift the way some populations respond to drought and water stress at low distances for some species, prompting future research to further investigate how neighbouring populations manage to adapt their survival strategies to local differences, delving into response mechanisms both from a morphological perspective (such as differences in leaf, xylem, and root morphology) and from a metabolic perspective (such as differences in stress-related response pathways, differences in hormone accumulations such as ABA, and secondary metabolites related to stress responses such



as carotenoids and anthocyanins). They also encourage biologists to take into consideration the possibility for local ecotypes when estimating drought resistance, as similar scenarios may also be supported by compatible observations made on several other Mediterranean plant species such as *Quercus faginea* (Solé-Medina et al. 2022), *Fagus sylvatica* (Aranda et al. 2015), *Pinus pinaster* (Aranda et al. 2010), and *Castanea sativa* (Míguez-Soto et al. 2019), highlighting the importance of ITV and local climatic heterogeneity in shaping plant ecology.

### CRedit authorship contribution statement

**Lorenzo Maria Iozia:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Laura Varone:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, 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 will be made available on request.

### Acknowledgements

Special thanks also go to Dr. Federica Porcu for helping us in growing seedlings and collecting data.

### Funding

This research was funded by PhD School XXXVI Cycle, Sapienza University of Rome.

### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.stress.2024.100556](https://doi.org/10.1016/j.stress.2024.100556).

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