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Leaf morpho-anatomical adjustments in a *Quercus pubescens* forest after 10 years of partial rain exclusion in the field

Justine Laoué^{1,*}, Guillermo Gea-Izquierdo², Sylvie Dupouyet¹, María Conde², Catherine Fernandez¹, Elena Ormeño^{1,*}

¹CNRS, Aix Marseille Univ, Univ Avignon, IRD, IMBE, University Campus Saint-Jérôme 13013 Marseille, France

²CIFOR-INIA, CSIC, Centro de Investigación Forestal Moncloa - Aravaca 28040 Madrid, Spain

*Corresponding authors: IMBE, University Campus Saint-Jérôme 13013 Marseille, France. Email: justine.laoue@gmail.com and elena.ormeno-lafuente@imbe.fr

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In the Mediterranean region, a reduction of annual precipitation and a longer and drier summer season are expected with climate change by the end of the century, eventually endangering forest survival. To cope with such rapid changes, trees may modulate their morpho-anatomical and physiological traits. In the present study, we focused on the variation in leaf gas exchange and different leaf morpho-anatomical functional traits of *Quercus pubescens* Willd. in summer using a long-term drought experiment in natura consisting of a dynamic rainfall exclusion system where trees have been submitted to amplified drought (AD) (~30% of annual precipitation) since April 2012 and compared them with trees under natural drought (ND) in a Mediterranean forest. During the study, we analyzed net CO₂ assimilation (A_n), stomatal conductance (g_s), transpiration (E), water-use efficiency (WUE), stomatal size and density, density of glandular trichomes and non-glandular trichomes, thickness of the different leaf tissues, specific leaf area and leaf surface. Under AD, tree functioning was slightly impacted, since only A_n exhibited a 49% drop, while g_s , E and WUE remained stable. The decrease in A_n under AD was regulated by concomitant lower stomatal density and reduced leaf thickness. Trees under AD also featured leaves with a higher non-glandular trichome density and a lower glandular trichome density compared with ND, which simultaneously limits transpiration and production costs. This study points out that *Q. pubescens* exhibits adjustments of leaf morpho-anatomical traits which can help trees to acclimate to AD scenarios as those expected in the future in the Mediterranean region.

Key words: acclimation, climate change, functional traits, long-term drought, Mediterranean forests, photosynthesis, stomata density, stomata size, trichomes.

Introduction

Climatic models forecast for the Mediterranean region a general warming between 1 and 5 °C by the end of the century, reaching up to 7 °C during summer, as well as a reduction in annual precipitation of about 30% (Guiot and Cramer 2016, Cramer et al. 2018, Lionello and Scarascia 2018, Zittis et al. 2019). Such changes will lead to longer and more frequent summer drought periods in Mediterranean climate areas (Lionello and Scarascia 2018), which should have a negative impact on forest productivity, health and regeneration (Cramer et al. 2018, Peñuelas et al. 2018).

To cope with water deficit, plants modulate their physiological as well as their morphological and anatomical traits (Basu et al. 2016, Li et al. 2023). For the former, rapid stomatal closure is well known to allow water saving during drought stress. Such response implies however a reduction in carbon assimilation through photosynthesis eventually constraining plant growth (Farquhar and Sharkey 1982, Chaves et al. 2002, Pirasteh-Anosheh et al. 2016, Saunier et al. 2018). Regarding morphological traits, stomatal density (SD) and stomatal size (SS) are involved in water loss regulation and thus, in plant adaptation to drought (Bertolino et al. 2019). Another important morphological adaptation to limit water loss is the development of leaf trichomes (Bickford 2016). Trichomes are appendages derived from aerial epidermal cells in leaves, stems and floral organs which act as a direct barrier against transpiration but also as a protection against herbivores and

UV-radiation (Wang et al. 2021). Drought stress increases leaf trichome density in many plant species (Shahzad et al. 2021, Chen et al. 2022), including *Quercus* species (Hernandez and Park 2022). Trichomes are classified as glandular trichomes (GT) and non-glandular trichomes (NGT) (Xiao et al. 2016). Glandular trichomes store and secrete large quantities of specialized metabolites including terpenoids (Mahmoud et al. 2021), flavonoids (Tattini et al. 2000) and phenolic acids (Zhou et al. 2021) which are involved in plant defense against environmental stresses such as drought (Haberstroh et al. 2018, Laoué et al. 2022) or herbivory (Peiffer et al. 2009). By contrast, NGT do not secrete specialized metabolites but act as a physical protection against high light and temperatures, playing a key role in limiting transpiration (Karabourniotis et al. 2020). Finally, regarding anatomical traits, leaf thickness plays an important role in plant adaptation to withstanding drought stress (Flexas et al. 2014, Niinemets 2015, Afzal et al. 2017) and more specifically, variation in mesophyll anatomy is of a great importance for photosynthetic capacity (Marchi et al. 2008, Somavilla et al. 2014). Usually, thicker leaves possess a better tolerance against water scarcity due to enlarged palisade mesophyll cells or increase in total leaf thickness which allows the leaf to store more water (Wright et al. 2004, Galmés et al. 2013). Moreover, a low specific leaf area (SLA)—owing to high tissue thickness—in evergreen sclerophyllous species, favors adaptation to arid and semi-arid climates (Vaz et al. 2010, Ramírez-Valiente et al. 2020) while

large leaves with high SLA—and thus thinner leaves—in non-sclerophyllous broad-leaf deciduous species reflects an acquisition resource-use strategy where light capture, photosynthesis and resource assimilation are maximized (Pérez-Ramos et al. 2013, Reich 2014).

Morpho-anatomical leaf traits are thus critical for leaf gas exchange since they permit plants to adjust their net CO₂ assimilation (A_n), stomatal conductance to water (g_s), transpiration (E) and water-use efficiency (WUE) (Franks and Farquhar 2007, Franks et al. 2009). Studies on morpho-anatomical adjustments in response to water scarcity are well documented in deciduous oak species (Thomas and Gausling 2000, Peguero-Pina et al. 2016, Robakowski et al. 2020, Hernandez and Park 2022). In the study of Peguero-Pina et al. (2016), significant morphological and physiological differences in response to water deficit according to oak species were seen where trees reducing the transpiring area appear to be an example of adaptation to water deficit as observed in *Quercus faginea* Lam.

Some tree species are more vulnerable than others to water deficit. *Quercus pubescens* Willd. is a widespread deciduous species in the North-Western part of the Mediterranean basin. It is a drought-resistant species (Levitt 1980, Struve et al. 2009) using a strategy to avoid leaf dehydration under water stress which is to develop a deep root system and high hydraulic conductivity that allows subsurface soil moisture uptake to leaves thus maintaining high relative leaf moisture (Nardini and Pitt 1999, Tognetti et al. 2007, Struve et al. 2009). Fotelli et al. (2000) showed that among other Mediterranean oak species (i.e. *Quercus frainetto* Ten., *Quercus macrolepis* Kotschy., and *Quercus ilex* L.), *Q. pubescens* maintained the highest water potential under drought conditions, reinforcing the idea that *Q. pubescens* has a greater efficiency in hydraulic transport compared to other *Quercus* species (Tognetti et al. 1998).

Our previous studies simulating future drought conditions in a *Q. pubescens* forest revealed that canopy capacity to fix CO₂ was limited in response to short, middle, and long-term amplified drought (AD; 1 year, 3–4 years, and 10 years after installation of a rain exclusion system, respectively) (Saunier et al. 2017, 2018, Genard-Zielinski et al. 2018, Laoué et al. 2023). Such limitation in net photosynthesis in response to long-term AD (>10 years) was accompanied by a higher accumulation of leaf central antioxidant defenses (i.e. carotenoids) to preserve *Q. pubescens* photosynthetic apparatus (Laoué et al. 2023) at the expense of specialized antioxidant defenses (i.e. phenolic compounds) whose production imposes an energetic cost to the plant because resources are diverted away from growth processes and related central metabolism (Paul-Victor et al. 2010, Agrawal 2011).

Although morpho-anatomical adjustments are crucial for plant acclimation to drought stress (Thomas and Gausling 2000, Peguero-Pina et al. 2016, Robakowski et al. 2020, Hernandez and Park 2022), no study has investigated such changes in *Q. pubescens* after recurrent and long-term AD. In order to fill this gap, this in natura study analyzed whether *Q. pubescens* deploys morpho-anatomical changes (i.e. high trichome density, low SD, thicker leaves) after 10 years of AD allowing the species to acclimate to future drier climatic conditions. Additionally, the present investigation explores the relationship between these morpho-anatomical adjustments and the limited net photosynthesis in trees submitted to AD (Saunier et al. 2018, Laoué et al. 2023) and highlights if

such changes could avoid water loss allowing *Q. pubescens* to withstand the increasing limited precipitation expected in the Mediterranean area.

Materials and methods

Study site and species

This study was conducted at O₃HP (Oak Observatory at the ‘Observatoire de Haute Provence’), an experimental forest site in natura mainly populated by ~85–100 years old *Q. pubescens*. This experimental site allows long-term monitoring of drought impact on a Mediterranean forest. It belongs to both AnaEE-France (Analysis and Experimentation on Ecosystems) and AnaEE-ERIC (European Research Infrastructure Consortium). It is located at 60 km north of Marseille in Southern France (5°42′44″E, 43°55′54″N) at an elevation of 650 m above sea level. Annual rainfall is 830 mm and annual mean temperature 11.9 °C (Laoué et al. 2023) which corresponds to a humid supra-Mediterranean bioclimate (Quézel and Médail 2003).

This experimental site is equipped since April 2012 with a dynamic rainfall exclusion device which is deployed during some rain events from April to October to exclude between 30% and 40% of annual precipitation (which corresponds on average to 20 days of rain exclusion) thus simulating future climate predictions in the Mediterranean region (Lionello and Scarascia 2018, Zittis et al. 2019). Two plots are monitored, namely the AD plot of 300 m² where trees are subjected to partial rain exclusion and the natural drought (ND) adjacent plot (300 m²) without rain exclusion where trees grow under ND conditions occurring in this Mediterranean forest, i.e. they suffer seasonal water deficit under summer drought conditions (see Fig. S1 available as Supplementary data at *Tree Physiology* Online). Twenty Downy oak (*Q. pubescens*) trees (10 trees under ND and 10 trees under AD; Fig. S1 available as Supplementary data at *Tree Physiology* Online) were selected within the plots to study their leaf physiological and morpho-anatomical traits in July 2021. Regarding climatic data, the year preceding leaf sampling (2020) was considered as a dry year since the annual cumulative precipitation was 499 mm which is well below the annual mean precipitation of 830 mm (Fig. 1a). Before our sampling dates in July 2021, the studied forest was exposed to 3 months of ND (in May, June and July) since temperature curves were above precipitation bars in the ombrothermic diagram. Such ND in 2021 was intensified in the AD plot since 27% of precipitation was excluded from April to July (Fig. 1b and c).

Physiological leaf traits

Leaf gas exchanges of CO₂ and H₂O were measured with an infrared open-gas analyzer (IRGA, CIRAS-3, PP Systems, Amesbury, MA, USA). Measurements were performed between 10 a.m. and 1 p.m. from 12 to 14 July 2021 on three full sun leaves from the top of each canopy (ND and AD) accessible using an elevated footbridge (see Fig. S1 available as Supplementary data at *Tree Physiology* Online). Within the leaf chamber, photosynthetically active radiation (PAR) was set to 1000 μmol m⁻² s⁻¹, leaf chamber temperature at 27 °C and CO₂ concentration at 400 μmol mol⁻¹. Physiological traits measured were the net CO₂ assimilation (A_n , μmol CO₂ m⁻² s⁻¹), stomatal conductance to water (g_s , mmol H₂O m⁻² s⁻¹), transpiration (E , mmol H₂O m⁻² s⁻¹) and WUE (mmol CO₂ mol⁻¹ H₂O) calculated as the ratio A_n/E .

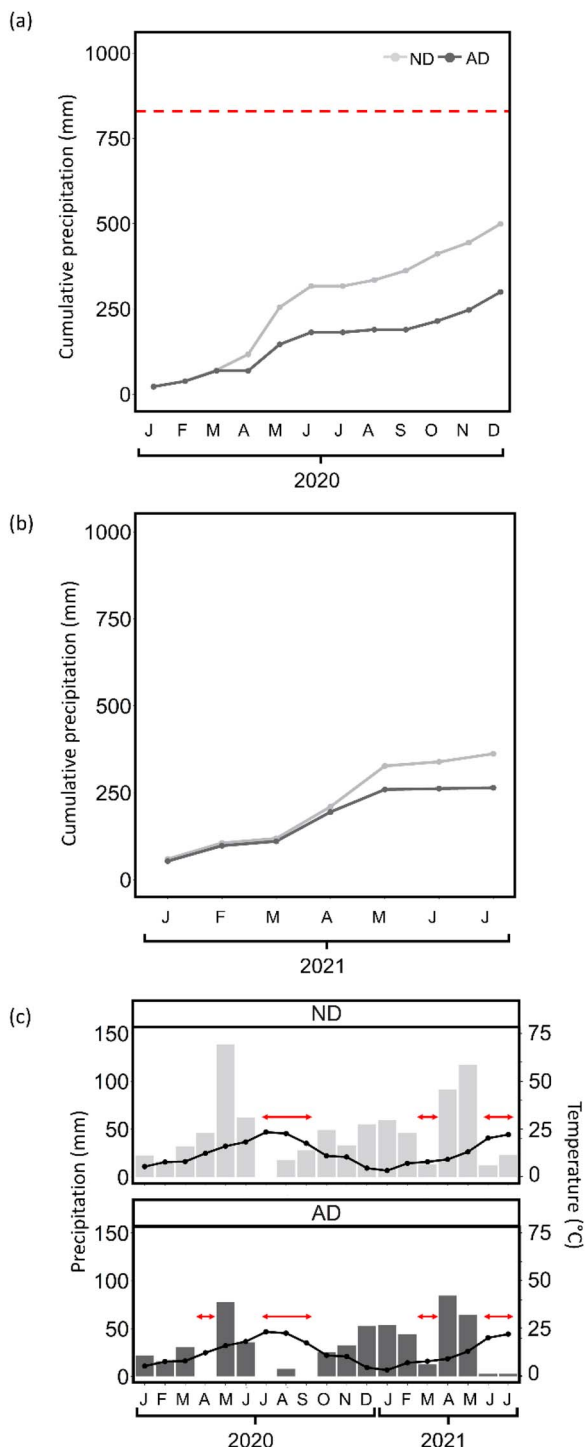


Figure 1. Climatic conditions at O₃HP. (a) Cumulative precipitation in the natural (ND) and amplified (AD) drought plots in 2020. The dotted line represents the annual mean precipitation calculated for the period 1967–2000 (830 mm). (b) Cumulative precipitation under ND and AD plots in 2021 (from January to July). (c) Ombrothermic diagram under natural ND and AD plots in 2020 and 2021 (until July) where bars represent monthly cumulative precipitation (P, mm) and curves represent mean monthly temperature (T, °C) with $P = 2 T$. When temperature curves are above precipitation bars (indicated by horizontal arrows), it corresponds to dry periods.

Morphological and anatomical leaf traits

Leaves were collected from the top of the canopy of each tree around midday and immediately frozen in liquid nitrogen then stored at -80°C until use. The SS, SD and trichome density of *Q. pubescens* leaves were measured from 30 leaves (3 leaves/tree, 10 trees/condition) to three different areas/leaf (totaling 180 analyses). Measurements were performed on the abaxial surface since the adaxial surface possessed a very low density of NGT and no GT (Safou and Saint-Martin 1989, Bussotti and Grossoni 1997, Fortini et al. 2009) (also see Fig. S2 available as Supplementary data at *Tree Physiology Online*). In *Q. pubescens*, GT are referred as simple seriate trichomes composed of a single column of two or more thin-walled cells. Their exact function (Fortini et al. 2015) and content have not been clarified but it is likely they store terpene-like compounds as in *Q. robur* (Engel et al. 1993). The leaf abaxial surfaces were observed through scanning electron microscope (Zeiss Evo 15 VP EP -SEM, Germany, 20 kV accelerating potential, 10 mm working distance) at $100\times$ magnification to calculate the density of NGT and GT and $500\times$ magnification for SS and density. Before microscopic observations, leaf sections were sputtered with gold. The SS was calculated as an ellipse from the measured guard cells length (Zhu et al. 2018, Caine et al. 2019). Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Leaf anatomical traits were characterized through leaf tissue thickness, leaf surface and SLA measured on three leaves/tree and three segments/leaf (1×0.5 cm) on either side of the main vein. Each segment was progressively dehydrated in ethanol series (70% to 100%, seven times) followed by xylene and then embedded with warm paraffin. Leaf transverse sections ($9\ \mu\text{m}$) were then cut using a rotary microtome (Leica biosystem RM235, Nussloch, Germany), then stained with safranin (lignified tissues) and Astra Blue (non-lignified tissues) and finally assembled in slides and sealed with neutral glue (Férriz et al. 2023). The thickness of both, adaxial and abaxial epidermis as well as palisade parenchyma and spongy parenchyma were determined using a light microscope (Leica DM2500, Germany) at a magnification of $100\times$. The SLA ($\text{cm}^2\ \text{g}^{-1}$) was calculated as the ratio of leaf surface (cm^2)—measured using ImageJ software—to dry mass calculated on 10–15 leaves by tree.

Statistical analyses

Differences in morpho-anatomical and physiological traits under drought conditions (ND and AD) were tested with principal component analysis (PCA), using FactoMineR and factoextra packages (Husson et al. 2010, Kassambara 2020), followed by permutational multivariate ANOVA (PERMANOVA) using vegan package (Oksanen et al. 2013) with 9999 permutations as well as with Student tests ($P < 0.05$). The relationship between physiological and morpho-anatomical traits within each drought condition was determined by Pearson correlation coefficients (r) using the ‘cor.test’ function. Pearson’s correlation matrices (Hmisc package with ‘rcorr’ function; Harrell 2014) were performed to assess relationship between physiological traits and between leaf thickness traits with drought conditions pooled together. Data were log-transformed when necessary to reach

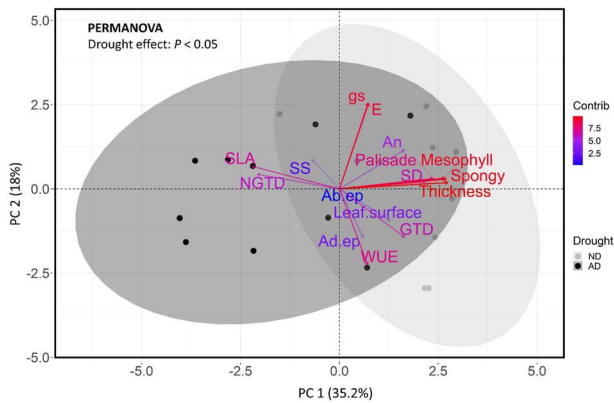


Figure 2. PCA for the first two PCA axes including all physiological and morpho-anatomical traits measured in this study. Color scale indicates the contribution of each variable. Natural drought (ND) and amplified drought (AD), with $n = 10$ for each condition and differences were tested with PERMANOVA. Ab. ep, abaxial epidermis; Ad. ep, adaxial epidermis; A_n , net CO_2 assimilation; E , transpiration; g_s , stomatal conductance to water; thickness, total leaf thickness.

normality and homoscedasticity. All statistical analyses were performed using R software v.4.0.3 (R Core Team 2020).

Results

Changes in leaf functional traits after 10 years of AD

When all traits (physiological and morpho-anatomical) were considered together, trees growing under ND and AD significantly differed in terms of morpho-anatomical traits, as explained by the first PCA component (PC1) (PERMANOVA, $P < 0.05$) which explained 35.2% of the variability (Fig. 2). The second PCA component (PC2) was more correlated with physiological traits (g_s , E and WUE) and explained 18% of the variation (Fig. 2). According to PC1, trees under long-term AD featured higher values of SLA and non-glandular trichome density (NGTD), whereas they exhibited lower leaf surface, SD and glandular trichome density (GTD) as well as lower leaf thickness-related traits (mesophyll, palisade, and spongy thickness), indicating a remarkable dichotomy between AD and ND conditions according to these traits. Correlation values of each variable with the two principal components are shown in Table S1 (available as Supplementary data at *Tree Physiology* Online).

Adjustments in leaf gas exchange after 10 years of AD

Among the physiological traits measured in July 2021, A_n was the only trait significantly reduced under AD ($P < 0.001$), dropping from 5.78 under ND to 2.96 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ under AD. The decreases also recorded in g_s , E and WUE under AD were not significant Table 1.

Adjustment of morphological traits after 10 years of AD

Stomata, only present on the abaxial side of *Q. pubescens* leaves (Figure 3a, Figure S2 available as Supplementary data at *Tree Physiology* Online), occurred in lower density under

AD (510 stomata mm^{-2}) than in trees under ND (601 stomata mm^{-2}) ($P < 0.01$; Fig. 3b). The SS ranged from 88 to 481 μm^2 with no difference between the two drought conditions compared (Fig. 3c). The SD and SS were not significantly correlated either under AD or ND (Fig. 4a).

Quercus pubescens abaxial leaf surfaces were covered by a crystal wax layer and presented two types of trichomes namely the NGT and the GT (Fig. 4a). The NGT were unicellular while GT were multicellular and uniseriate (i.e. they possessed a single vertical row of cells) (Fig. 4a). The NGT density (NGTD) was on average 3 and 7 trichomes mm^{-2} under ND and AD, respectively, therefore increased under AD ($P = 0.05$; Fig. 4b). By contrast, GT density (GTD) significantly dropped from 33 (under ND) to 18 trichomes mm^{-2} under AD ($P < 0.05$; Fig. 4c). Interestingly, we observed a negative correlation between GTD and NGTD, reflecting a trade-off between the trichomes secreting specialized metabolites and those that do not (Fig. 5b).

Adjustment of anatomical traits after 10 years of AD

Leaf thickness ranged from 157 to 205 μm , and leaves were significantly thinner (-7%) in trees growing under long-term AD ($P < 0.05$; Fig. 6a and b) because leaves had thinner mesophylls ($P < 0.05$; Fig. 6a and b). The change in mesophyll thickness was due to a thinner spongy parenchyma thickness which significantly dropped from an average of 154 μm under ND to 141 μm under AD ($P < 0.05$; Fig. 6a and b). Thickness of most tissues positively correlated with each other (Fig. S3 available as Supplementary data at *Tree Physiology* Online). For example, spongy and palisade parenchyma were highly correlated ($r = 0.73$, $P < 0.001$; Fig. S3 available as Supplementary data at *Tree Physiology* Online). In addition, trees under AD possessed a higher SLA in line with thinner leaves since both traits (SLA and leaf thickness) were negatively correlated ($r = 0.41$, $P < 0.01$). The leaf surface did not differ according to drought conditions, although it was slightly lower under AD (Fig. 6c).

Concomitant morpho-anatomical and physiological adjustments in response to 10 years of AD

Regarding morphological traits related to the leaf surface, higher SD was associated to lower A_n under ND ($P < 0.05$) while lower SD was associated to lower A_n under AD ($P = 0.06$) (Fig. 7a). Additionally, higher NGTD under AD was associated with low E ($r = 0.43$, $P < 0.05$; Fig. 7c) and high WUE ($r = 0.39$, $P < 0.05$; Fig. 7d) but was not associated with g_s (Fig. 7b). The total leaf thickness was positively associated with physiological traits (A_n , g_s and E) under AD (Fig. 8a–c). Especially, the decrease in A_n under AD was correlated with a decrease in the spongy parenchyma thickness ($r = 0.56$, $P < 0.01$; Fig. 8d). Also, the spongy parenchyma thickness positively correlated with g_s ($r = 0.54$, $P < 0.01$; Fig. 8e) and E ($r = 0.48$, $P < 0.05$; Fig. 8f). However, the palisade thickness was not associated to physiological traits (Fig. 8g–i). Lower leaf surface under AD was associated to higher WUE ($r = 0.39$, $P < 0.05$; Fig. 9).

Discussion

Previous studies on the same site indicated that production of leaf specialized chemical defenses in *Q. pubescens* was slightly reduced (e.g. some phenolic compounds) or remained stable

Table 1. Physiological leaf traits measured in July 2021 under ND and AD.

Physiological traits	ND		AD		
	Mean	SE	Mean	SE	
Net assimilation (A_n ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	5.78	±	0.56	±	0.36 ***
Stomatal conductance to water (g_s ; $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	28.27	±	4.89	±	3.46 Ns
Transpiration (E ; $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	0.50	±	0.10	±	0.07 Ns
WUE ($\text{mmol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)	15.63	±	2.85	±	2.14 Ns

Significant differences are tested with Student tests and are denoted using asterisks (***: $P < 0.001$). Ns: not significant. Values are mean \pm SE ($n = 10$).

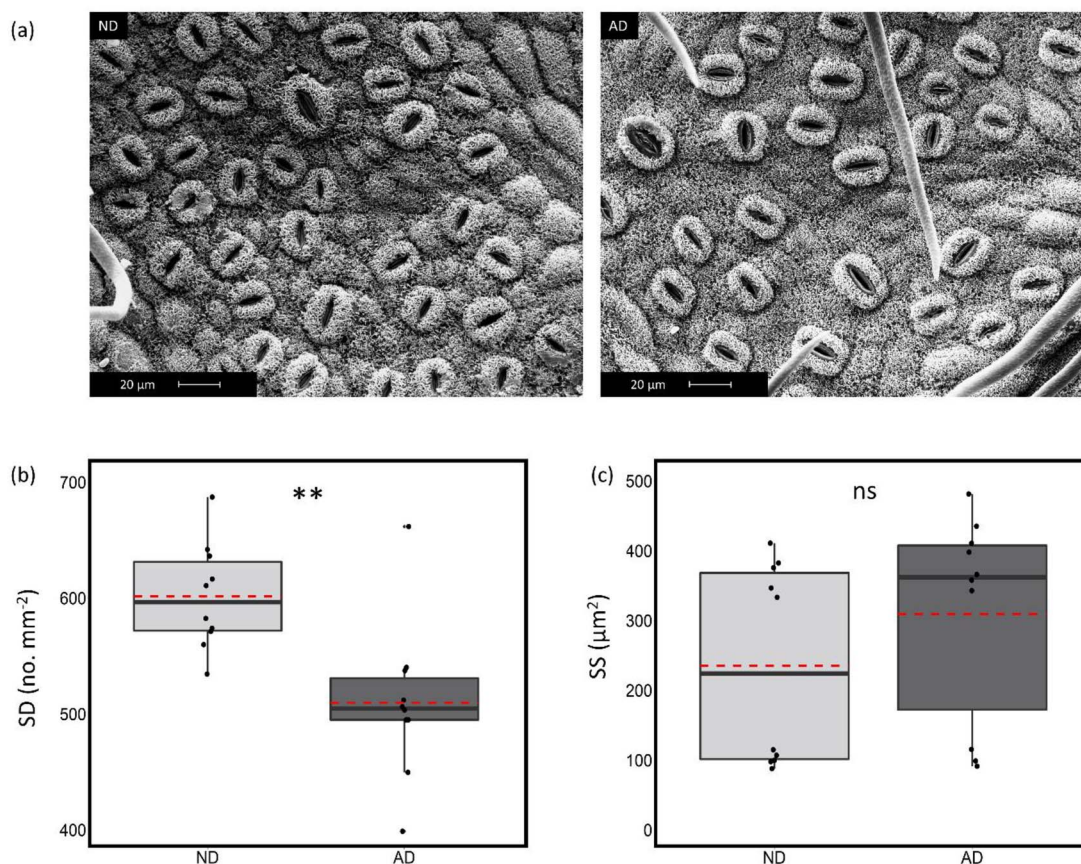


Figure 3. Leaf morphological traits of *Q. pubescens* including SD and size on the abaxial leaf surface according to ND and AD conditions. (a) Scanning electron microscopy (SEM) images of the abaxial leaf surface under ND (left) and AD (right). (b) SD. (c) SS. The horizontal lines inside the boxes are the medians and the horizontal dashed lines are the means ($n = 10$ trees). Differences between ND and AD were tested with Student tests with asterisk denoting significant differences between conditions (**: $0.001 < P < 0.01$; ns: not significant).

in response to long-term (10 years) AD (Laoué et al. 2023). Only some central metabolites (plastochromanol-8, lutein, and neoxanthin), were more accumulated after 10 years of rain withholding indicating some capacity of *Q. pubescens* to cope with increasing drought-related oxidative pressure. In order to understand the physical strategies deployed in response to prolonged rainfall deficit, we investigated whether *Q. pubescens* exhibited morpho-anatomical adjustments and how they could contribute to tree acclimation to drought.

After 10 years of partial rain exclusion, trees exhibited signs of physiological slow down through a 49% decrease of A_n measured in summer, indicating a significant impact on the capacity of *Q. pubescens* to fix CO_2 . This result confirms results from Saunier et al. (2018) after 3 and 4 years of partial rain exclusion on the same experimental site. Stomatal conductance remained stable under amplified long-term drought which is explained by the capacity of *Q. pubescens* to

maintain the hydraulic conductivity and relative leaf moisture content (Nardini and Pitt 1999, Tognetti et al. 2007, Struve et al. 2009). In addition, the decline in net photosynthesis while stomatal conductance was stable under AD, revealed that non-stomatal limitations to photosynthesis were predominant at this level of stress, as highlighted in our previous study (higher internal CO_2 concentration in summer under AD) (Laoué et al. 2023). In addition, the decoupling between photosynthesis and stomatal conductance has also been reported under both water and heat stress combined for other deciduous trees and partially for *Q. pubescens* (Haldimann et al. 2008, Marchin et al. 2023). It is worth noting that stomatal conductance stability despite increasing aridity is also typical in anisohydric plant species such as *Q. pubescens* where stomata remain partially opened even under severe water stress conditions (Damesin and Rambal 1995, Poyatos et al. 2008). This strategy can be considered advantageous since it allows

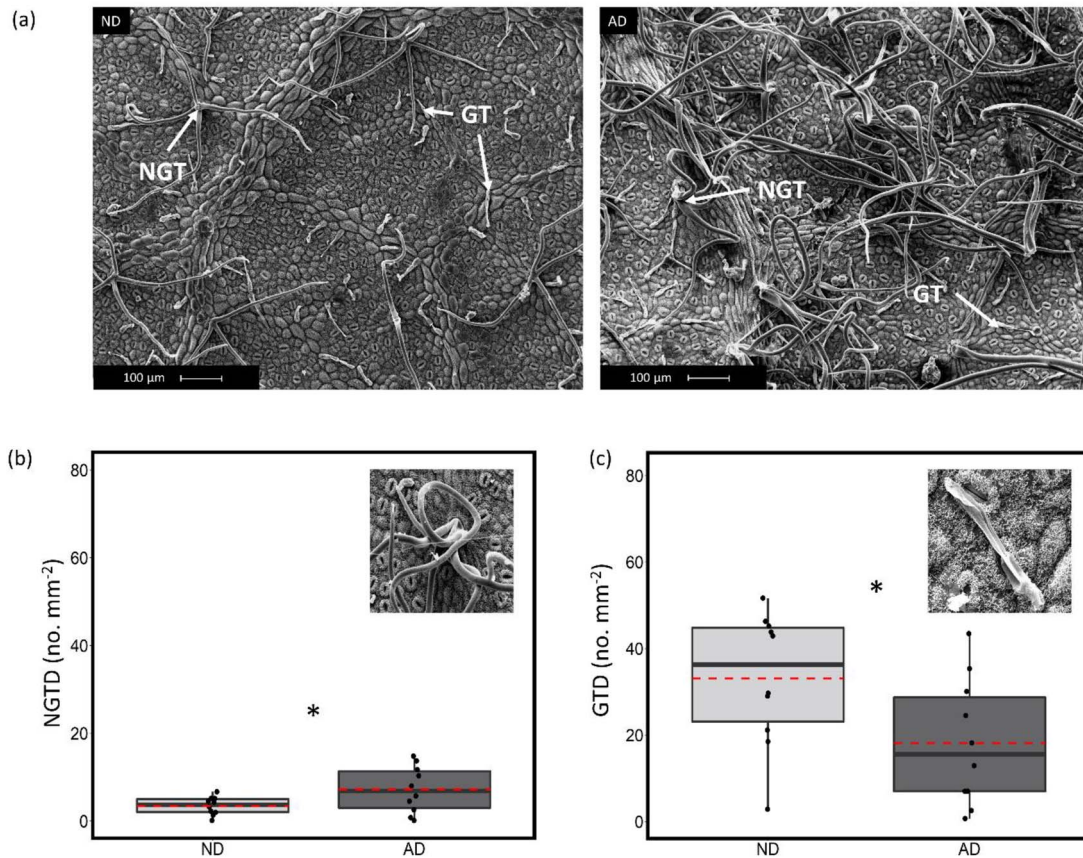


Figure 4. Leaf morphological traits of *Q. pubescens* including trichome density on the abaxial leaf surface under ND and AD conditions. (a) SEM images of the abaxial leaf surface under ND (left) and AD (right). The arrows indicate NGT and GT. (b) NGTD. (c) GTD. The horizontal lines inside the boxes are the median and the horizontal dashed lines are the mean ($n = 10$ trees). Differences between ND and AD were tested with student tests with asterisk denoting significant differences between conditions (*: $0.01 < P < 0.05$).

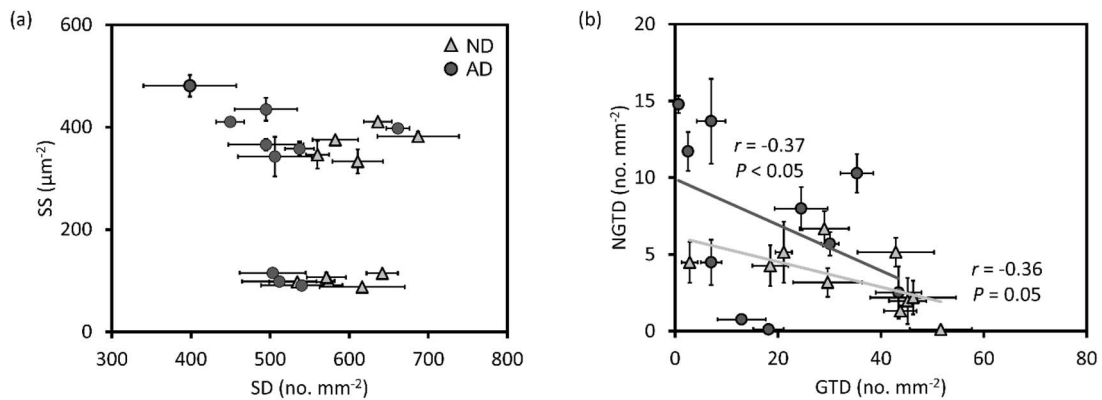


Figure 5. Linear Pearson correlations between leaf surface morphological traits. (a) Correlation between SD and SS under both drought conditions. (b) Correlation between GTD and NGTD under both drought conditions. In b, solid lines represent the regression line for ND and AD. Pearson coefficient of determination (r) and P -values are indicated. Each point represents the means \pm SE ($n = 10$ trees).

stomata to stay partially opened thus maintaining certain CO₂ assimilation (Levitt 1980, Genard-Zielinski et al. 2014). Deciduous oak species (*Q. faginea* Lam. and *Q. pyrenaica* Willd.) do not usually show a conservative water-use strategy and tend to maintain photosynthetically active leaves during most of the summer (Mediavilla and Escudero 2003). Such characteristic allows these species to compensate for the shorter leaf longevity compared with evergreen oak species (*Q. ilex* and *Q. suber*), which avoid irreversible leaf damage during summer drought through extremely conservative water use (Mediavilla and Escudero 2003).

Two morpho-anatomical traits (spongy parenchyma thickness and SD) were positively correlated with photosynthesis in trees submitted to AD. Given that spongy parenchyma and stomata provide the physical basis for CO₂ conduction for photosynthesis and could modulate the entry and diffusion of CO₂ to fixation sites (Flexas et al. 2012, Borsuk et al. 2022), poor spongy parenchyma thickness and SD under AD could have resulted in limited photosynthesis rates. Although reducing SD has been described as an advantageous strategy allowing to limit both g_s and E (Liu et al. 2015, Hughes et al. 2017, Morales-Navarro et al. 2018), SD did not correlate

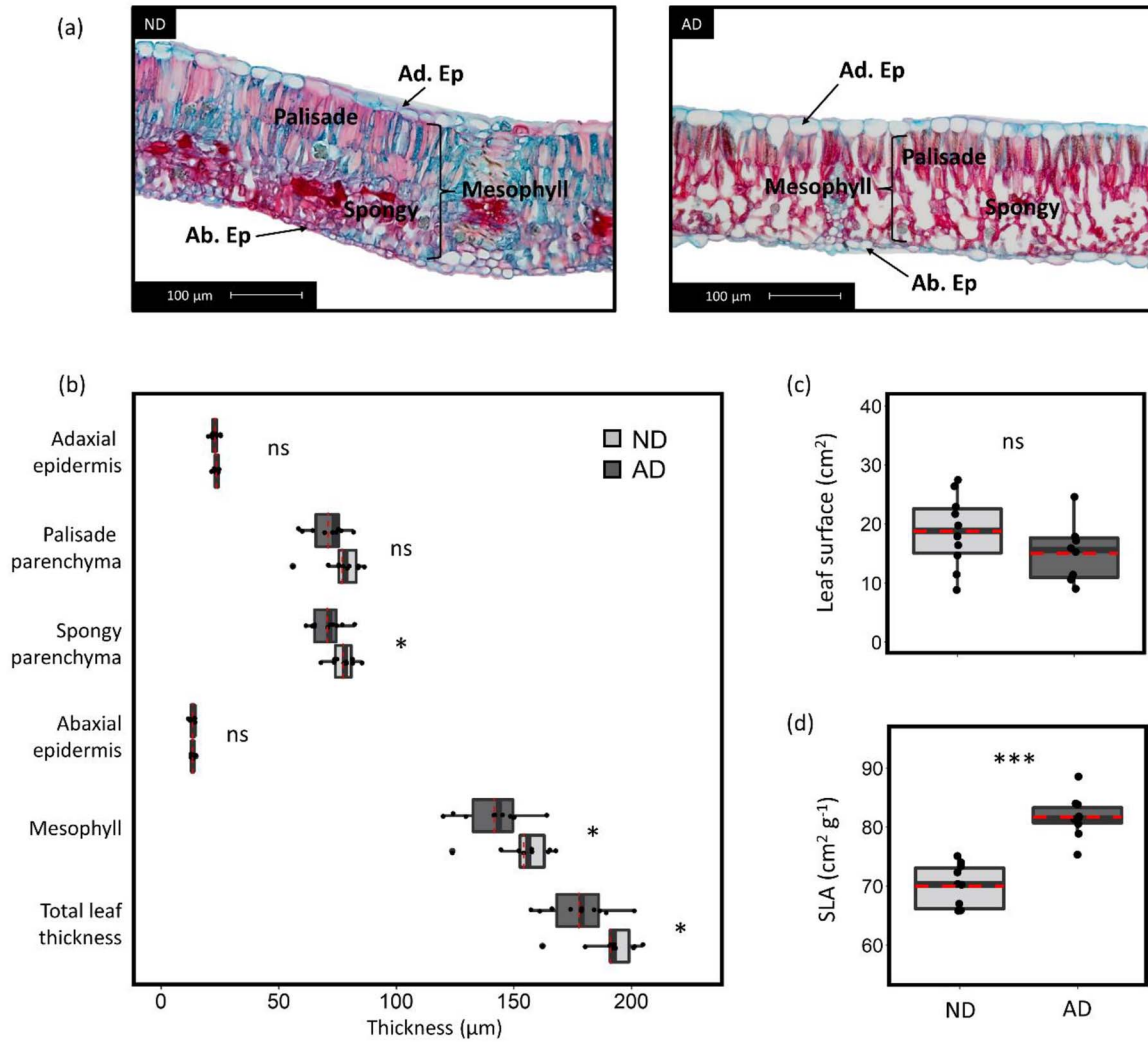


Figure 6. Leaf anatomical traits of *Q. pubescens* according to ND and AD. (a) Optical microscopy images of leaf cross sections under ND (left) and AD (right). Ad. Ep, adaxial epidermis; Ab. Ep, abaxial epidermis. (b) Leaf thickness of the different tissues. (c) Leaf surface. (d) SLA. The horizontal lines inside the boxes represent the median and the horizontal dashed lines represent the mean ($n = 10$ trees). Differences between ND and AD were tested with Student tests. Asterisks denote significant differences between drought conditions (*: $0.01 < P < 0.05$, ***: $P < 0.001$; ns: not significant).

with these physiological traits in our study. It is worth noting that only SD changes do not necessarily limit gas exchange, but rather the combination with SS. Indeed, smaller and denser stomata are associated with higher maximal stomatal conductance (Henry et al. 2019). In our study, the absence of changes in SS could explain that stomatal traits did not influence either stomatal conductance or transpiration. Unexpectedly, SD negatively correlated with A_n under ND (Fig. 7a) which differs from several previous studies (Xu and Zhou 2008, Tanaka et al. 2013, Zhang et al. 2014). Also, the relationship between photosynthesis and stomatal traits is not only determined by mechanical process but is more likely a combination of diffusive and biochemical process, limited by both stomatal and non-stomatal factors (Nadal and Flexas 2018). The recent work of Trueba et al. (2022) puts some light into this relationship by demonstrating that the ratio of stomatal number to mesophyll volume, rather than the stomata number alone, is positively correlated with g_s and negatively with WUE. Such result indicates that the combination of the whole three-dimensional leaf structure combined to SD has a direct impact on leaf gas exchange and thus on

physiological performance of the plant. In addition, the mesophyll conductance (g_m) can also impose the same level of limitation to photosynthesis than stomata traits or biochemistry (Gago et al. 2020). Indeed, the modification in leaf structure can influence the gas- and liquid-phase conductance and thus the CO₂ diffusion efficiency for photosynthesis (Niinemets et al. 2009, Gago et al. 2020).

As a whole, leaves under AD exhibited a lower leaf thickness (-7%) associated to higher SLA ($+14\%$). The opposite response to increasing water stress has, however, been more documented (that is, SLA decrease is associated to higher leaf thickness), particularly in sclerophyllous species like *Q. coccifera* as a strategy to limit water transpiration and favor drought resistance (Peguero-Pina et al. 2016, Ormeño et al. 2020). *Quercus pubescens* growth should have been halted in trees submitted to harsher drought conditions because there is always a negative relationship between growth and drought (Weber et al. 2007, Vodnik et al. 2019, Gea-Izquierdo et al. 2021). Indeed, in our previous study, SLA was lower in summer leaves compared with spring leaves, but such decrease was less marked under amplified (Laoué et al. 2023). This

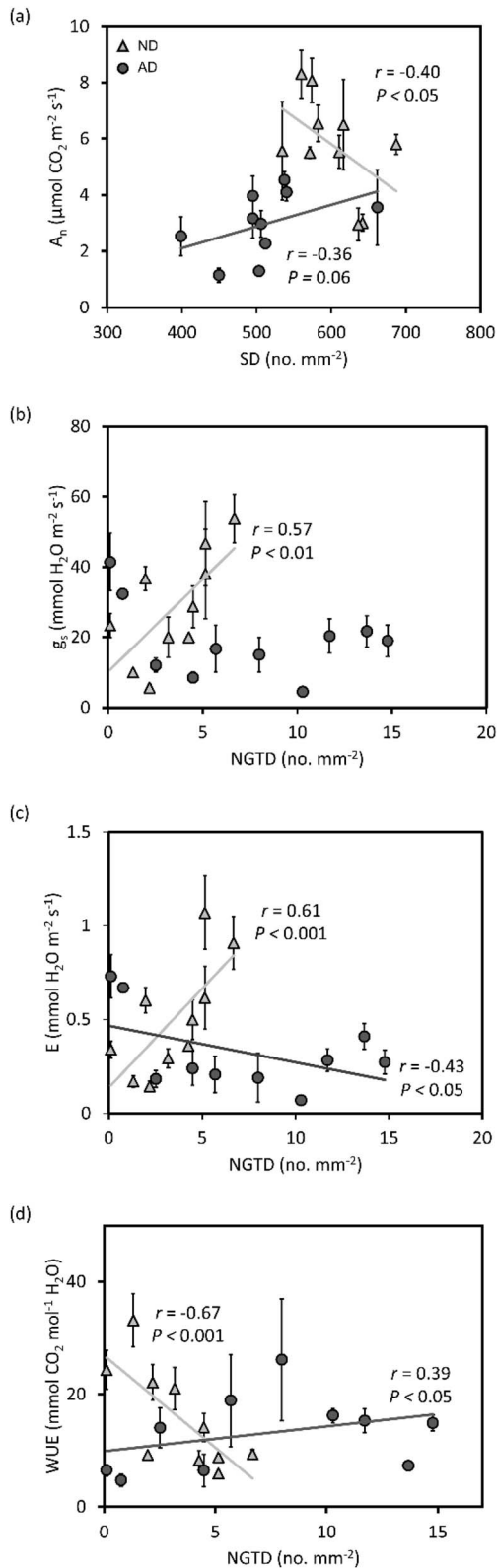


Figure 7. Linear Pearson correlations between physiological and morphological traits. A_n , net CO₂ assimilation; E , transpiration; g_s , stomatal conductance to water; NGTD, non-glandular trichome density; SD, stomatal density and WUE, water-use efficiency. Solid lines represent the regression line for ND and AD. Pearson coefficient of determination (r) and P -values are indicated. Values are means \pm SE ($n = 10$ trees).

result indicates that trees growing under AD—and related limited carbon uptake—will experience limited leaf growth compared to trees growing under ND conditions. When leaves possess not only higher SLA but also larger leaves, this is regarded as an acquisition resource-use strategy where light capture, photosynthesis and resource assimilation are maximized (Pérez-Ramos et al. 2013, Reich 2014). In our study, leaves under AD conditions showed higher SLA (as also observed in *Q. pubescens* seedlings; Genard-Zielinski et al. 2014), but also a lower A_n and potentially smaller leaf surface. This result suggests a potential loss of competitive ability—due to prolonged water stress, negatively affecting CO₂ fixation and thus leaf growth—rather than an optimal adaptation strategy as already suggested for *Q. petraea* when submitted to water stress (Robakowski et al. 2020). Despite the fact that *Q. pubescens* reduces both its capacity to fix CO₂ and potentially its leaf surface under AD, this could be partially offset by enhancement in WUE—as evidenced through a negative correlation between leaf surface and WUE (also previously reported in Ackerly et al. 2002)—and could also be advantageous by reducing tissue construction costs. A reduction in leaf size has already been proposed as one of the key traits that allow different Mediterranean oaks to cope with drier conditions (Baldocchi and Xu 2007, Peguero-Pina et al. 2016).

Despite the lower SD under AD, transpiration rate did not drop (as also reported in spring and autumn; Laoué et al. 2023), which could be an indicator of *Q. pubescens* capacity to acclimate to long-term water stress. This could be explained by both partial stomatal closure to preserve hydraulic conductivity (Nardini and Pitt 1999) and by a higher NGTD (+52%), which negatively correlated with transpiration in trees under AD. These trichomes have indeed been suggested to limit transpiration by reducing the boundary layer conductance, thereby minimizing water loss (Buckley et al. 2022). The higher density of NGT under AD is consistent with several studies where water stress increased the density of leaf trichome to limit water loss eventually conferring drought resistance in *Quercus* species (Mediavilla et al. 2019, Hernandez and Park 2022) and other plant species (González et al. 2008, Galdon-Armero et al. 2018, Chen et al. 2022).

Unlike unicellular NGT, multicellular GT—identified in the abaxial leaf surface, as previously reported (Safou and Saint-Martin 1989, Fortini et al. 2009)—did not seem to provide any advantage against long-term AD. The GTD was on average two-fold lower in trees under AD compared with trees under ND and correlated negatively with the NGTD, which indicates a trade-off between these two types of trichomes when trees are subjected to carbon-uptake-limiting conditions (as suggested by the drop in A_n). When water resources are limited during drought periods, the production of simple unicellular structures (i.e. NGT) may be prioritized over multicellular GT, since the former is less costly (Hare et al. 2003).

Another plausible explanation for the poorer density of GT under AD is the limited capacity of *Q. pubescens* to produce terpene-like defense compounds under pronounced water scarcity. Although further research is required to confirm this hypothesis, *Q. pubescens* leaves contain small amounts of monoterpene compounds (Santonja et al. 2015) which are likely to be stored within GT as in *Q. robur* leaves (Engel et al. 1993). Furthermore, alike isoprene, whose synthesis was impacted since the third year of rain restriction in our

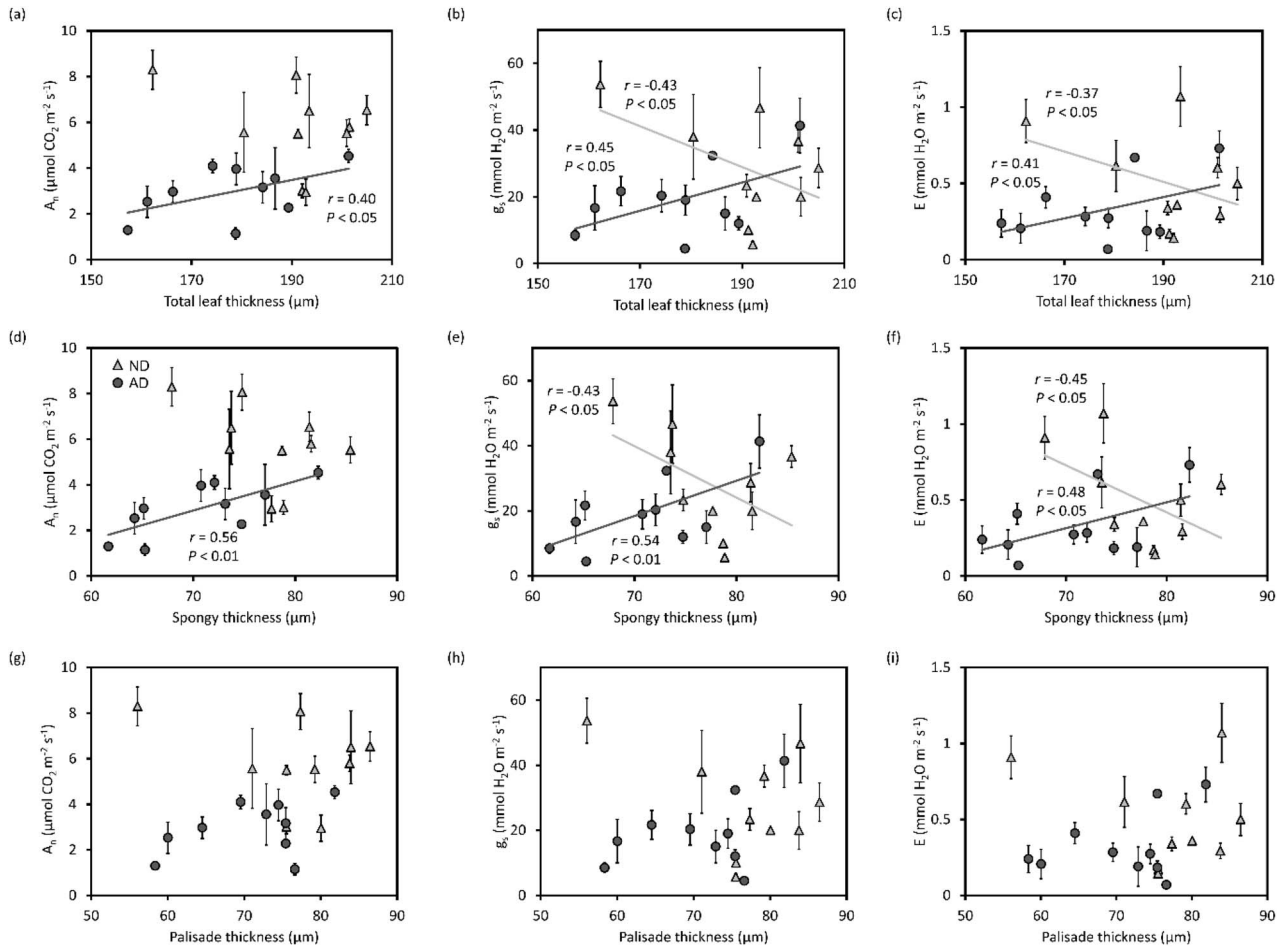


Figure 8. Linear Pearson correlations between physiological and anatomical traits. A_n , net CO_2 assimilation; E , transpiration; and g_s , stomatal conductance to water. Solid lines represent the regression line for ND and AD. Pearson coefficient of determination (r) and P -values are indicated. Values are means \pm SE ($n = 10$ trees).

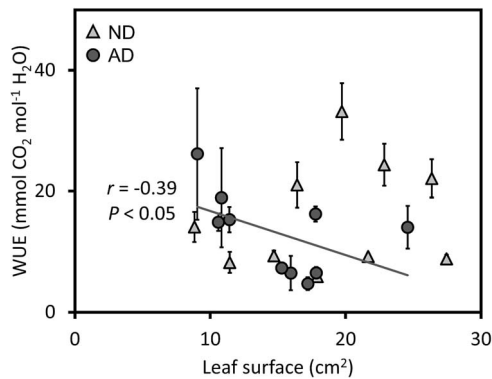


Figure 9. Linear Pearson correlations between WUE and leaf surface. Solid lines represent the regression line for AD. Pearson coefficient of determination (r) and P -values are indicated. Values are means \pm SE ($n = 10$ trees).

field site (Saunier et al. 2018), monoterpene synthesis and storage could also be reduced after 10 years of partial rain exclusion since isoprene and monoterpenes are produced from the same precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (Zhao et al. 2013). Producing a poor density of GT under AD would thus avoid an

unnecessary extra-cost production for *Q. pubescens* when it grows under long-term AD.

All these results suggest that the trade-off between both types of trichomes in favor of low-cost NGT confers a competitive advantage in terms of resistance to long-term drought (and more particularly, limiting leaf transpiration) despite the partial loss in the production of specialized defenses (isoprene, phenolic compounds) under AD observed in previous studies (Saunier et al. 2017, 2022, Laoué et al. 2023). Altogether, the decrease observed in all the different leaf traits analyzed express carbon limitation in trees under long-term AD.

Conclusion

In conclusion, *Q. pubescens* functioning was clearly limited under long-term partial rain exclusion since net photosynthesis was two-fold lower (as a result of lower SD and spongy thickness). Several leaf morphological and anatomical adjustments—including a high NGTD, a low SD and a potential reduction in leaf surface—can contribute to maintain physiological functioning of *Q. pubescens* and confer protection under future drier Mediterranean climate. Another remarkable result was the trade-off between non-glandular and GT reflecting an important adjustment where low-cost NGT are favored providing both, a more limited transpiration rate (also

provided by partial stomatal closure) and lower production costs. Together with thinner and potentially smaller leaves, all these adjustments could contribute to minimize tissue construction cost and allow *Q. pubescens* to resist against more arid summer conditions expected in the future in the Mediterranean region.

Supplementary data

Supplementary data are available at *Tree Physiology* online.

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Authors' contributions

E.O. was the project leader. E.O. and C.F. conceived the study with input from J.L. J.L. performed fieldwork and collected the data with the help of E.O. and S.D. J.L. did all the statistical and image analysis. M.C. helped with tissue coloration and microscopic observations. J.L. wrote the manuscript with input and advice from E.O., G.G.-I., S.D., M.C., and C.F.

Conflict of interest

The authors declare that they have no conflict of interest.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request.

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