TESIS DE LA UNIVERSIDAD
DE ZARAGOZA
2023
128

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ISSN 2254-7606

Prensas de la Universidad
Universidad Zaragoza

Servicio de Publicaciones

ISSN 2254-7606

Tesis Doctoral

DECIDUOUS AND EVERGREEN OAKS: UNRAVELLING THE CO-OCCURRENCE OF DIFFERENT FUNCTIONAL STRATEGIES IN QUERCUS L. SPECIES UNDER MEDITERRANEAN TYPE CLIMATES

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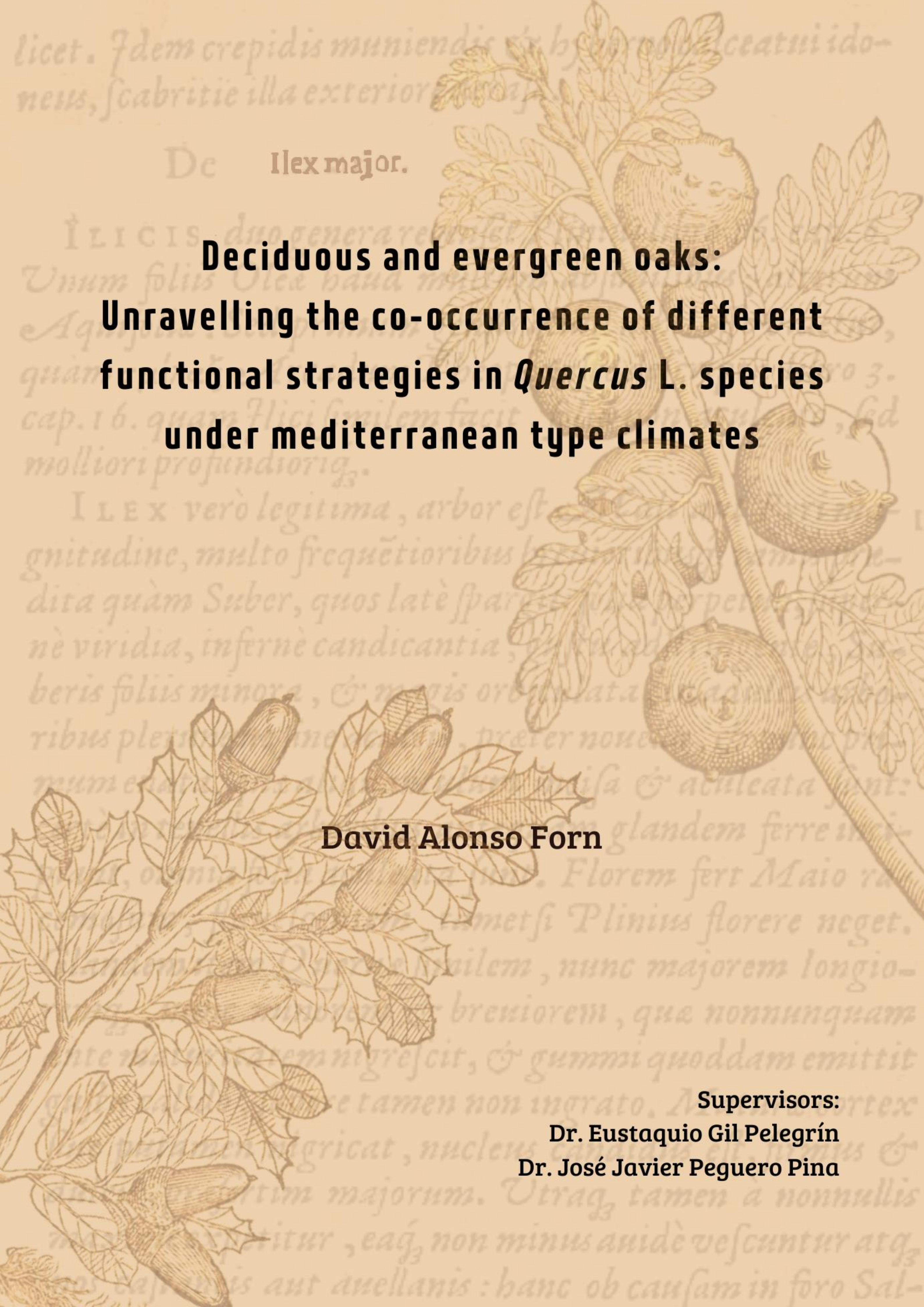
UNIVERSIDAD DE ZARAGOZA
Escuela de Doctorado

Programa de Doctorado en Ciencias Agrarias y del Medio Natural

2023



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Universidad Zaragoza

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CENTRO DE INVESTIGACIÓN Y TECNOLOGÍA
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Compendio de publicaciones

La presente tesis doctoral se ha elaborado como un compendio de cinco trabajos, ya sean publicados en revistas científicas o en fase de revisión. A continuación, se muestra la lista de publicaciones que constituyen el cuerpo de la tesis.

Alonso-Forn D, Sancho-Knapik D, Ferrio JP, Peguero-Pina JJ, Bueno A, Onoda Y, Cavender-Bares J, Niinemets Ü, Jansen S, Riederer M, Cornelissen JHC, Chai Y and Gil-Pelegrín E. 2020. Revisiting the Functional Basis of Sclerophylly Within the Leaf Economics Spectrum of Oaks: Different Roads to Rome. **Current Forestry Reports** 6: 260–281. doi: 10.1007/s40725-020-00122-7

Sancho-Knapik D, Escudero A, Mediavilla S, Scoffoni C, Zailaa J, Cavender-Bares J, Álvarez-Arenas TG, Molins A, **Alonso-Forn D**, Ferrio JP, Peguero-Pina JJ and Gil-Pelegrín E. 2021. Deciduous and evergreen oaks show contrasting adaptive responses in leaf mass per area across environments. **New Phytologist** 230: 521-534. doi: 10.1111/nph.17151

Alonso-Forn D, Sancho-Knapik D, Fariñas MD, Nadal M, Martín-Sánchez R, Ferrio JP, Resco de Dios V, Peguero-Pina JJ, Onoda Y, Cavender-Bares J, Álvarez Arenas T, Gil-Pelegrín E. Disentangling leaf structural and material properties in relation to their anatomical and chemical compositional traits in oaks (*Quercus* L.). **En revisión** y publicado como *preprint* en bioRxiv. doi: 10.1101/2022.10.21.513225

Alonso-Forn D, Peguero-Pina JJ, Ferrio JP, García-Plazaola JI, Martín-Sánchez R, Niinemets Ü, Sancho-Knapik D, Gil-Pelegrín E. 2022. Cell-level anatomy explains leaf age-dependent declines in mesophyll conductance and photosynthetic capacity in the evergreen Mediterranean oak *Quercus ilex* subsp. *rotundifolia*. **Tree Physiology** 42: 1988–2002. doi: 10.1093/treephys/tpac049

Alonso-Forn D, Peguero-Pina JJ, Ferrio JP, Mencuccini M, Mendoza-Herrer Ó, Sancho-Knapik D, Gil-Pelegrín E. 2021. Contrasting functional strategies following severe drought in two Mediterranean oaks with different leaf habit: *Quercus faginea* and *Quercus ilex* subsp. *rotundifolia*. **Tree Physiology** 41: 371–387. doi: 10.1093/treephys/tpaa1353

Agradecimientos

La presente tesis doctoral fue financiada por una ayuda para contratos predoctorales para la formación de doctores (BES-2017-081208) del Ministerio de Economía, Industria y Competitividad del Gobierno de España, la Agencia Estatal de Investigación y el Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA). La tesis se enmarca en el proyecto de investigación "Aplicación de nuevas tecnologías al desarrollo de sensores para el seguimiento no destructivo del estado hídrico de la encina (*Quercus ilex* L.) en plantaciones truferas" (INIA-2017-0008/RTA2015-00054-C02-0). Además, la misma ayuda (BES-2017-081208), también financió una estancia breve de tres meses en la Universidad de Kioto (Japón) bajo la supervisión del Dr. Yusuke Onoda. Así mismo, el proyecto "CO_2PPICE" (Proyecto Retos Investigación del Ministerio de Ciencia e Innovación/ Agencia Estatal de Investigación [PID2019-106701RR-I00 /AEI/ 10.13039/501100011033]) y el grupo de investigación del Gobierno de Aragón H09_20R han financiado parte de los trabajos que forman parte del cuerpo de esta tesis.

A continuación, me gustaría expresar mi más sincero agradecimiento a todas aquellas personas que han dedicado parte de su tiempo y dedicación a la elaboración de la presente tesis doctoral. En especial a los directores de esta; Eustaquio Gil Pelegrín y José Javier Peguero Pina. A ellos les debo este trabajo por guiarme desde el inicio, por compartir conmigo mil ideas y alentar las mías por poco sentido que tuvieran. Me han ayudado en los numerosos obstáculos que se han presentado durante la elaboración de la tesis y siempre he tenido la certeza de estarían cuando los necesitara. Con sus consejos, enseñanzas y calidez humana, sin duda, me han hecho crecer como científico y como persona, al tiempo que me han transmitido su pasión por la ecofisiología.

Este trabajo tampoco hubiera sido posible sin las valiosas aportaciones y numerosas discusiones científicas del grupo de Ecofisiología Forestal del Departamento de Sistemas Agrícolas, Forestales y Medio Ambiente (SAFMA) del CITA y colaboradores. Desde un primer momento me han hecho sentir como en casa y como uno más del grupo. Así, me gustaría agradecer a Domingo Sancho, Juan Pedro Ferrio, Fernando Martínez, Mercedes Vico, José Sánchez, Edurne Martínez, Rubén Martín, Miquel Nadal, Lola Fariñas, Ana López, Rubén Escribano, Elena Granda, Enrique Murria, Alexis Sánchez y Guillermo Ferrando por todo el apoyo, el esfuerzo y el tiempo que han invertido en hacer posible esta tesis doctoral.

Además, el doctorado me ha permitido viajar y conocer científicos a los que quiero mostrar mi agradecimiento: en especial, a Yusuke Onoda, Kaoru Kitajima y todo el laboratorio de *Tropical Forest Resources and Environments* de la Universidad de Kioto en Japón, donde me acogieron estupendamente e hicieron de mi estancia un periodo muy enriquecedor; a Marta Ruiz y Pablo Acedo del grupo de Sensores y Técnicas de Instrumentación de la Universidad Carlos III de Madrid; a Juan Losada del grupo de Fruticultura Subtropical en la Estación Experimental IHSM "La Mayora" en Málaga; y a Jeannine Cavender-Bares del Departamento de Ecología, Evolución y Comportamiento de la Universidad de Minnesota en EEUU. Asimismo, quiero agradecer a Alexandra Elbakyan por "eliminar las barreras en el camino de la ciencia" facilitando el libre acceso a la literatura científica.

Finalmente, me gustaría dar las gracias a mi familia, amigos y todas aquellas personas que me han brindado los ánimos y la fuerza necesaria para terminar esta tesis. Sin su apoyo incondicional este trabajo nunca se habría escrito y, por eso, es también suyo.

Listado de abreviaturas

A_{area}	Area-based net CO_2 assimilation
A_{chl}	Total chloroplast area
AI	Arid intensity (sum of (2 t_m – p_m) for months with tm > 10°C and 2 t_m > p_m)
$\mathbf{AI}_{\mathbf{Lang}}$	Lang aridity index
A_{mass}	Photosynthetic capacity per leaf dry mass
A_N	Net photosynthesis
AP	Arid period (number of months with t_m > 10°C and 2 t_m > p_m)
A_{plg}	Total area occupied by plastoglobuli grains
ARID	Arid species
A_{stg}	Total area occupied by starch grains
BSE_cp	Bundle sheath extension cover percentage
BSE_d	Bundle sheath extension density
BSE_w	Bundle sheath extension width
C	Shoot capacitance
C.LD	Carbon per unit volume
C_a	CO_2 concentration surrounding the leaf
CC	Cellulose content
C_c	Chloroplastic CO2 concentration
CC	Cellulose content
Ccw_Ctotal	Carbon in cell wall:Carbon
CD	Cellulose per unit volume
Chl	Chlorophyll
C_i	Substomatal CO_2 concentration
Ctotal	Carbon content
Ctotal_Ntotal	Ratio carbon:nitrogen

DEC	Winter-deciduous oaks
EVE	Evergreen oaks
ε_{max}	Maximum bulk modulus of elasticity
F_0	Initial Chl fluorescence in the dark adapted state
F'_0	Minimum Chl fluorescence in the light-adapted state
f_{ias}	Fraction of the mesophyll tissue occupied by the intercellular air spaces
F_M	Maximal Chl fluorescence in the dark-adapted state
F'_M	Maximum Chl fluorescence in the light-adapted state
FPG	Period of full plant growth (number of months with t_m > 10°C)
F_S	Chl fluorescence at steady state photosynthesis
F_V / F_M	The dark-adapted, maximum quantum efficiency of PSII
g_{ias}	Gas phase conductance from substomatal cavities to outer surface of cell walls
g_{liq}	Conductance in the liquid and lipid phases from the outer surface of cell walls to chloroplasts
g_m	Mesophyll conductance
$g_{m,a}$	Mesophyll diffusion conductance estimated using the measured leaf anatomical traits
g_{min}	Minimum leaf conductance
g_s	Stomatal conductance for H_2O
GS	Growing season (number of months with t_m > 5°C)

g_{tot}	Total conductance to CO2 from leaf surface to carboxylation sites in the chloroplasts
HC	Hemicellulose content
HC	Hemicellulose content
HCD	Hemicellulose per unit volume
IVD	Interveinal distance
iWUE	Intrinsic water-use efficiency
J_F	Photosynthetic electron transport rate
J_{flu}	Photosynthetic electron transport rate
J_{max}	Photosynthetic electron transport capacity
K_m	Michaelis–Menten constant
K_{ox}	Outside-xylem component of leaf hydraulic conductance
K_{shoot}	Shoot hydraulic conductance
K_{stem}	Hydraulic conductivity
l_b	Biochemical limitation to net CO_2 assimilation
LCC	Lignin and cutin content
LCC	Lignin and cutin content
LCD	Lignin and cutin per unit volume
L_{chl}	Chloroplast length
LD	Leaf density
LE_latw	Lower epidermis lateral wall
LE_lu	Lower epidermis lumen cell
LE_lul	Lower epidermis lumen length
LE_luw	Lower epidermis lumen width
LE_ow	Lower epidermis outer wall
LES	Leaf economic spectrum
LET	Lower epidermis thickness
L_{ias}	Average gas-phase thickness
LL	Leaf life span
l_m	Mesophyll limitation to net CO_2 assimilation
LMA	Leaf dry mass per unit area
l_s	Stomatal limitation to net CO_2 assimilation
LSC	Leaf specific conductivity
LT	Leaf thickness
MAP	Mean annual precipitation
MAT	Mean annual temperature
MED	Mediterranenan species
N.LD	Nitrogen per unit volume
N_{area}	Nitrogen content per leaf area
$N_{cell\ wall}$	Nitrogen content of the cell wall fraction
Ncw_Ntotal	Nitrogen in cell wall:Nitrogen
$N_{cytoplasm}$	Fraction of nitrogen allocated in the cytoplasm
NDF	Neutral fiber detergent
NDVI	Normalized difference vegetation index
NPQ	Non-photochemical quenching
N_{total}	Total leaf nitrogen
Ntotal	Nitrogen content
p	Cuticular water permeability
P or MAP	Mean annual precipitation
P_{50} **or** PLC_{50}	Stem water potential inducing a loss of 50% of xylem hydraulic conductivity
P_{88} **or** PLC_{88}	Stem water potential inducing a loss of 88% of xylem hydraulic conductivity
PLC	Percentage loss of conductivity
p_m	Monthly precipitation
PM_cl	Palisade mesophyll cell length

PM_cw	Palisade mesophyll cell width
PM_nl	Palisade mesophyll number of layers
PMT	Palisade mesophyll thickness
PNUE	Photosynthetic nitrogen-use efficiency
PPFD	Photosynthetic photon flux density
PS	Punch strength
PSII	Photosystem II
PV	Pressure-volume
$r_{\mathrm{cel,tot}}$	Liquid phase inside the cell
r_{cw}	Serial resistances of the cell wall
R_{L}	respiration rate in the light
S_{c}/S	Chloroplast surface area exposed to intercellular air space
SI	Sclerophylly Index
S_{m}/S	Mesophyll surface area exposed to intercellular air space
SM_p	Spongy mesophyll porosity
SMT	Spongy mesophyll thickness
SPS	Specific Punch Strength
SWC	Soil water content
SWF	Specific Work to Fracture
T or MAT	Mean annual temperature
T_{chl}	Chloroplast thickness
T_{cw}	Cell wall thickness
T_{cyt}	Cytoplasm thickness
TDRY	Mean temperature of driest quarter
TEM	Temperate species
t_{m}	Mean monthly temperature
TMIN	Minimum temperature of coldest month
TRO	Tropical species
UE_latw	Upper epidermis lateral wall
UE_lu	Upper epidermis lumen cell
UE_lul	Upper epidermis lumen length
UE_luw	Upper epidermis lumen width
UE_ow	Upper epidermis outer wall
UET	Upper epidermis thickness
V_{cmax}	Maximum velocity of carboxylation
VS	Visual scoring
WF	Work of fracture
$\pi 0$	Osmotic potential at full turgor
$\pi \mathrm{tlp}$	Water potential at turgor loss point
Φ_{exc}	Intrinsic PSII efficiency
Φ_{PSII}	Actual PSII efficiency
Ψ_0	Water potential before rehydration
Ψ_{f}	Water potential after rehydration
Ψ_{MD}	Midday leaf water potential
Ψ_{PD}	Predawn leaf water potential
Γ^*	Chloroplastic CO_2 compensation point in the absence of mitochondrial respiration

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Abstract

Mediterranean-type climates present mild or cold and humid winters, while the summer is affected by high pressures that bring dry and hot air (summer aridity), the latter is considered the genuine characteristic that differentiates it from other types of climates. In this way, the vegetative period is affected by summer drought, which, together with the low temperatures in winter, can limit vegetative growth to essentially two seasons: spring and autumn. Even with these restrictions, Mediterranean ecosystems are highly species-rich and represent one-sixth of the planet's flora. This is the case of the genus *Quercus*, which, with more than 400 species distributed throughout the northern hemisphere, is the dominant element in a wide variety of habitats, especially, in Mediterranean-type climates.

Nowadays, two well-differentiated types of oaks coexist in Mediterranean regions: the sclerophyllous evergreen oak with an origin in the Paleotropical Flora and the deciduous malacophilous of Arctotertiary origin. These two models may be very different at morphological, phenological and physiological levels. In this way, the two types of oaks must constitute two very different operating models in the Mediterranean climate. Thus, the main objective of this thesis is the comparative study of the functioning of the two types of oak species with different foliar habits and degrees of sclerophylly that coexist in the Mediterranean climate. Therefore, this thesis consists of five chapters corresponding to five articles already published or under review that aim to shed light on the abovementioned issue.

Thus, a review of sclerophylly in the genus *Quercus* was carried out to define the mechanisms behind it and its economic consequences for the leaf. Despite the wide range of variation in the leaf functional traits and their contrasting adaptive strategies, it cannot be explained by itself by any of the ecological factors considered (drought, nutrient scarcity, low temperatures and physical damage). The study suggests that these restrictions may have a synergistic effect, and from a functional point of view, we can conclude that leaf habit greatly modulates the physiological implications of sclerophylly in oaks.

Using a phylogenetic approach, anatomical, compositional, and climatic variables were quantified in 85 deciduous and evergreen *Quercus* species that

showed fundamental leaf morphological differences revealing a diverse adaptive response. While LMA in deciduous seems to have diversified in coordination with thickness modulated mainly by aridity, LMA diversification in evergreen seems to depend on the infrageneric group. Thus, in the latter, leaf thickness diversification was modulated by both aridity and cold, while leaf density diversification is only modulated by aridity.

Furthermore, the relative importance of each leaf characteristic on the mechanical properties of 25 Quercus species was studied. It was suggested that Quercus sclerophyllous leaves are tougher and stronger due to the thicker external wall of their epidermis and/or a higher concentration of cellulose. moreover, the species in section Ilex shared common traits although they occupy quite different climates. Similarly, evergreen species living in Mediterranean-type climates shared common leaf traits regardless of their different phylogenetic origin.

In addition, changes dependent on leaf age in morphological, anatomical, chemical and photosynthetic traits were evaluated in *Quercus ilex* subsp. *rotundifolia* Lam., an evergreen oak with a high leaf life span. Decreases in all photosynthetic traits were observed with increasing leaf age. Changes in leaf anatomy and biochemistry were responsible for age-dependent changes in net CO_2 assimilation and CO_2 conductance through the mesophyll. These findings revealed a gradual physiological deterioration related to the dismantling of the photosynthetic apparatus in older leaves of *Q. ilex* subsp. *rotundifolia*.

Finally, the photosynthetic, photochemical and hydraulic characteristics of different organs of *Q. faginea* and *Q. ilex* subsp. *rotundifolia* under severely dry conditions. The strong decrease in the photosynthetic traits of *Q. faginea* was accompanied by a strong decrease in the hydraulic conductance of the leaves in response to drought. This fact probably prevented a xylem embolism in the stems ("vulnerability segmentation"). On the contrary, the leaves of *Q. ilex* subsp. *rotundifolia* showed effective photoprotective mechanisms and high resistance to cavitation induced by drought, which would be related to the greater longevity of the leaves of Mediterranean oaks.

Resumen

El clima mediterráneo presenta inviernos suaves o fríos y húmedos, mientras que el verano está afectado por las altas presiones que traen aire seco y caluroso (aridez estival), siendo esta última, la característica genuina que lo diferencia de otros tipos de clima. De este modo, el periodo vegetativo se ve afectado por la sequía en verano, que, junto con las bajas temperaturas en invierno, pueden limitar el crecimiento vegetativo esencialmente a dos estaciones: primavera y otoño. Aún con estas restricciones, los ecosistemas mediterráneos tienen una alta riqueza de especies que representan la sexta parte de la flora del planeta. Este es el caso del género *Quercus*, que, con más de 400 especies distribuidas por todo el hemisferio norte, es el elemento dominante de una amplia variedad de hábitats y en especial, del clima mediterráneo.

En la actualidad es posible hablar de dos tipos de robles bien diferenciados que coexisten en las regiones mediterráneas: el roble perennifolio esclerófilo con un origen en la Flora Paleotropical y el malacófilo caducifolio de origen Arctoterciario. Estos dos modelos, que influyen en la adecuación de la planta al ambiente, pueden ser muy diferentes a nivel morfológico, fenológico y fisiológico. De este modo, los dos tipos de robles deben constituir dos modelos de funcionamiento muy distintos frente al clima mediterráneo. Es por eso por lo que el principal objetivo de esta tesis es el estudio comparado del funcionamiento de los dos tipos de especies de robles con diferentes hábitos foliares y grado de esclerofilia que coexisten en el clima mediterráneo. Por consiguiente, esta tesis consta de cinco capítulos que corresponden a cinco artículos ya publicados o en fase de revisión que pretenden arrojar luz al tema planteado anteriormente.

Así, se realizó una revisión bibliográfica sobre la esclerofilia en el género *Quercus* para definir los mecanismos que hay detrás y sus consecuencias económicas para la hoja. Pese a al amplio rango de variación en los rasgos funcionales de las hojas y sus estrategias adaptativas contrastantes, no puede explicarse por sí sola por ninguno de los factores ecológicos considerados (sequía, escasez de nutrientes, bajas temperaturas y daño físico). El estudio sugiere que estas restricciones pueden tener un efecto sinérgico, y desde un punto de vista funcional, podemos concluir que, en los robles, el hábito foliar modula en gran medida las implicaciones fisiológicas de la esclerofilia.

Usando un enfoque filogenético, se cuantificaron las variables anatómicas, composicionales y climáticas en 85 especies de *Quercus* caducifolios y

perennifolios que mostraron diferencias morfológicas foliares fundamentales revelando una respuesta adaptativa diversa. Mientras que la LMA en los caducifolios parece haberse diversificado en coordinación con el grosor modulado principalmente por la aridez, la diversificación de la LMA en los perennifolios parece depender del grupo infragenérico. Así, en estos últimos, la diversificación en el grosor de la hoja fue modulada tanto por la aridez como por el frío, mientras que la diversificación en la densidad de la hoja es sólo modulada por la aridez.

Asimismo, se estudió la importancia relativa de cada característica de la hoja sobre las propiedades mecánicas en 25 especies de *Quercus*. Se sugiere que las hojas de *Quercus* esclerófilos son más duras y fuertes debido a la pared externa de su epidermis más gruesa y/o a una mayor concentración de celulosa. Además, las especies de la sección *Ilex* comparten rasgos comunes independientemente de que ocupen climas bastante diferentes. De modo similar, las especies de hoja perenne que viven en climas de tipo mediterráneo comparten rasgos foliares comunes independientemente de su diferente origen filogenético.

Además, se evaluaron los cambios dependientes de la edad foliar en los rasgos morfológicos, anatómicos, químicos y fotosintéticos en *Quercus ilex* subsp. *rotundifolia* Lam., un roble perennifolio con hojas de alta longevidad. Se observan disminuciones de todos los rasgos fotosintéticos con el aumento de la edad de la hoja. Los cambios en la anatomía y bioquímica de la hoja fueron responsables de las modificaciones dependientes de la edad en la asimilación neta de CO_2 y la conductancia del CO_2 a través del mesófilo. Estos hallazgos revelaron un deterioro fisiológico gradual relacionado con el desmantelamiento del aparato fotosintético en hojas más viejas de *Q. ilex* subsp. *rotundifolia.*

Por último, se analizaron las características fotosintéticas, fotoquímicas e hidráulicas de diferentes órganos de *Q. faginea* y *Q. ilex* subsp. *rotundifolia* bajo condiciones de sequía severa. La fuerte disminución de los rasgos fotosintéticos de *Q. faginea* estuvo acompañada de una fuerte disminución de la conductancia hidráulica de las hojas en respuesta a la sequía. Este hecho probablemente evitó una embolia xilemática en los tallos ("segmentación de la vulnerabilidad"). Por el contrario, las hojas de *Q. ilex* subsp. *rotundifolia* mostraron mecanismos fotoprotectores efectivos y alta resistencia a la cavitación inducida por la sequía, lo que estaría relacionado con la mayor longevidad de las hojas de las encinas mediterráneas.

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Capítulo I

Introducción general

1.1. El género *Quercus*: origen y distribución

Quercus L. (*Fagaceae*) es uno de los géneros de plantas leñosas más diverso en número de especies, habiendo despertado un gran interés en la botánica por su complejidad y riqueza desde los orígenes de esta ciencia. El considerado como su fundador, Teofrasto de Ereso (371-286 a. C.), en su "Historia de las Plantas" ya ofreció una descripción general de aquellos robles que por proximidad pudo estudiar. Distinguió varias especies, tanto de hoja caduca como de hoja persistente, fácilmente identificables en la actualidad. Aunque Teofrasto les asignó nombres vernáculos, podemos distinguir especies como *Q. cerris*, *Q. frainetto*, *Q. ithaburensis*, *Q. ilex* o *Q. coccifera* (Thanos, 2005).

Un intento más sistematizado de abundar en la diversidad dentro del género *Quercus* se lo debemos a Charles de l'Ecluse (1576) que reseñó y dibujó con bastante precisión ocho de sus especies. Sin embargo, todavía no aglutinó todas ellas en un mismo género en el sentido que actualmente atribuimos a esta categoría taxonómica. Este autor las agrupó en "géneros" diferentes, tales como *Robur*, *Ilex* o *Suber.* Llama la atención el hecho de que sí agrupó en la misma categoría genérica especies que hoy sabemos están muy próximas filogenéticamente. Son ejemplo de esto mencionado; *Q. ilex* y *Q. coccifera* a las que llamó *Ilex maior* e *Ilex coccigera*, respectivamente. Fue necesario esperar a la publicación del "*Species Plantarum*" de Linneo (1753), para ver agrupadas en un único género, definitivamente *Quercus* L., 14 especies que derivaban de la aplicación del *Systema Naturae* a este grupo de plantas.

En la actualidad, sabemos que este género comprende más de 400 especies de árboles y arbustos (Kremer y Hipp, 2020), que presentan una serie de características comunes como son la monoicidad, hojas simples, dispersión por el viento (anemocoria) y la producción de bellotas. Las últimas clasificaciones basadas en estudios filogenéticos moleculares sugieren que el género *Quercus* se clasifica en dos subgéneros y ocho secciones (Denk *et al*., 2017; Hipp *et al*., 2019). Por una parte, se encuentra el subgénero *Quercus*, también conocido como "clado del Nuevo Mundo" (Manos *et al*., 2001) o "clado de alta latitud" (Grimsson *et al*., 2015; Simeone *et al*., 2016) que comprende cinco secciones (*Protobalanus*, *Ponticae*, *Virentes*, *Quercus* y *Lobatae*). Por otra parte, el subgénero *Cerris*, llamado también "clado del Viejo Mundo" o "clado de latitud media" que incluye tres secciones (*Cyclobalanopsis*, *Ilex* y *Cerris*).

En cuanto a su historia evolutiva parecen tener dos orígenes contrastados: (i) los robles procedentes de la Flora Arctoterciaria de naturaleza caducifolia y de climas templado-húmedos y (ii) los robles procedentes de la Flora Paleotropical, perennifolios y que evolucionaron en climas de naturaleza tropical (Barbero *et al*., 1992; Damesin *et al*., 1998; Mediavilla y Escudero, 2004). En el presente, ambas paleofloras han ocupado fitoclimas equivalentes en sus extremos evolutivos, desde la ocupación de los biomas neotropicales o mediterráneos (Hipp *et al*., 2019; Jiang *et al*., 2019).

Desde su aparición hace aproximadamente 56 millones de años, los robles se han expandido ampliamente por el hemisferio norte (Hofmann *et al*., 2011; Barrón *et al*., 2017; ver **Fig. 1**). De este modo, en la actualidad ocupan zonas desde el ecuador hasta las regiones boreales, y desde el nivel del mar hasta los más de 4000 m de altitud (Manos y Stanford, 2001 y referencias incluidas). Debido a esta amplia distribución, el género *Quercus* se encuentra en diversos fitoclimas muy contrastados, desde los bosques templados y subtropicales hasta los bosques mediterráneos perennifolios (Gil-Pelegrín *et al*., 2017).

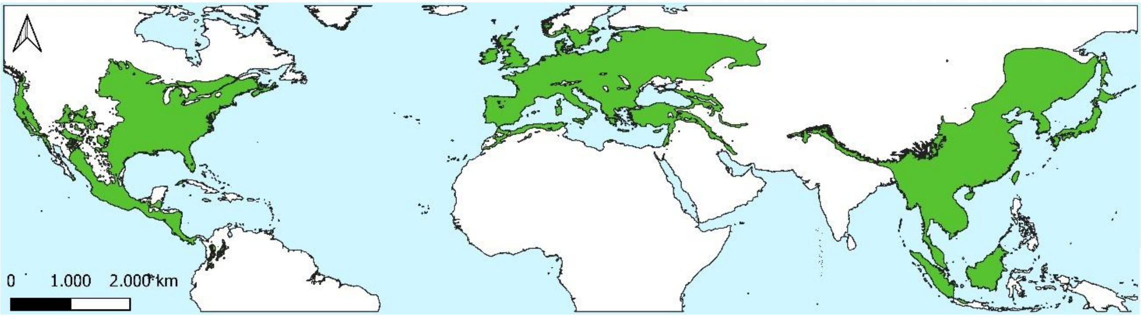

Figura 1. Área de la distribución del género *Quercus* en la actualidad según registros de presencia de especies de "Global Biodiversity Information Facility" (GBIF, 2022).

Según la clasificación de la geobiosfera en nueve zonobiomas propuesta por Walter (1985), encontramos robles en cinco de ellos. Así, a modo de ejemplo, *Quercus urbanii* habita el zonobioma II o "zonobioma de las savanas, bosques caducifolios y pastizales"; *Q. ilex* es característico del zonobioma IV o "zonobioma de bosques esclerófilos"; *Q. nigra* se encuentra en el zonobioma V o "zonobioma de laurisilva"; *Q. robur*, en el zonobioma VI o "zonobioma de bosques caducifolios" y *Q. balloot*, en el zonobioma VII o "zonobioma de estepas y desiertos fríos" (Breckle, 2002). Sin embargo, uno de estos zonobiomas es clave en cuanto a la presencia de robles en su transición a otros zonobiomas: es el caso del zonobioma IV, considerado el propio de la región mediterránea (Walter, 1985).

1.2. El género *Quercus* en los ecosistemas mediterráneos

El clima mediterráneo presenta inviernos suaves o fríos y húmedos, mientras que el verano está afectado por las altas presiones que traen aire seco y caluroso (aridez estival), siendo esta última, la característica genuina que lo diferencia de otros tipos de clima (Walter, 1985; Lionello *et al*., 2006). Más del 50% de la precipitación anual se produce durante el invierno y menos del 20% durante los meses de estío (Deich *et al.*, 2017). De este modo, el periodo vegetativo se ve afectado por la sequía en verano, que, junto con las bajas temperaturas en invierno, pueden limitar el crecimiento vegetativo esencialmente a dos estaciones: primavera y otoño (Mitrakos, 1980). Aún con estas restricciones, los ecosistemas mediterráneos tienen una alta riqueza de especies que representan la sexta parte de la flora del planeta (Cowling *et al*., 1996; Rundel *et al*., 2016). En especial, solo en la cuenca mediterránea se encuentran unas 25 000 especies de plantas nativas, siendo más de la mitad endemismos (Rundel *et al*., 2016).

Este clima recibe el nombre del mar Mediterráneo, región donde es típico este clima y en la que adquiere mayor extensión geográfica, abarcando más de 2.3 millones de km^2 (Cowling *et al*., 1996). Sin embargo, también está presente en otras zonas del planeta localizadas entre los 30° y 40° de latitud en ambos hemisferios (Lionello *et al*., 2006). Estas zonas presentan cambios en cuanto en a la distribución temporal de la precipitación, la duración del período de aridez, la amplitud térmica anual o la frecuencia de eventos extremos. Así, el clima mediterráneo genuino ocurre en cinco regiones que son: (i) la cuenca mediterránea, desde el océano Atlántico hasta Afganistán; (ii) la costa del Pacífico en América del Norte, California y Baja California; (iii) la costa central de Chile; (iv) la región del Cabo de Sudáfrica y (v) el oeste y sur de Australia (Walter, 1985). Si tenemos en cuenta la distribución de los robles en este tipo de clima a nivel global, cabe destacar las dos primeras regiones mencionadas (la cuenca mediterránea y la costa del Pacífico en América del Norte), ya que son las únicas en las que se encuentra el género *Quercus*.

A pesar de la característica común de un período de sequía estival, el clima mediterráneo es bastante diverso, con una marcada influencia en la fisonomía de la vegetación. Desde una perspectiva fitoclimática, y teniendo en cuenta la presencia de robles, el clima mediterráneo genuino puede evolucionar hacia (i) subclimas más cálidos y secos, en transición a los áridos; (ii) más templados y húmedos, en transición a climas templados o (iii) variantes más secas y frías, más cercanas a los climas típicos de estepa (Gil-Pelegrín *et al.*, 2017).

En el primer apartado de esta introducción se explicó cómo Walter (1985) le asignó a la región mediterránea el zonobioma IV y cómo este es clave en cuanto a la presencia de robles en su transición a otros zonobiomas. Respecto a esto, Breckle (2002) consideró los bosques perennifolios esclerófilos con predominancia de *Q. ilex* como la vegetación original de esta región. Así, se puede considerar que cuando las condiciones climáticas empeoran (i.e. aumento de la temperatura y reducción de la precipitación), *Q. ilex* se vería sustituido por *Q. coccifera* en las áreas de transición entre el zonobioma IV y el zonobioma III o "zonobioma de los desiertos cálidos" (**Fig. 2**). Por otra parte, cuando la temperatura y la aridez disminuyen, en la transición hacía el zonobioma VI, especies de caducifolios submediterráneos como *Q. faginea* o *Q. pyrenaica* son las que sustituyen a *Q. ilex* **(Fig. 2)**. Finalmente, en la transición al zonobioma VII (i.e. cuando baja la temperatura y la precipitación se mantiene), solo la subespecie *Q. ilex* subsp. *ballota* podría vivir en estas condiciones (Gil-Pelegrín *et al.*, 2017; Martín-Sánchez *et al.*, 2022; **Fig. 2**).

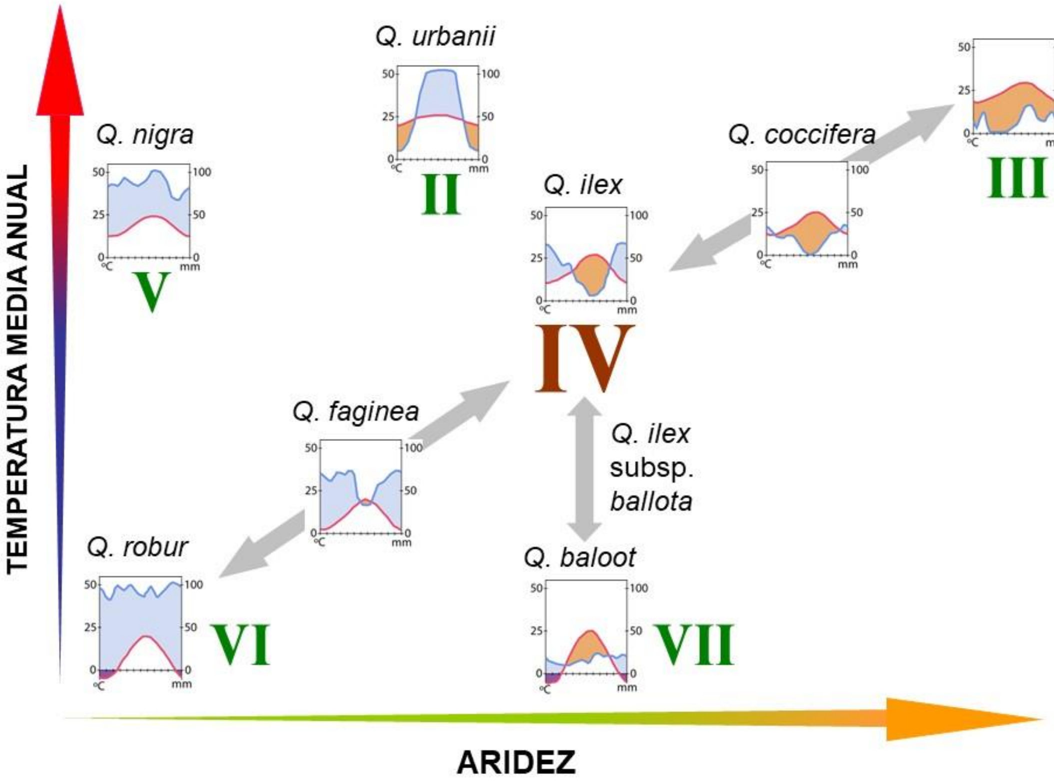

Figura 2. Transiciones entre el zonobioma IV y condiciones más áridas (zonobioma III), condiciones húmedas y frías (zonobioma VI) y condiciones más frías (zonobioma VII). Se muestran las especies de robles representativas de cada zonobioma de Walter (1985) y las transiciones mencionadas. Figura modificada de Gil-Pelegrín *et al.* (2017).

1.3. Tipos de robles en el clima mediterráneo

Debido a la diversidad que presenta el clima mediterráneo, así como la gran variabilidad fisionómica y funcional de los robles de estos ecosistemas, definir un prototipo de roble mediterráneo genuino no es nada sencillo. Históricamente, los árboles perennifolios esclerófilos de hoja ancha de las regiones mediterráneas se han considerado como un ejemplo de evolución convergente de la fisionomía vegetal en relación con el clima (Schimper, 1903; Cody y Mooney, 1978; Box, 1981). En este sentido, Schimper (1903) propuso: *"The mild temperate districts with winter rain and prolonged summer-drought are the home of evergreen xerophilous woody plants, which, owing to the stiffness of their thick, leathery leaves, may be termed sclerophyllous woody plants"*.

Sin embargo, el propio Schimper (1903) ya reconoció que en la cuenca mediterránea no solo existen robles perennifolios esclerófilos, pues también habitan en ella robles malacófilos (*i.e.*, de hoja blanda) caducifolios como *Q. lusitánica*. Así, observó: *"*Quercus Ilex*, Linn. (…), resembles the olive closely in habit. In the Mediterranean countries, this species with some others, such as* Q. coccifera *(…) and* Q. Suber*, represents the sclerophyllous type of the genus* Quercus*, whilst other common species of the district, such as* Q. lusitanica*, remain green in summer. The sclerophyllous oaks have small stiff leaves, which in* Q. Ilex *are usually entire, but in other species are sharply toothed"* (Schimper, 1903).

Hoy en día, se conocen especies caducifolias frecuentemente dominantes en lugares donde la sequía es severa. Este es el caso de *Q. douglasii*, que habita en la costa californiana y se distribuye en zonas que tienden a ser demasiado secas para otras especies (Barbour, 1988; Blumler, 1991). Incluso *Q. lobata*, un malacófilo caducifolio endémico de California que crece en los valles cálidos y piedemontes interiores, puede llegar a ser más tolerante a la sequía estival que otras especies perennifolias (Griffin, 1971, 1976). Además, en áreas de la cuenca mediterránea que experimentan veranos relativamente secos o, incluso muy secos, como en el sur de España, Italia, sudoeste de Turquía y el norte de África, se encuentran *Quercus* caducifolios malacófilos. Este es el caso de especies como *Q. faginea*, *Q. pyrenaica*, *Q. cerris* o *Q. ithaburensis*. De hecho, esta última especie es habitual en algunas de las localidades más áridas de la cuenca mediterránea, situadas al este de la misma (Dufour-Dror y Ertas, 2004).

La perspectiva paleontológica puede ayudar a entender mejor la complejidad a la hora de definir un prototipo de roble mediterráneo genuino. A principios del Terciario, mucho antes de la aparición del clima mediterráneo, las regiones mediterráneas presentaban un clima cálido y húmedo con precipitaciones que se distribuían a lo largo de todo el año (Verdú *et al*., 2003; Valiente-Banuet *et al*., 2006; Millar, 2012). En estas zonas del hemisferio norte habitaba la Flora Paleotropical, que como se ha comentado más arriba, estaba formada por abundantes bosques perennifolios (Valiente-Banuet *et al*., 2006) mientras que los taxones esclerófilos constituían en la mayoría de los casos el sotobosque de estos bosques (Valiente-Banuet *et al*., 2006).

Una gran cantidad especies se extinguieron cuando la sequía estival característica del clima mediterráneo se estableció en el hemisferio norte, hacia el Terciario Superior o principios del Cuaternario (Suc, 1984; Verdú *et al*., 2003; Millar, 2012). Fue entonces cuando esos arbustos perennifolios esclerófilos del sotobosque se expandieron geográficamente (Valiente-Banuet *et al*., 2006). En ese periodo de desarrollo de los ambientes secos, algunos autores ya observaron la presencia de hojas esclerófilas correspondientes a robles de tipo "ilex" o "suber" en el sur de Europa (Kovar-Eder, 2003) y al tipo "chrysolepis" en la costa oeste de Norte América (Retallack, 2004). Así, la evolución de los robles esclerófilos con anterioridad al desarrollo del clima mediterráneo pone de manifiesto que sería más preciso hablar de una preadaptación a este clima (Minnich, 1985).

Sin embargo, las regiones mediterráneas del hemisferio norte no solo fueron ocupadas por perennifolios esclerófilos. A principios del Terciario, existió una vegetación caducifolia que ocupó latitudes septentrionales, conocida como Flora Arctoterciaria (Mai, 1991). Esta vegetación migró hacia latitudes meridionales debido a la disminución global de las temperaturas que ocurrió durante el Terciario Medio y Superior (Mai, 1991; Kovar-Eder, 2003). Esto derivó en la aparición de especies arctoterciarias en áreas mediterráneas antes de la formación del clima mediterráneo (Axelrod, 1973; Kovar-Eder, 2003; Ackerly, 2009). Este pudo ser el caso de *Q. faginea*, cuyos primeros registros fósiles, encontrados en el sur de Francia, coinciden con el desarrollo de la estacionalidad mediterránea durante el Terciario Superior (Roiron, 1983; Barrón *et al*., 2010).

Quercus faginea es el roble de la sección *Quercus* más abundante y ampliamente distribuido en la Península Ibérica (Olalde *et al*., 2002). Algunos estudios sobre su resistencia a la sequía se basan en la comparación con el perennifolio *Q. ilex*, con el que coexiste en la mayoría de las áreas mediterráneas continentales de la Península Ibérica (Corcuera *et al*., 2002; Mediavilla y Escudero, 2003). Estas especies representan dos ejemplos de hábitos foliares contrastados con estrategias funcionales diferentes (Kikuzawa, 1995). Así, se ha propuesto que la condición perennifolia de *Q. ilex* permitiría a esta especie asimilar carbono durante un período de tiempo más prolongado (Acherar y Rambal, 1992; Ogaya y Peñuelas, 2007; van Ommen Kloeke *et al*., 2012). Por el contrario, la condición caducifolia de *Q. faginea* limita la actividad fotosintética de las hojas a un período más corto. Esto implicaría la necesidad de mayores tasas de ganancia de carbono en condiciones favorables (van Ommen Kloeke *et al*., 2012).

El hecho de que en la actualidad existan robles caducifolios en zonas con clima mediterráneo puede deberse a una adaptación de las especies arctoterciarias que incursionaron a latitudes más bajas (Suc, 1984; Blumler, 1991;

Peguero-Pina *et al.*, 2016). Teniendo en cuenta todos estos factores, en la actualidad es posible hablar de dos tipos de robles bien diferenciados que coexisten en las regiones mediterráneas: el roble perennifolio esclerófilo con un origen en la Flora Paleotropical y el malacófilo caducifolio de origen Arctoterciario (Barbero *et al.*, 1992; Damesin *et al.*, 1998; Mediavilla y Escudero, 2004).

Aunque esta diferenciación es clara, aún no ha sido posible dar una explicación definitiva a la coexistencia de los dos tipos de roble en el clima mediterráneo. La explicación mayormente aceptada hasta la fecha es la que sugiere que esta coexistencia se debe a cambios en la fertilidad del sustrato, en el clima a nivel local o la competencia con pantas herbáceas (Blumler, 1984, 1991). Así, los robles caducifolios mediterráneos dominarían aquellos sitios con inviernos más fríos y húmedos (Chabot y Hicks, 1982), suelos fértiles (Salleo *et al.*, 2002) y con alta competencia de la vegetación herbácea (Blumler, 1991). Por el contrario, los robles perennifolios se verían favorecidos en los lugares más secos, con suelos infértiles y con menor competencia con las herbáceas (Blumler, 1991; Manes, 2006). De este modo, no es difícil pensar que ambos tipos de robles coexistirán en ambientes heterogéneos con variaciones en los factores mencionados anteriormente, mientras que en los ambientes homogéneos dominará un solo tipo (Gil-Pelegrín *et al.*, 2017).

1.4. Adaptaciones de los robles al clima mediterráneo

Como se ha mencionado anteriormente, el frío invernal y la aridez estival limitan el funcionamiento de los robles mediterráneos a lo largo del año, produciendo una fragmentación del periodo vegetativo (Mitrakos, 1980; Castro-Díez y Montserrat-Martí, 1998). Los caducifolios pierden las hojas en otoño, de modo que así evitan las condiciones impuestas por el invierno (bajas temperaturas, sequía fisiológica) que sí deben soportar las hojas de los perennifolios (García-Plazaola *et al.*, 1999; Corcuera *et al.*, 2005). Sin embargo, las condiciones de sequía impuestas por el verano mediterráneo afectan al desempeño y crecimiento de los dos tipos de robles coexistentes, aunque con respuestas muy distintas (Gil-Pelegrín *et al.*, 2017). De este modo, los dos tipos de robles deben constituir dos modelos de funcionamiento muy distintos frente al clima mediterráneo. Estos modelos, que influyen en la adecuación de la planta al ambiente (Violle *et al.*, 2007), pueden ser muy diferentes a (i) nivel morfológico, (ii) fenológico y (iii) fisiológico.

1.4.1. Adaptaciones morfológicas

Uno de los aspectos más inmediatos que surgen del estudio de los *Quercus* mediterráneos es la textura foliar, existiendo robles de hojas duras y robles de hojas blandas. En el estudio de la botánica se han usado diferentes términos como "dura", "coriácea" o "rígida" entre otros para describir la textura foliar. Sin embargo, fue Schimper (1903), quien agrupó todas estas características en un solo concepto al que llamó esclerofilia. Este término está formado por la conjunción de los vocablos griegos *sklēros* (duro) y *phyllon* (hoja), significando literalmente "hoja dura". Originalmente, se usó para diferenciar la vegetación xeromórfica (plantas adaptadas a la sequía) con hojas coriáceas de las plantas suculentas. No obstante, el término esclerofilia ha sufrido cambios sustanciales desde que fue acuñado, ya que en la actualidad se sabe que puede encontrarse en una gran variedad de entornos y su significado funcional es incierto (Read y Sanson, 2003).

Entre las funciones hipotéticas se incluyen la resistencia a la sequía (Schimper, 1903; Oertli *et al.*, 1990), una respuesta a los suelos bajos en nutrientes (Loveless, 1961; Beadle, 1966), la resistencia a bajas temperaturas (Koppel y Heinsoo, 1994; Lamontagne *et al.*, 1998) o el incremento de la longevidad foliar mediante su protección (Chabot y Hicks, 1982; Turner, 1994).

A pesar de la evidente importancia ecológica de la esclerofilia, se sabe poco sobre este rasgo foliar. Para obtener un valor cuantitativo de la esclerofilia, se han usado diferentes estimadores y variables representativas, ejemplo de ello es la *leaf mass per unit area* (LMA), ampliamente usada en ecofisiología (Witkowski y Lamont, 1991; Osone *et al.*, 2008; Poorter *et al.*, 2012). La LMA es el producto de la densidad y el espesor foliar, por lo que se ha sugerido que las variaciones en este rasgo tienen dos consecuencias principales para el funcionamiento de la hoja asociadas a la teoría del *leaf economics spectrum (*Wright *et al.*, 2004; Shipley *et al.*, 2006*)*. Por una parte, se puede esperar que un aumento de LMA, asociado al espesor del mesófilo, propicie una mayor fotosíntesis por unidad de área debida al incremento de tejido fotosintético (Niinemets, 1999; Niinemets, 2001). Por otra parte, los cambios en LMA asociados a la densidad implican una variación en la distribución de la biomasa foliar o en la cantidad de tipos celulares (Hassiotou *et* *al.*, 2010; Niinemets, 2001). Esto provocaría una disminución de la fracción de tejidos fotosintéticamente activos, reduciendo así la asimilación de carbono instantánea que podría ser compensada con una mayor longevidad foliar (Kikuzawa, 1995; Niinemets, 2001; Kikuzawa y Lechowicz, 2011).

En el contexto de los dos tipos de *Quercus* mencionados anteriormente, existe una diferencia clara en su LMA. Mientras que los robles mediterráneos perennifolios presentan valores promedios de 150-160 g m^2 (Villar y Merino, 2001; Corcuera *et al.*, 2002) llegando a sobrepasar los 200 g m^2 en especies como *Q. ilex* (Serrano *et al.*, 2005) o *Q. coccifera* (Rubio de Casas *et al.*, 2009), los caducifolios muestran valores promedio de 80-112 g m^2 (Villar y Merino, 2001; Corcuera *et al.*, 2002). Por lo tanto, LMA es uno de los parámetros funcionales que separan de manera más clara los dos tipos de robles mediterráneos entre sí.

Sin embargo, aunque la LMA representa un buen rasgo representativo de la esclerofilia, no es necesariamente una medida de la resistencia mecánica de la hoja (Onoda *et al.*, 2011). Para estudiar las propiedades mecánicas existen estimadores directos como los relacionadas con la fractura, como son la fuerza y la resistencia (Onoda *et al.*, 2011). Aunque existen múltiples rasgos que pueden estar relacionados con la resistencia a la fractura de la hoja aún no se conoce su importancia relativa a la esclerofilia. Este es el caso grosor del mesófilo y la epidermis, la densidad del tejido, el contenido de fibra de la pared celular y la dureza y disposición de las venas (Choong *et al.*, 1992; Westbrook *et al.*, 2011). La resistencia mecánica puede proteger las hojas de los daños bióticos (Wright *et al.*, 2007; Peeters *et al.*, 2007) y abióticos (Poudyal *et al.*, 2004; Niklas, 1993). En consecuencia, una mayor dureza de la hoja debería incrementar la vida útil de la hoja y la supervivencia de toda la planta.

1.4.2. Adaptaciones fenológicas

La relación entre el aumento en la esclerofilia y la mayor longevidad foliar se ha estudiado desde una perspectiva global de las plantas. En este sentido, varios autores han encontrado relaciones positivas entre LMA o propiedades físicas con la vida útil de la hoja (Wright *et al.*, 2001, 2004; Onoda *et al.*, 2011). Un ejemplo de estas relaciones se da en los esclerófilos perennifolios mediterráneos, entre los que se encuentra *Q. ilex* con cohortes de hojas que llegan a durar varios años (Niinemets *et al.*, 2005). Además, aunque las hojas con varios años tienen tasas bajas de fotosíntesis, contribuyen a una mayor asimilación de CO_2 a nivel de toda la copa (Escudero y Mediavilla, 2003).

Numerosos estudios ponen de manifiesto que las hojas viejas de diferentes plantas presentan una mayor limitación de la fotosíntesis, influenciada por la difusión del CO_2 desde las cavidades subestomáticas hasta el interior del cloroplasto (conductancia del mesófilo, g_m) (Niinemets *et al.*, 2005, 2006; Flexas *et al.*, 2007). El paso del CO_2 dentro del mesófilo se ve comprometido por una serie de rasgos anatómicos, incluyendo el tamaño del cloroplasto, el grosor de la pared celular y la superficie de mesófilo y de cloroplasto expuesta a espacios intercelulares (Terashima *et al.*, 2011; Peguero-Pina *et al.*, 2016, 2017). Además, la senescencia que sufren las hojas viejas puede llevar a desmantelar su aparato fotosintético, lo que se ve reflejado en un menor contenido de nitrógeno por la pérdida de enzimas fotosintéticos (Rubisco). Sin embargo, el papel de la anatomía y el contenido de nitrógeno en la capacidad fotosintética y g_m en hojas con varios años de vida aún no está claro.

1.4.3. Adaptaciones fisiológicas

Como se ha visto anteriormente, la incidencia de un periodo de sequía estival afecta severamente el crecimiento y desempeño de los dos tipos de robles que coexisten en el clima mediterráneo. Por eso, han desarrollado diferentes estrategias para sobrevivir a la insuficiente disponibilidad de agua. Además, en condiciones de sequía, se suele originar un estrés múltiple ya que frecuentemente el estrés hídrico suele ir acompañado de altas temperaturas y fuerte radiación solar. Entre estas respuestas fisiológicas, se encuentran (i) aquellas relacionadas con el control estomático y el transporte de agua y (ii) las asociadas con la regulación de la actividad fotosintética (Medrano y Flexas, 2003).

El cierre estomático responde principalmente a la necesidad de la planta de reducir las pérdidas de agua por transpiración a expensas de una reducción en la fotosíntesis (Lambers *et al.*, 1998). Este mecanismo se produce principalmente cuando disminuye el potencial hídrico de las hojas y ocurre en ambos tipos de robles (Acherar y Rambal, 1992; Mediavilla y Escudero, 2003; Peguero-Pina *et al.*, 2009). Durante un periodo de sequía, el gradiente de potencial entre las raíces y las hojas se incrementa y puede producirse un embolismo que reduzca la capacidad de transporte de agua o conductividad hidráulica. Así, muchos de los robles mediterráneos desarrollan un xilema altamente resistente al embolismo inducido por sequía que soporta grandes gradientes de potencial hídrico (Corcuera *et al*. 2005; Gil-Pelegrín *et al*., 2017). De este modo, el cierre estomático temprano y la alta resistencia del xilema al embolismo son factores claves en la supervivencia de los robles mediterráneos (Vilagrosa *et al.,* 2010). Esta resistencia puede ser alta en el xilema del tallo, de la hoja o de ambos (Hochberg *et al.,* 2017; Creek *et al*., 2018). La combinación de una baja tolerancia en hojas y alta en tallos constituye un ejemplo de "segmentación de la vulnerabilidad" que confina el daño por sequia a los extremos más "prescindibles" del continuo raíz-tallo-hoja (Peguero-Pina *et al.,* 2015; Skelton *et al*., 2018).

Estudios previos han establecido que el vínculo entre la fotosíntesis y la hidráulica de la hoja surge de la conductancia estomática (g_s) (Brodribb *et al*., 2007; Scoffoni *et al*., 2016), ya que la pérdida de agua y la absorción de CO_2 comparten una vía común a través de los estomas (Boyer, 2015). Además, varios estudios también han señalado la existencia de una coordinación entre la conductancia del mesófilo y la hidráulica foliar (Flexas *et al*., 2013; Lu *et al*., 2019; Xiong *et al*., 2017, 2018; Xiong y Nadal, 2020). Estos autores sugirieron que el movimiento del agua en los tejidos del mesófilo también comparte una vía común con el transporte de CO_2 a través de la fase gaseosa que conectaría g_m y el componente de la conductancia hidráulica de la hoja por fuera del xilema (K_{ox}).

En cuanto a las respuestas al estrés hídrico asociadas a la regulación a la baja de la actividad fotosintética se encuentran diferentes procesos. Este es el caso de la disminución en la conductancia del mesófilo (Flexas *et al*., 2012; Niinemets y Keenan, 2014) y la disminución de la captación de luz para proteger el aparato fotosintético (Peguero-Pina *et al*., 2008, 2009). Así, cuando la luz es excesiva bajo condiciones de cierre estomático y la clorofila excitada no es capaz de impulsar la fotoquímica, puede volver a su estado inicial emitiendo fluorescencia o disipando calor. Este último es un mecanismo fotoprotector llamado extinción no fotoquímica (NPQ) de la fluorescencia de la clorofila que se asocia al ciclo de las xantofilas (interconversiones de carotenoides en la membrana del tilacoide) y está presente en *Quercus* mediterráneos (García-Plazaola *et al*., 2017 y referencias incluidas). Sin embargo, existen pocos estudios comparados que analicen la respuesta de la planta a un periodo de sequía severa a nivel fotosintético, fotoquímico y de rasgos hidráulicos de diferentes órganos en los dos tipos de robles mediterráneos.

1.5. Objetivos y estructura de la tesis

1.5.1. Objetivos

El principal objetivo de esta tesis es el estudio comparado del funcionamiento de los dos tipos de especies de robles con diferentes hábitos foliares y grado de esclerofilia que coexisten en el clima mediterráneo. Para la consecución de este objetivo general, se han establecido una serie de objetivos específicos, detallados a continuación:

i) La esclerofilia en el género *Quercus* a partir de la información disponible en la literatura y bases de datos públicas: factores que la promueven, su papel funcional y las consecuencias para la hoja en el contexto del "*Leaf Economics Spectrum*".

ii) Cuantificación y estudio de la diversificación en la morfología, anatomía y composición química de las hojas de robles caducifolios y perennifolios, analizando el papel del clima en la diversificación diferencial de LMA dentro de ambos tipos de robles.

iii) Estudio de la resistencia mecánica foliar en especies caducifolias y perennifolias del género *Quercus*: determinación de los rasgos anatómicos y composicionales que explican la variabilidad dentro del género.

iv) Evaluación de los cambios en los rasgos morfológicos, anatómicos, químicos y fotosintéticos relacionados con la edad de las hojas en un roble mediterráneo perennifolio con una alta longevidad foliar.

v) Análisis de la diferente respuesta a la sequía en los rasgos fotosintéticos, fotoquímicos e hidráulicos en diferentes órganos de dos especies mediterráneas con diferente habito foliar (*Q. faginea* y *Q. ilex* subsp. *rotundifolia*).

1.5.2. Estructura de la tesis

Esta tesis consta de siete capítulos: la introducción general (primer capítulo), cinco artículos de investigación (capítulos del segundo al sexto) y la discusión general, que, junto a las conclusiones, cierran este trabajo en el séptimo capítulo.

Cuatro de los cinco capítulos de investigación han sido publicados en revistas internacionales pertenecientes al primer decil (D1) dentro de las categorías de *Journal Citation Reports* (JCR) catalogadas como *Forestry* o *Plant Sciences*. En el momento finalización de esta tesis, el cuarto capítulo aún no ha sido publicado en una revista, pero sí como *preprint* en bioRxiv (doi: 10.1101/2022.10.21.513225). Además, este ha sido enviado para su revisión a la revista *Annals of Botany*, situada en el primer cuartil *(*Q1) en *Plant Sciences*. Para su presentación en este documento, se ha conservado el formato de edición de cada una de las revistas en la que se han publicado los artículos y se mantiene el idioma en el que fueron publicados, que en todos los casos es el inglés.

El segundo capítulo fue publicado en 2020 en la revista *Current Forestry Reports* bajo el título de *Revisiting the Functional Basis of Sclerophylly Within the Leaf Economics Spectrum of Oaks: Different Roads to Rome*. En él se aborda el primer objetivo específico de la tesis realizando una revisión bibliográfica sobre la esclerofilia en el género *Quercus* para definir los mecanismos que hay detrás y sus consecuencias económicas para la hoja. Pese a al amplio rango de variación en los rasgos funcionales de las hojas y sus estrategias adaptativas contrastantes, no puede explicarse por sí sola por ninguno de los factores ecológicos considerados. Así, la sequía, la escasez de nutrientes, las bajas temperaturas durante el período vegetativo, y daño físico, no pudieron ser descartado por completo. Nuestro estudio sugiere que estas restricciones pueden tener un efecto sinérgico, y desde un punto de vista funcional, podemos concluir que, en los robles, el hábito foliar modula en gran medida las implicaciones fisiológicas de la esclerofilia.

El tercer capítulo de la tesis fue publicado en 2021 en la revista *New Phytologist* con el título *Deciduous and evergreen oaks show contrasting adaptive responses in leaf mass per area across environments*. En este capítulo se aborda el segundo objetivo específico. Para ello, usando un enfoque filogenético, se cuantifican las variables anatómicas, composicionales y climáticas en 85 especies de *Quercus* caducifolios y perennifolios de todo el hemisferio norte. Estos robles mostraron diferencias morfológicas foliares fundamentales que revelaron una respuesta adaptativa diversa. Mientras que la LMA en los caducifolios parece haberse diversificado en coordinación con el grosor modulado principalmente por la aridez, la diversificación de la LMA en los perennifolios parece depender del grupo infragenérico. Así, en estos últimos, la diversificación en el grosor de la hoja fue modulada tanto por la aridez como por el frío, mientras que la diversificación en la densidad de la hoja es sólo modulada por la aridez.

El cuarto capítulo, que fue publicado en 2022 como *preprint* en bioRxiv (doi: 10.1101/2022.10.21.513225), lleva por título *Disentagling leaf structural and material properties in relation to their anatomical and chemical compositional traits in oaks (*Quercus *L.)*. En este capítulo se acomete el tercer objetivo específico estudiando la importancia relativa de cada característica de la hoja sobre las propiedades mecánicas en 25 especies de *Quercus*. Se sugiere que las hojas de *Quercus* esclerófilos son más duras y fuertes debido a la pared externa de su epidermis más gruesa y/o a una mayor concentración de celulosa. Además, las especies de la sección *Ilex* comparten rasgos comunes independientemente de que ocupen climas bastante diferentes. De modo similar, las especies de hoja perenne que viven en climas de tipo mediterráneo comparten rasgos foliares comunes independientemente de su diferente origen filogenético.

El quinto capítulo se publicó en 2022. en la revista *Tree Physiology* bajo el título *Cell-level anatomy explains leaf age dependent declines in mesophyll conductance and photosynthetic capacity in the evergreen Mediterranean oak* Quercus ilex *subsp.* rotundifolia. En él se evalúan los cambios dependientes de la edad foliar en los rasgos morfológicos, anatómicos, químicos y fotosintéticos en *Quercus ilex* subsp. *rotundifolia* Lam., un roble perennifolio con hojas de alta longevidad. Se observan disminuciones de todos los rasgos fotosintéticos con el aumento de la edad de la hoja. Los cambios en la anatomía y bioquímica de la hoja fueron responsables de las modificaciones dependientes de la edad en la asimilación neta de CO_2 y la conductancia del CO_2 a través del mesófilo. Estos hallazgos revelaron un deterioro fisiológico gradual relacionado con el desmantelamiento del aparato fotosintético en hojas más viejas de *Q. ilex* subsp. *rotundifolia.*

El sexto capítulo de la tesis se publicó en 2021 en *Tree Physiology* con el título *Contrasting functional strategies following severe drought in two Mediterranean oaks with different leaf habit:* Quercus faginea *and* Quercus ilex *subsp.* rotundifolia. En este capítulo se aborda el quinto objetivo de la tesis. Para ello, se analizan las características fotosintéticas, fotoquímicas e hidráulicas de diferentes órganos de

Q. faginea y *Q. ilex* subsp. *rotundifolia* bajo condiciones de sequía severa. La fuerte disminución de los rasgos fotosintéticos de *Q. faginea* estuvo acompañada de una fuerte disminución de la conductancia hidráulica de las hojas en respuesta a la sequía. Este hecho probablemente evitó una embolia xilemática en los tallos ("segmentación de la vulnerabilidad"). Por el contrario, las hojas de *Q. ilex* subsp. *rotundifolia* mostraron mecanismos fotoprotectores efectivos y alta resistencia a la cavitación inducida por la sequía, lo que estaría relacionado con la mayor longevidad de las hojas de las encinas mediterráneas.

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Capítulo II

Revisiting the functional basis of sclerophylly within the Leaf Economics Spectrum: Different roads to Rome

Current Forestry Reports
https://doi.org/10.1007/s40725-020-00122-7

ECOLOGICAL FUNCTION (M WATT, SECTION EDITOR)

Revisiting the Functional Basis of Sclerophylly Within the Leaf Economics Spectrum of Oaks: Different Roads to Rome

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Abstract

Purpose of Review Defining the mechanisms behind and the leaf economic consequences of the development of sclerophylly in woody plants will allow us to understand its ecological implications, anticipate the potential for adaptation of different tree species to global change, and define new woody plant ideotypes for stress tolerance.

Recent Findings Sclerophylly has evolved independently in different woody plant genera and has been traditionally considered as a stress-tolerance trait. However, the underlying drivers for this functional trait are still a matter of debate; it has been proposed as an adaptive response to miscellaneous stress factors, such as nutrient scarcity, drought stress, herbivory, and cold tolerance, and due to the large investment costs of sclerophylly, it is generally associated with a longer leaf life span.

Summary The genus *Quercus* constitutes a unique living laboratory to understand global adaptive patterns along the leaf economic spectrum in forest trees. With more than 400 species, oaks are distributed along six zonobiomes and its versatility has resulted in a wide range of variations in leaf functional traits and contrasting adaptive strategies. However, although this wide variability cannot be explained alone by any of the ecological factors considered, such as drought, nutrient scarcity, low temperatures during vegetative period, and physical damage, neither any of them could be fully discarded. Noteworthy, our study also suggests that these constraints may have a synergistic effect, and from a functional point of view, we can conclude that in oaks leaf habit largely modulates the physiological implications of sclerophylly.

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This article is part of the Topical Collection on *Ecological Function*

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s40725-020-00122-7) contains supplementary material, which is available to authorized users.

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Keywords Sclerophylly · *Quercus* · Stress factors · Drought · Nutrient scarcity · Leaf life span

General Introduction

The term sclerophylly comes from the Greek *skleros* (hard) and *phyllon* (leaf) literally meaning "hard-leaved." According to Schimper [1], sclerophyllous species have common characteristics, such as leaves with entire margins, oriented obliquely or parallel to the incidence of sun rays, thick cell walls, abundance of sclerenchyma, and highly developed cuticle. Thus, Schimper concluded that "these qualities in the aggregate give the leaf its characteristic, stiff, leathery consistency."

Thus, as the concept of sclerophylly is a perception, it is difficult to obtain an accurate measure. Different methods and proxies have appeared for estimating this characteristic, such as the Loveless sclerophylly index [2, 3] or leaf mass per area (LMA) [4–7] and others that will be explained more in depth.

However, the underlying drivers for this functional trait are still a matter of debate; it has been proposed as an adaptive response to miscellaneous stress factors, such as drought [8, 9], nutrient scarcity [2, 3, 10], low temperatures during the vegetative period [11, 12], and physical damage [13, 14]. On the other hand, it has been argued that the higher construction costs of sclerophyllous leaves tend to be compensated by a longer leaf life span, although the mechanisms behind are controversial [15].

In this review, we expose the main factors that promote sclerophylly and its functional implications using the genus *Quercus* as a living model. This genus comprises more than 400 species, is widely distributed along six well-differentiated zonobiomes, and its versatility and diversity has resulted in a wide range of variation in leaf functional traits and adaptive strategies [16••].

How to Measure Sclerophylly?

Direct Measurement of Leaf Strength: Mechanical Approaches

As sclerophylly means "hard-leaved," it is crucial to quantify mechanical properties of leaves in order to understand this trait. There are a number of methods available to evaluate mechanical properties of leaves (Table 1). According to the concepts, "strength" and "toughness" of a material refers to its ability to resist applied force and applied work, respectively, that could lead to fracture or permanent failure, while "elasticity" refers to its ability to deform (without fracture or permanent failure) under applied force. On the one hand, fracture properties, including strength and toughness, are indicators of the ability to protect leaves against the risks of fracture caused by piercing, chewing and browsing herbivores, wind tear, etc. On the other hand, elastic properties become important when leaves are exposed to deformation, such as bending under gravitational force, rotating around the petiole, and streamlining under dynamic load such as wind or avoidance of strong solar radiation under water stress (e.g., wilting) etc.

There are four major types of tests that are frequently used to measure leaf mechanical properties (Table 1; [17, 18]): (1) shearing tests (also called scissoring, cutting tests) measure how much work is required to cut across a leaf with a single blade (against an anvil) or with a pair of blades (i.e., instrumented scissors) [19–21]; (2) punch tests, including punch-and-die and penetrometer tests, measure the maximum load required for the punch rod to penetrate a leaf [22–25]; (3) tensile tests (also called tearing tests) measure the force required to stretch and eventually tear a strip of leaf lamina [26–28]; and (4) bending tests (or flexural tests) measure the force required to bend a strip of leaf lamina that is placed on two supports [17, 29] or entire leaf lamina with attached petiole [30, 31]. These tests can measure mechanical properties of a leaf specimen (often rectangular strip) in different directions (vertical or horizontal to the lamina surface) and

Table 1 A list of leaf mechanical properties and their units

Type of tests	Fracture properties			Elastic properties	
	Structural level	Material level	Mass level	Structural level	Material level
Shearing test	Work to shear (J m ^{1})	Fracture toughness (J m ^{2})	Specific toughness (J m Kg ^{1})	NA	Shear modulus
Punch test	Force to punch (N m ^{1})	Punch strength (N m ^{2})	Specific punch strength (N m Kg ^{1})	NA	NA
Tensile test	Force to tear (N m ^{1})	Tensile strength (N m ^{2})	Specific tensile strength (N m Kg ^{1})	Tensile stiffness (N)	Young's modulus (N m ^{2})
Bending test	NA	NA	NA	Bending stiffness (N m^{2})	Young's modulus (N m ^{2})

different tissue types (e.g., the major leaf veins or the lamina between veins).

Leaf mechanical properties can be expressed on a different basis of expression depending on the purpose (Table 1; [18]). Structural properties are affected by both chemical properties and lamina thickness, whereas chemical properties are normalized to cross-sectional area (thickness × width) of the test specimen [32]. It is also possible to evaluate how efficiently leaf mass is exploited for fracture strength or toughness from strength or toughness divided by tissue density of the specimen [18, 33].

Although sclerophylly has been recognized as a key functional trait for many years [1], direct quantification of mechanical properties of sclerophylly had rarely been achieved until the 1990s. Turner et al. [34] measured and compared the fracture toughness of several plant communities, including three sclerophyllous communities, by the cutting test. They found that the mean fracture toughness of the sclerophyllous communities was 2.1 to 4.6 times greater than the soft-leaved pioneer communities. Read & Sanson [32] made a detailed investigation of leaf chemical as well as mechanical traits using the punch, shear, tensile, and bending tests for a diverse set of 33 plant species grown in a botanical garden. On average, sclerophyllous leaves were stronger, tougher, and stiffer than non-sclerophyllous leaves in all mechanical tests, but more importantly, they found that structural properties (such as structural strength or bending stiffness) correlated more strongly with sclerophylly than material properties. This means that sclerophylly is characterized by not only high material strength or toughness but also high lamina thickness.

As discussed later, plant cuticle plays an essential role in preventing passive water loss. While less appreciated, cuticles are also important for mechanical defense and stability [14]. Cuticle layers are composed by cutin, polysaccharides, and waxes, and their Young's moduli (material stiffness) can be an order of magnitude higher than the corresponding values of the leaf lamina [35]. Furthermore, the farther the leaf material is located from the neutral axis, the more it contributes to bending stiffness (second moment of area). Therefore, leaves with thicker cuticles often have higher lamina strength and stiffness [29]. Thicker and stiff cuticles are conducive to long leaf life span by protecting leaves from external mechanical stresses and may contribute to keeping the leaf surface intact and sustaining high desiccation tolerance over longer life spans.

Chemical Proxies

An increase in sclerophylly is associated with a decrease in the percentage of protein content and with an increase in the percentage of fiber content [3]. Loveless [2, 3] proposed an estimation method of sclerophylly expressed as the ratio of crude protein content (calculated as nitrogen content multiplied by 6.25) per unit of leaf tissue.

$$(\text{Crude fiber dry mass} \times 100)/(\text{Crude protein dry mass})$$

Loveless [2, 3] suggested that the use of crude protein content has some advantages since it is a useful indicator to measure functionally active leaf tissue (protoplasm), does not vary throughout the day, and does not define fiber content in terms of another measure that already includes the fiber in it. However, the estimation of sclerophylly using the method proposed by Loveless has some drawbacks. Firstly, the Sclerophylly Index (SI) cannot be used to correlate the degree of sclerophylly with the fiber or protein content, since these components are already part of the ratio. Secondly, it seems that the increase in SI is mainly due to a decrease in the denominator (protein content, not related to sclerophylly), rather than to an increase in fiber content (related to sclerophylly) [36]. Furthermore, there is an increasing body of evidence that a large fraction of leaf nitrogen can be incorporated in cell wall proteins, especially in more sclerophyllous species [37, 38•] suggesting that the "crude protein" content is not a good measure of the protoplasm.

Leaf Mass per Area as a Global Proxy for Sclerophylly

Leaf mass per area (LMA), is the most widely used proxy for sclerophylly in ecology (see e.g., [4–7, 18, 39, 40]). The main advantage of LMA is that it can be easily determined and stands as a good indicator of the carbon investment per unit of leaf area. Hence, it is a reflection of the cost-benefit between light interception and plant carbon balance, and hence of the leaf economic spectrum (LES; [6, 40]). At least on a global scale, LMA shows a good correlation with mechanical measures of sclerophylly [18] and, to a lesser extent, with chemical proxies [3, 41]. LMA is indeed a composite of leaf thickness (LT) and leaf density (LD), which can vary independently along environmental gradients [5, 41, 42]. Although the relative contribution of LT and LD to sclerophylly depends on the underlying strategy, both factors are known to contribute to leaf toughness [18]. When we compare oaks in the context of woody angiosperms from temperate and tropical zones, LMA of winter-deciduous oaks (DEC) did not differ significantly from other DEC species, whereas evergreen oaks (EVE, those retaining their leaves during the whole year) showed higher LMA than EVE plant species (Fig. 1a, data from the Leaf Biomechanics Dataset [18]). Oaks in general seem to have relatively thin leaves (low LT, Fig. 1b), and dense tissues (high LD, Fig. 1c), with EVE falling within the uppermost range of LD (Fig. 1c). As we will discuss later in detail in section 4.1, large LMA values may have contrasting physiological implications, depending

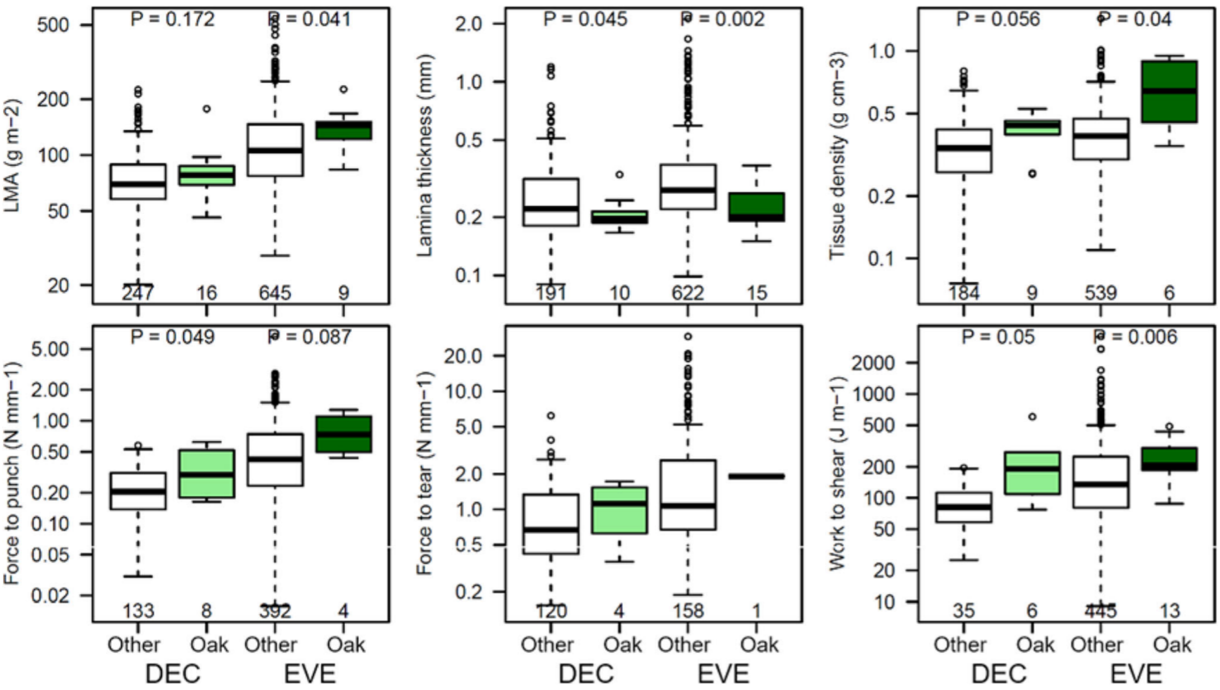

Fig. 1 Measurements of leaf attributes (leaf mass per area (LMA), leaf thickness, and leaf density) and mechanical strength (force to punch, force to tear, work to shear) in deciduous (DEC) and evergreen (EVE) oak species, as compared with other genera of woody angiosperms, all from temperate and tropical biomes. Data from the Leaf Biomechanics Dataset [18]

on the relative contribution of LT and LD. In particular, we will see how the high LD of EVE constitutes a major physiological constraint by restricting CO_2 diffusion.

Despite a general association between LMA and leaf structural strength, the two variables may vary independently. On the one hand, leaves with higher concentrations of fibers can have higher leaf structural strength without affecting LMA (i.e., higher specific strength) [18]. On the other hand, sclerophyllous leaves often have thicker cuticles, which also contribute to leaf strength and stiffness [29, 35], but have a comparatively small effect on LMA (for further discussion about the association between LMA and cuticles, see "Functional Mechanisms Associating Sclerophylly and Drought Tolerance"). While we are aware of these constraints, throughout this review, we will focus on LMA as the most general proxy for sclerophylly, and the only one that has been determined in a wide range of oak species.

Key Factors Promoting Sclerophylly

Sclerophylly as a Xeromorphic Trait to Cope with Drought

The "Evidence" Given by Mediterranean Woody Plants

The development of hard leaves as a response to drought is one of the earlier interpretations of sclerophylly [14, 43, 44], still current [45–48] but under debate ([16••] and references therein). The association between sclerophylly (the development of specially hard or tough leaves) and xeromorphism (all the anatomical and morphological traits common in plants living in dry or physiologically dry habitats) partially depends on phytogeographical evidence, such as the predominance of sclerophyllous shrubs and trees in all five Mediterranean regions of the world, with dry and hot summers [1, 14, 49–52].

Schimper [1] described the features of a typical xeromorphic leaf, as a way of coping with limited water-supply in their habitats. Among several adaptations, he included the presence of sclerenchyma although an explicit function of it was not provided. Schimper also suggested that the increase in "physiological dryness" (a term indicating the lack of available water for plants) should induce some concomitant changes in the leaf anatomy or morphology. Among others, the development of smaller, thicker, and sclerophyllous leaves was reported in his analysis. This response, common of many plant species of dry habitats, was put at the same level as leaf succulence or aphylly by Schimper [1], among the different plant responses to drought. He argued that the regions of the Earth with "prolonged summer drought" (or Mediterranean-type areas in other words) are the habitat of "evergreen xerophyllous" woody plants that show sclerophyllous leaves. Oppenheimer [53] gave a list of anatomical features that were common in xeromorphous leaves and included the increase in mechanical tissues as "a general principle" in those plants from dry habitats. Moreover, the leaf thickening—or the reduction of surface to volume, as literally expressed by this author—was also proposed as a key trait in plants living in dry conditions. Both characteristics should imply a higher LMA, linking xeromorphism and sclerophylly. In fact, he included the Mediterranean evergreen sclerophyllous plants among the six main xerophyte types according to their physiognomy or phytogeography. Kummerow [50] insisted that regions with Mediterranean-type climates show vegetation with a similar physiognomy, which could be interpreted as xerophytic—plants of dry habitats—and, specifically, "sclerophyllous xerophytes." Those plants were again the evergreen hard-leaved trees and shrubs previously described by Schimper [1] or Mooney & Dunn [49]. Both studies clearly supported the idea that the leaf anatomy of these sclerophyllous plants was responsible for the adaptation for withstanding water stress during the summer. The anatomical modifications suggested by Kummerow [50] coincide with those proposed by Oppenheimer [50], including a reduction in the external leaf area and presence of a group of traits inducing high leaf density, such as smaller cell size with thick cell walls, a high vein density, or the presence of reinforcement tissues. The presence of sclerenchyma among the leaf tissues in these leaves was proposed to serve as protection when subjected to intense water deficit. Mooney [54] recognized similar features in the leaves of different species of shrubs from the areas under Mediterranean-type climate of California as a response to water stress. The development of dense mesophyll tissue—constituted by small cells with relatively thick walls and low presence of intercellular spaces and a higher proportion of palisade to spongy mesophyll thickness—is still proposed as an anatomical response to water shortage [55].

What Can Be Concluded From Other Phytogeographical Data? Sclerophyllous Oaks Under Non-Mediterranean Conditions

However, this association between the Mediterranean-type climates and sclerophylly conflicted with other phytogeographical evidence, namely the presence of sclerophyllous woody plants in wet climatic regions of the Earth [14, 53, 56]. In fact, their massive presence in areas with wet tropical climatic regimes is well-established [44, 57], with some areas clearly dominated by evergreen, sclerophyllous oak species [58]. Palaeobotanical studies would suggest that the possible ancestors of Mediterranean species of holly oaks (*Quercus* Group Ilex) lived in fully humid conditions [59], confirming the pre-adaptive conditions of a high LMA in the present Mediterranean woody plants [60].

In a common garden study, Gil-Pelegrín et al. [16••] concluded that evergreen oaks from tropical (zonobiome II), arid (zonobiome III), Mediterranean (zonobiome IV), and temperate (zonobiome VI) did not differ significantly in LMA, in spite of the extreme differences in their respective phytoclimates in origin. Similarly, the two groups of winter-deciduous species, namely Mediterranean (zonobiome IV) and temperate (zonobiome VI) did not show differences in LMA values between them but did show significantly lower values than all the evergreen oaks. These data confirm (i) the existence of sclerophyllous *Quercus* species in contrasting phytoclimates and (ii) the existance of both sclerophyllous and malacophyllous species in the same phytoclimatic conditions (zonobiomes IV and VI specially). In order to take into account the possible relationship between aridity and LMA, the values of all of these groups have been plotted against an index of aridity for oak species of all of the considered groups (Fig. 2). We used the Lang aridity index (AI_{Lang})—the ratio of the mean annual precipitation (MAP) and the mean annual temperature (MAT) [61]—in several native locations as

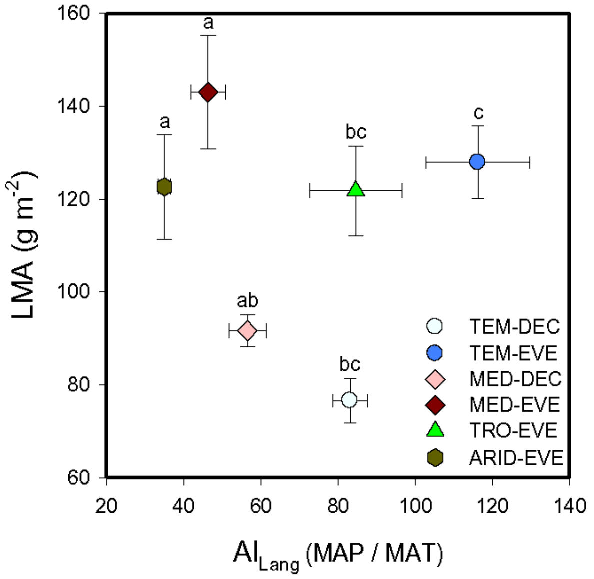

Fig. 2 Relation between Lang aridity index (AILang) and leaf mass area (LMA) for oak species belonged to different groups. AILang calculated as the ratio between mean annual precipitation (MAP) and mean annual temperature (MAT). DEC: deciduous, EVE: evergreen, TEM: temperate, MED: Mediterranean, TRO: tropical, ARID: arid. Data as mean ± se values of 73 oak species obtained from Gil-Pelegrín et al. [16••]. Letters indicate significant differences (Tukey test, $p < 0.05$) in AILang between groups of species

confirmed by herbarium data (see [16••]). On the one hand, evergreen oak species from Arid and Mediterranean phytoclimates showed the lowest values for AI_{Lang}, confirming that both inhabit dry to very dry areas. Conversely, the AI_{Lang} for temperate and tropical evergreens ranged from humid to perhumid values, in spite of the similarity in LMA (see above). Otherwise, deciduous species, which do not differ statistically in LMA, do differ in terms of AI_{Lang}. This is because LMA values of Mediterranean deciduous species (MED-DEC) overlap to some extent with the values of MED-EVE species in xeric locations and with those of deciduous temperate (TEM-DEC) species found in wetter locations. Obviously, a simple phytogeographical association between the overall climatic aridity and sclerophylly cannot be immediately derived.

Concerning sclerophyllous oaks in tropical environments, several different studies have ruled out the influence of water deficit as the main factor explaining their abundance in these humid habitats [62–64]. Grubb [14], in fact, considered the term sclerophyllous to be quite ambiguous, in contrast to leaf anatomical characters that can be described for the so-called hard leaves. He proposed the distinction between "pycnophylls" (from the ancient Greek *puknós*, dense, compact) for the Mediterranean plants with dense mesophylls, and "pachyphylls" (from ancient greek *pakhús*, thick) for the tropical sclerophyllous plants, with thick but lacunous mesophylls. Grubb [14] attributed to the Mediterranean pycnophylls a higher ability to withstand water deficit than tropical pachyphylls, breaking a uniequivocal link between developing hard leaves and coping with drought. However, the suspicion of a possible influence of water shortage in the development of these tropical sclerophyllous leaves has also been considered [65]. The possibility of leaf water shortage in tropical climates could arise from low soil water retention and strong radiation in some of these habitats [14, 65, 66], or from severe physiological stress imposed on the canopy by root anoxia during long-lasting inundation periods [67]. Unfortunately, the lack of sufficient functional studies related to this topic, and especially concerning oaks under tropical climates, precludes our ability to reach unambiguous conclusions.

However, several studies about the ecology and ecophysiology of a group of evergreen and sclerophyllous oak species of the *Ilex* group, also known as section *Heterobalanus* (Oerst.) Menitsky [59], provide an insight into the development of hard leaves with high LMA under climates that differ from those in arid or Mediterranean zonobiomes. These oaks can be dominant species in many forests of the Himalaya-Hengduan Mountains [68, 69], at an altitudinal range roughly from 1000 to 3500 m a.s.l. [70]. In spite of the genetic proximity with their circum-Mediterranean relatives (e.g., *Q. ilex*), with evident leaf morphological similarities, the species of Himalaya-Hengduan inhabit areas within climates that differ substantially from the summer drought period of the Mediterranean-type climates [71]. In fact, their wide altitudinal range results in a high-temperature gradient, such that climates range from subtropical at the base to warm or cool temperate at mid-altitudes and to cold subalpine climates at the upper distribution limit of these species [68, 72, 73].

The whole area is influenced by the monsoon rhythm, which implies the existence of a clear seasonality and a warm rainy season from mid-June to September, preceded by a cold and dry season (from October to February) and a pre-monsoon dry summer (March to May). More than 80% of the annual rainfall—between 1000 and 3000 mm [74, 75]—can be accounted during the warm rainy season [72, 76]. Compared with this situation, the precipitation in most areas under Mediterranean-type climates registers annual values around less than half in average, with a minimum account during the summer [16••]. Therefore, these two contrasting phytoclimatic conditions for the circum-Mediterranean and the Himalayan-Hengduan oak species of the *Ilex* group can apparently question the seminal idea of a link between sclerophylly and xeromorphy in these species. In other words, are these sclerophyllous oaks of the Himalaya-Hengduan affected by drought to a similar extent as their circum-Mediterranean relatives? In fact, the extreme seasonality mentioned above induces a long dry period in the monsoonal climates where these sclerophyllous oaks predominate [73, 76]. The combination of a temperature rising with scarce precipitations during the pre-monsoon months induces a specially critical period for the vegetation in terms of water deficit [77, 78], when the minimal values of water potential reported in these sclerophyllous species have been registered [69, 72, 76]. Moreover, the intra-annual variation in water deficit due to the inherent seasonality of the climates affected by the monsoon runs parallel to an inter-annual incidence of severe drought that induces periodic extreme water deficit in these oak species.

In fact, Singh et al. [69] reported that such intense droughts affect the Central Himalaya in 5–6-year cycles. Such periodicity in the incidence of severe droughts has been associated with the influence of global phenomena, such as El Niño [79], justifying the periodicity of the inter-annual variability. The incidence of such variability in the ecophysiology of different sclerophyllous oaks of the *Ilex* group is confirmed in different studies. However, during 1999, Singh et al. [69] studied the effect of a severe drought in other two oak species of the *Ilex* group, *Q. floribunda* and *Q. leucotrichophora* in Kumaun, the Central Indian Himalaya. During the peak of drought, they registered mean values of predawn water potential of − 4.4 MPa, but the value fell to − 5.5 MPa in the specimens more severely affected (showing dead leaves). These extreme values are close to the lowest values reported for *Q. ilex* (around − 5 or even − 6 MPa; [80]. The ability to survive after reaching these low water potential values, preserving their buds to produce leaves again, would imply that these species show a high resistance to drought, establishing a direct correspondence with other circum-Mediterranean oaks of the *Ilex* group [81, 82].

The Co-occurrence of Sclerophyllous and Malacophyllous Oaks in Mediterranean-Type Climates: Any Difference to Cope With Drought?

As above suggested, another phytogeographical fact that can question the role of a high LMA as a direct functional mechanism to cope with drought is the presence of winter-deciduous oak with relatively low LMA values in many areas under Mediterranean-type climates of the Northern Hemisphere [83]. Some of these winter-deciduous species occupy dry habitats of the Eastern Mediterranean Basin [84] or coexist with evergreen congeneric oaks in the western Mediterranean Basin [52, 85].

This coexistence has been a matter of discussion. Coexistence of these winter-deciduous oaks (MED-DEC) with evergreen oaks, although being the aim of different ecophysiological studies [86, 87] is not easily explained from an ecophysiological perspective [52] and further research is needed to fully understand this fact.

On the one hand, winter-deciduous Mediterranean oaks (MED-DEC) are considered elements of the so-called “sub-Mediterranean” vegetation [88], where the influence of the Mediterranean summer aridity is mitigated by a higher annual precipitation and a lower mean temperature. This fact is supported by the intermediate values of $\mathrm{AI}_{\mathrm{Lang}}$, which does not show significant differences both with those for MED-EVE and TEM-DEC. Comparative ecophysiological studies concerning both types of Mediterranean oaks have reflected different strategies to cope with water limitations [83, 86], and some of them concluded that evergreen species are able to resist a higher level of extreme water stress [52, 87, 89, 90]. The co-occurrence of these sub-Mediterranean oaks with MED-EVE in many areas of the western Mediterranean Basin should be the consequence of the alteration of the habitat affecting the water storage capability of the degraded soils, where evergreens tend to be more competitive [91].

Corcuera et al. [83] compared different species of oaks growing in a common garden taking into account the leaf area, LMA, and different characteristics derived from the analysis of PV curves. In this study, a clear coincidence in the value of osmotic potential at full turgor (π0) between Mediterranean species, independently of their leaf habit, was reported, against the lower value found for with TEM-DEC. Concerning oaks, the finding of higher π0 in oaks living in dry habitats have been previously reported (see [83] and references therein). MED-DEC showed the higher values for leaf water potential at turgor loss (πtlp), slightly but significantly higher than the mean value for MED-EVE and much higher than the one for TEM-MED. Bartlett et al. [92] concluded that πtlp was the trait more closely related to drought tolerance, with π0 as the main characteristic influencing πtlp, which is related with (i) the minimum soil water potential for water uptake and (ii) with the ability for maintaining gas exchange at lower soil water potential. Both facts can be crucial for surviving and growing under Mediterranean climates, especially when leaf life span is limited to the dry summer in MED-DEC. So, MED-DEC could be interpreted as clearly able of coping with the summer drought as well as the genuine Mediterranean MED-EVE, which should be in accordance with the results of Damesin et al. [52] when compared with the responses of co-occurring *Q. ilex* and *Q. pubescens* under Mediterranean conditions in southern France.

Corcuera et al. [83] also found a clear statistical difference concerning the maximum bulk modulus of elasticity ($\varepsilon_{\max}$), whith MED-EVE showing higher values of $\varepsilon_{\max}$ than MED-DEC and, specially, for TEM-DEC. The rigidity of the cell walls has been studied by several authors in terms of coping with drought (see [16••] and references therein). Bartlett et al. (2012) did not attribute any relevance in terms of coping with drought to $\varepsilon_{\max}$, but a low cell wall elasticity plays a role in water conservation, as a large change in turgor can be achieved with a minor change in symplasmic water [93]. This may constitute a mechanism to reduce water loss, through the effect of turgor loss in stomatal closure [94], reducing the diurnal and seasonal water loss [93]. A high $\varepsilon_{\max}$ in MED-EVE would be interpreted as a conservative mechanism, allowing them to avoid rapid soil water consumption in extremely dry areas or in locations with poorly developed soils or degraded soils and to develop a higher water potential gradient that would enable these species to obtain water from drier soils. On the contrary, the lower $\varepsilon_{\max}$ found in MED-DEC would allow the maintenance of positive turgor and, consequently, plant gas exchange and growth but at the expense of higher water consumption and the risk of xylem cavitation [87].

Woody plants from dry areas seem to develop a higher resistance to water stress-induced cavitation than those native from mesic habitats [95]. So, the comparison of the specific vulnerability to drought-induced xylem cavitation may serve as a way to compare the relative tolerance to climatic drought between MED-EVE and MED-DEC.

The quantification of this resistance can be done using different traits derived from the so-called cavitation curve, with P_{50} and P_{88} [96, 97]. Figure 3 evidences a tendency of P50 and P88 to increase as the aridity increases (lower AILang values), with an exponential decay for higher AILang where TEM-DEC predominate. The higher values for aridity and cavitation thresholds have been reported for some species of the MED-EVE (e.g., [81]) but not for all (see high dispersion in *y* axis). Moreover, very negative values for P50 and, specially, for P88 are reported for species of the MED-DEC

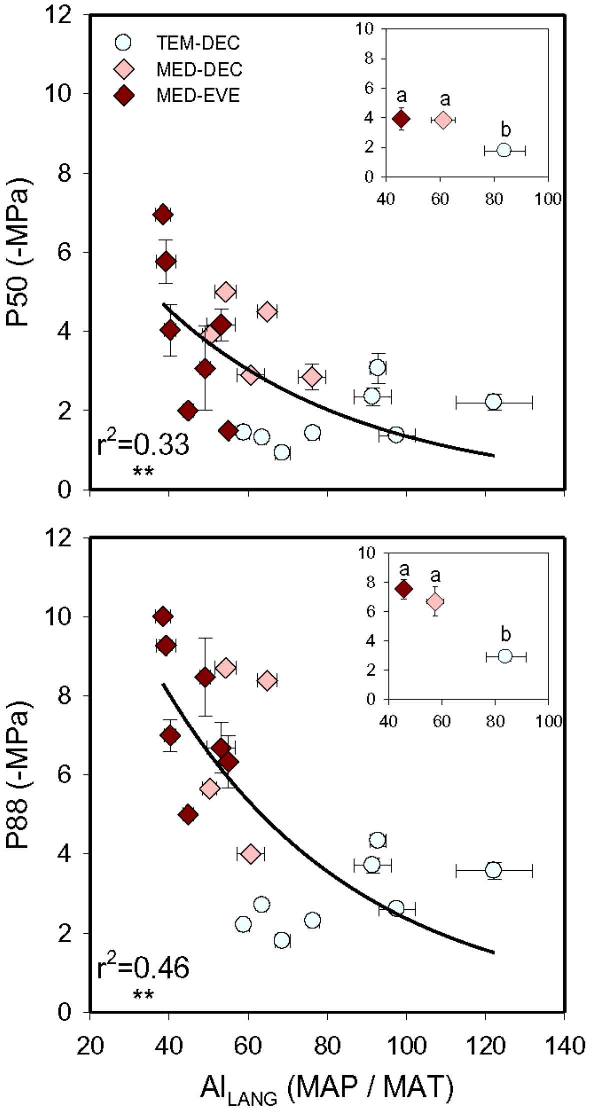

Fig. 3 Relationship between Lang aridity index (AILang) and the stem water potential inducing a loss of 50% (P50) and 88% (P88) of xylem hydraulic conductivity for oak species belonged to different groups. AILang calculated as the ratio between mean annual precipitation (MAP) and mean annual temperature (MAT). Group abbreviations as in Fig. 2. Data as mean ± se. ** means a p value < 0.01. P50 and P88 values obtained from literature (see Supplementary. MAP and MAT obtained from WorldCLim database according to Gil-Pelegrín et al. [16••]. Mean group values ± se of these parameters are plotted in the upper right side of each figure. Letters indicate statistically significant differences (Tukey test, $p < 0.05$) in the mean values of P50 and P88

group. Therefore, P50 and P88 values do not seem to differ among Mediterranean oaks with contrasting leaf habits. Therefore, assuming P88 is a reasonable proxy for an overall ability of coping with aridity in *Quercus* species, sclerophyllous oaks do not have more drought-resistant stem xylem than other oaks. It should be noted, however, that oaks are long-vesseled species and thus subject to artifacts in the production of vulnerability curves. The relative P50 and P88 values are still likely meaningful, but this is an area of continued debate within the literature.

Sclerophylly in Response to Limited Nutrient Resources

Since the first studies of Schimper in 1903 [1] in which a relationship between sclerophylly and xeromorphism was proposed, numerous studies have arisen questioning this relationship, such as those carried out by Beadle [98, 99]. This author, observing the Australian flora, showed that sclerophylly was not necessarily a xerophytic adaptation, since he perceived the existence of the humid sclerophyllous forest. Moreover, he concluded that in the Sydney district, the community structure was explained directly or indirectly by the phosphate content of the soil. In this area, rainforest composed of plants with laureate leaves occurs in the most fertile soils, whereas dry-looking sclerophyllous forests, composed of highly xeromorphic plants, occur in less fertile soil but, according to Beadle, this is not as a response to lack of water. Furthermore, Beadle [10] also studied the number of tropical and subtropical rainforest genera and observed that it correlates with the level of phosphate in the soil, independently of precipitation. He also found that the adaptation to low fertile soils accentuates xeromorphic characters through a reduction in leaf size, and his experiments indicated that the degree of xeromorphy could be reduced in many cases by the addition of phosphorus and nitrate.

Although these early studies were based in Australia, where the presence of soils with low nutrient content, mainly phosphorus, is common [100], other authors have also observed the existence of sclerophyllous species in nutrient-limited tropical rainforests [44, 62].

Loveless [2] also found that there was a high correlation between phosphorus content in leaves and the fiber/protein ratio. In addition, he showed that below a phosphorus content of 0.3%, the fiber/protein ratio increases with the decrease in phosphorus and suggested that "phosphate deficiency of the soil might be an important factor in sclerophylly." Moreover, in later studies, it has been observed that sclerophylly is a response to nutrient-poor soils [57, 101], and especially to phosphorus deficiency [102] or nitrogen stress [39, 103]. However, Sereneski-de Lima et al. [104], studying the highly sclerophyllous mangrove tree species from South Brazil, concluded that mangrove trees were not limited by soil nitrogen nor phosphorus; thus, the high LMA observed was not determined by soil oligotrophy.

Focusing on genus *Quercus*, there are studies that support Beadle's postulates, since they find sclerophyllous species inhabiting oligotrophic conditions. Cork-oak (*Q. suber*) forests that inhabit the Northeast Iberian Peninsula had a mineral content of the litter fall composition with low macronutrients such as nitrogen and phosphorus [105]. Furthermore, Castro & Fernandez-Nuñez [106] showed that in three monospecific *Quercus* forests of Northeastern Portugal there is a soil-fertility gradient among the three ecosystems, coinciding with the degree of sclerophylly. From the malacophyllous *Q. pyrenaica*, found in more favored areas, to the highly sclerophyllous *Q. ilex* subsp. *ballota*, developed in oligotrophic conditions, going through *Q. suber* in intermediate zones.

Conversely, other studies found no significant relationships between the degree of sclerophylly and low nutrient content. Cavender-Bares et al. [107] studied 17 *Quercus* species growing in Florida across different habitats, and found only weak associations between LMA and nutrient-related soil traits (inorganic nitrogen, soil pH, phosphorus and calcium). García et al. [108] found mixed forests of *Q. suber* (sclerophyllous) with *Q. faginea* or *Q. canariensis* (malacophyllous) in areas of southern Spain and Portugal, where phosphorus availability was very low. Similarly, Aranda & Comino [109] found poor-nutrient soils in the oak forests of Sierra Nevada National Park (southern Spain), where *Q. pyrenaica* (malacophyllous) and *Q. ilex* subsp. *ballota* (highly sclerophyllous) coexist. However, in Cazorla-Segura-Las Villas Natural Park, also dominated by mixed forests (*Q. faginea* and *Q. ilex* subsp. *ballota*), soils with high levels of nutrients were found.

Furthermore, sclerophyllous oaks are not the only group found in environments with Mediterranean climate and high nutrient content [109, 110]; malacophyllous oaks, such as *Q. robur*, *Q. infectoria*, *Q. brantii*, or *Q. saii*, also persist with low phosphorus availability [111]. Moreover, it has even been observed that this element can control the primary production of these forests [111, 112]. Zhang et al. [113] found a wide range of variation in LMA (ca. 40–80 g m^{-2}) across provenances of *Q. acutissima*, a malacophyllous species distributed along a wide range of soil conditions in China [114]. However, the LMA range was kept constant across the three provenance trials included in the study, despite contrasting soil conditions.

Overall, the potential link between sclerophylly and soil nutrient availability still remains controversial, due to the interrelation among soil traits (e.g., water availability and nitrogen content may be both affected by soil depth and texture) and with other environmental drivers (e.g., the concomitant increase in nutrient and water availability in deep valley soils).

As a proof of concept for the potential association between soil nutrients and the prevalence of sclerophylly, we took advantage of the information on species occurrence and soil traits, available from the US Forest Inventory and Analysis (FIA) database (http://www.fia.fs.fed.us/; accessed march 2019). Following a similar approach to that described by Rueda et al. [115], we first compiled information on soil conditions and species occurrence from all plots with presence of oaks (for further details on the methodology, see Supplementary Material). This resulted in 124,687 sites with soil data across the USA, with information on 36 oak species. With these data, we could determine average values per species for all soil and site variables across their distribution range in the USA (Fig. 4). We then compared these soil traits with the mean LMA for each species, based on literature data. Although this approach obviates plastic and genotypic intraspecific variability along the distribution range, it provides a first insight into the soil conditions most commonly associated with sclerophyllous species.

Across the USA, soil quality showed an effect on LMA, although it came associated with other geographic gradients. We found higher LMA values in shallower and more mineral, dense soils (Fig. 4a), with lower soil water content (Fig. 4b) and lower total nitrogen content (Fig. 4c). However, this could be linked to the high LMA prevailing in the dry mountain regions (ARID-EVE, MED-EVE in Fig. 4), as opposed to the low LMA prevailing in the cold-temperate planes (TEM-EVE), leading to a positive association with elevation (Fig. 4d). The altitudinal trend was partly disrupted by the low LMA of the winter-deciduous (TEM-DEC) *Q. gambelii*, which shows the highest mean elevation, and the moderately high LMA of the southern-temperate evergreens (TEM-EVE; e.g., *Q. virginiana*, *Q. minima*. Overall, we found the largest LMA values in the arid and Mediterranean mountain ranges in the Southwest and the Pacific coast, largely dominated by evergreen and sub-evergreen sclerophyllous oaks (e.g., *Q. agrifolia*, *Q. chrysolepis*, *Q. rugosa*; ARID-EVE and MED-EVE). Conversely, the lowest values were found in the North Central and Northeastern cold-temperate regions, dominated by temperate deciduous oaks (TEM-DEC), such as *Q. muehlenbergii*, *Q. macrocarpa*, *Q. rubra*, or *Q. alba*.

Notably, phosphorous availability did not show any association with LMA (Fig. 4e), opposing the postulates by Beadle [10, 98, 99] Furthermore, although a weak association with pH (Fig. 4f) was found, the highest LMA were found within the less nutrient-limiting range of pH (6–7). LMA also showed a negative association with some exchangeable cations ($r^2 = 0.32$, $p < 0.001$ for magnesium, $r^2 = 0.23$, $p < 0.01$ for aluminum), but was not significantly correlated with neither calcium nor potassium. A similarly weak, negative trend was found for other metals, such as iron ($r^2 = 0.22$, $p < 0.01$) or zinc ($r^2 = 0.22$, $p < 0.01$), but none of them appear to be particularly limiting. Conversely, nitrogen content emerges as the soil trait most strongly associated with LMA in our survey (Fig. 4e), confirming the negative association between soil nitrogen content and LMA reported in previous studies [39, 103]. In this regard, despite the emphasis put on phosphorus since the early studies of Beadle (1953, 1954), nitrogen may have a more critical role than phosphorus in shaping plant adaptive responses [110]. A recent study on model plants highlighted that phosphate starvation genes are largely controlled by nitrogen availability, but not vice versa [116].

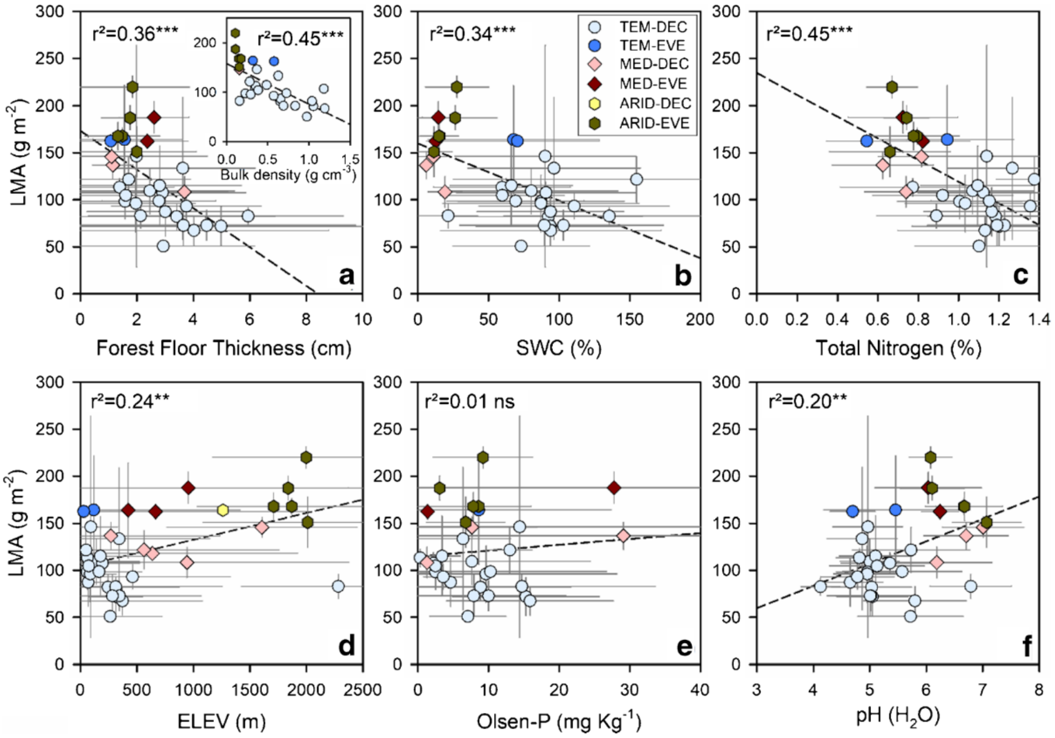

Fig. 4 Association between typical leaf mass per area (LMA) and average site conditions across 36 oak species found in the US Forest Inventory (see Supplementary Material). Species were classified according to their most typical biome and leaf habit, following [16••]. Circles, diamonds, and hexagons stand for temperate, Mediterranean, and arid biomes. Light and dark colors denote deciduous and evergreen species, respectively. ELEV: elevation, SWC: soil water content. Soil characteristics in this figure refer to the forest floor, except Olsen-P and pH, which correspond to soil horizons

Despite the apparent response of LMA to nitrogen availability, at least in our case, this trend cannot be isolated from the large-scale geographic gradients in environmental and soil physical traits. Eroded, shallow soils prevail in the dry mountain areas in the West (MED-EVE, ARID-EVE), contrasting with the deep, fertile, and wet soils in Central and Northeastern plains (TEM-DEC). Besides, the association between LMA and soil nitrogen could be further enhanced by the higher decomposability (and eventually faster turnover) in deciduous trees, as compared with the evergreens [117, 118]. Furthermore, LMA tends to be negatively correlated with both leaf nitrogen content and leaf digestibility, and positively correlated with fiber content ([2, 6, 117]; see also section 4.1). Hence, low nitrogen content in the *soils* might be partly an indirect consequence of sclerophylly, rather than a driving force for the selection for this trait. In support of the influence of tree litter on soil nitrogen, the negative association between LMA and nitrogen was less consistent in the deeper soil horizons ($r^2 = 0.35$, $p < 0.001$) than in the forest floor ($r^2 = 0.45$, $p < 0.001$; Fig. 4). Although this point is hard to disentangle based on standard field surveys, it may deserve further assessment through experimental, manipulative approaches.

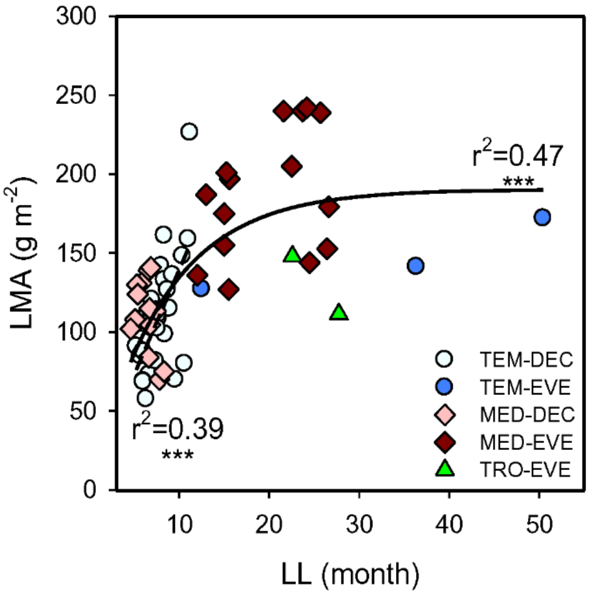

Fig. 5 Relationship between leaf lifespan (LL) and leaf mass per area (LMA) for species of Genus *Quercus*. Black continuous line is the correlation ($r^2 = 0.47$, $p < 0.001$) with all species. Dash line represents the correlation ($r^2 = 0.39$, $p < 0.001$) for temperate deciduous species. Data from literature [6, 120–124] and personal measurements

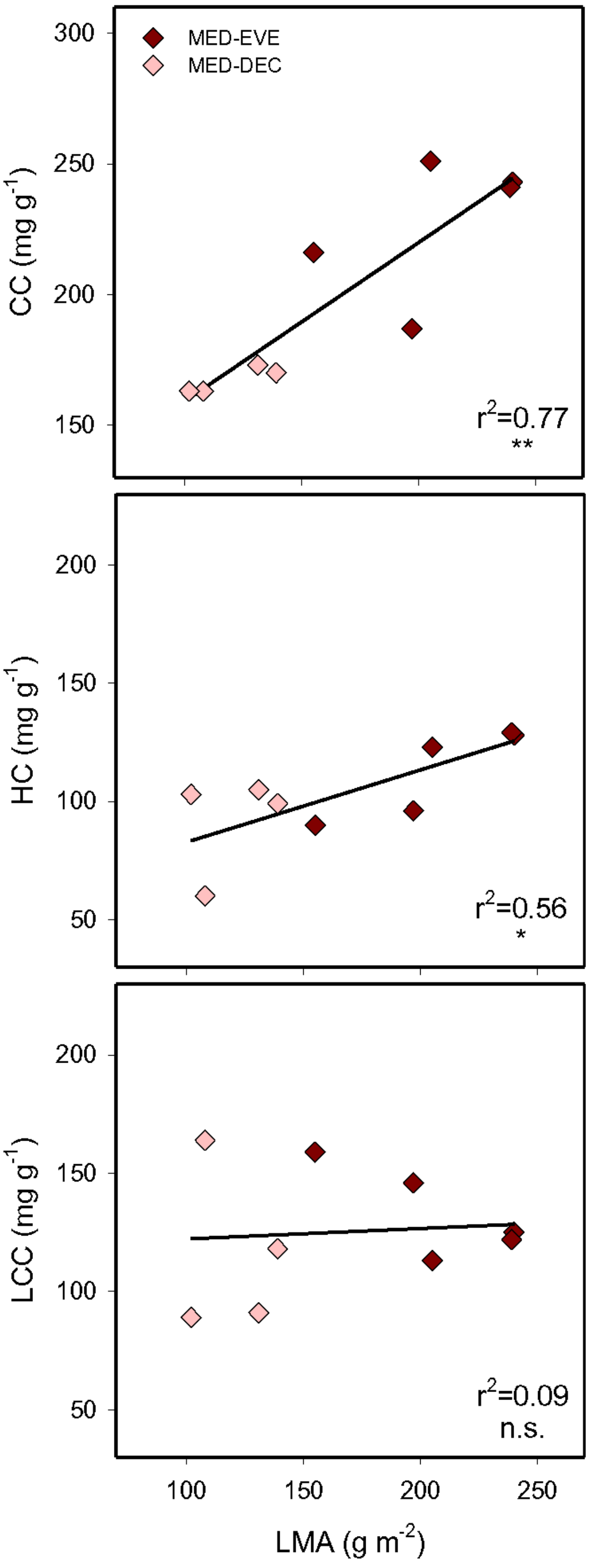

Fig. 6 Relationship between leaf mass per area (LMA) and cellulose content (CC), hemicellulose content (HC), and cutin and lignin content (LCC) for Mediterranean evergreen and deciduous *Quercus* species. Data obtained from [120]

Sclerophylly to Increase Leaf Longevity by Reducing Mechanical Damage

From a global plant perspective, increases in leaf sclerophylly can be associated with increases in leaf longevity, as several authors have reported positive relationships between LMA or physical properties with leaf life span (LL) (e.g., [4, 6, 18, 21, 119, 120]. Focusing on the genus *Quercus*, a relationship between LL and LMA obtained from literature and personal measurements follows the same positive trend (Fig. 5). In this genus and independently of the species leaf habit and climate, an increase in LL from 5 to 15 months is associated with a strong increase in LMA from 85 to 170 g m^{-2}. However, a further increase in oak LL to 50 months is not related to a significant increase in LMA, indicating the existence of an oak LMA threshold (c.a. 185 g m^{-2}) towards higher values of LL. Additionally, within leaf habit and climate, we only found a significant correlation ($r^2 = 0.39$ $p < 0.001$) for the temperate deciduous (TEM-DEC) group (Fig. 5).

The higher LMA found in longer lived leaves has been explained through a greater leaf reinforcement by the accumulation of structural carbohydrates, mainly due to the increase in cellulose (CC) and hemicellulose (HC) content [32, 120] (Fig. 6). By contrast, lignin and cutin content (LCC) does not seem to have a direct correlation with LMA and leaf duration, suggesting other explanations for the LCC interspecific differences such as water transport functions associated with the venation system of the leaf [120].

The accumulation of structural carbohydrates might enhance leaf persistence through the enhanced leaf protection [15, 37, 125]. This would help to resist the tear and wear due to physical [76, 126] and biotical [127, 128] interactions with the environment [15]. In this sense, leaf herbivory by insects has been the most common mechanical stress factor studied in the literature [127, 129, 130]. Generally, insects avoid eating sclerophyllous (high-LMA) leaves, both under laboratory [41] and field experiments [121, 131]. Accordingly, *Quercus* species with lower LMA suffer a higher percentage of leaf area lost per branch due to insect herbivory (see supplementary material Figure 1). The influence of mechanical leaf properties on insect herbivory can also determine the organization of the community composition of herbivores, including their diversity and density [128, 132]. However, in other studies the relationship between the level of sclerophylly and herbivory is not so evident [101]. Some species with low LMA values can be eaten as little as species with high values of LMA [41]. In those cases, small concentrations of secondary defense compounds, such as tannins [133], may significantly curb the set of herbivores able to feed on given leaves without hardly depending on the level of sclerophylly [125], although the degree of herbivory in relation to LMA and secondary chemical content has not been studied across oak species.

Sclerophylly as a Cold Protection Mechanism in Evergreen Oaks

Winter is a season that can constitute stressful conditions due to low temperatures and frost in evergreen species [11]. Among other effects, plants can suffer photoinhibition, xylem embolism, or cuticular abrasion, and consequently, plants may reduce their physiological activity [11, 12, 91, 134]. To mitigate cold stress effects, plants may adjust several structural and physiological traits related to LMA. According to González-Zurdo et al. (2016), *Q. ilex* and *Q. suber*, two evergreen Mediterranean oaks, presented higher leaf thickness in those sites with lower values of mean annual temperature. Increasing leaf thickness both by increasing the number of cell layers or by increasing individual cell layer, reduces the average intensity of light reaching the interior of the leaf, thereby reducing the probability for photodamage [135, 136•]. Being thicker also results in a greater water content per unit leaf area [137, 138]. As water has a higher heat capacitance, the increase in water content per area may increase the buffering of temperature changes and so reduces the rates of leaf freezing [139] and leaf thawing [140], reducing the physiological damage by frost, especially in leaves exposed to multiple freeze-thaw cycles [136•]. Frost acclimation may also lead to increases in cell wall thickness and cell wall rigidity [141]. This acclimation may help to avoid the collapse of cells under low water potentials that may occur when frozen soils prevent water delivery to transpiring leaves. A higher cell wall rigidity also implies that for a given change in water potential, there is a lower degree of water migration from cells to extracellular spaces in comparison with more elastic cell walls [136•]. Intracellular water can remain supercooled during low temperatures, whereas extracellular water freezes faster; therefore, a higher rigidity would delay water freezing [142]. The higher cell wall rigidity may be due to an increase in fiber contents [143, 144]. In fact, González-Zurdo et al. [145] observed higher contents of cellulose + hemicellulose in individuals of *Q. ilex* and *Q. suber* inhabiting sites with higher number of days with frost per year.

These adjustments in leaf traits in response to low temperatures might promote an increase in the LMA of evergreen oaks, at least within species [145, 146]. However, among species, it is still unclear whether the main driver of changes in LMA is severity of cold itself (e.g., characterized by mean annual temperature, MAT) or it is related to the length of the cold season. Most of the studies only focus on MAT and other temperature indicators, not analyzing the effect of the length of the unfavorable cold season. In this sense, Kikuzawa et al. [7] found a strong positive relationship between MAT and the portion of the year for favorable growth. Furthermore, taking into account the temperature in natural conditions of 30 oak species [16••], we have found a strong correlation ($r^2 = 0.84$, $p < 0.0001$) between MAT and the number of months with mean temperature below 5 °C (Fig. 7), i.e., the unfavorable season for growth, according to Wypych et al. [147]. These correlations suggest that LMA variations among species attributed to MAT could be also attributed and explained by the length of the unfavorable season. Therefore, further research is needed in order to clarify whether variations in LMA among oak species actually respond to the intensity of low temperatures or, by contrast, to the length of the cold season.

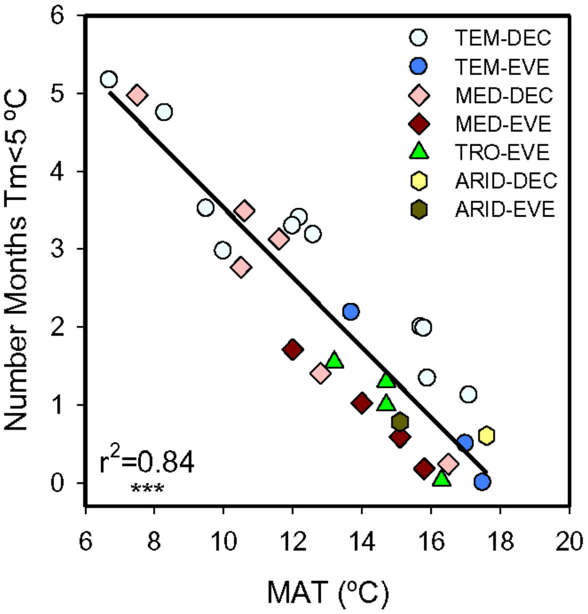

Fig. 7 Relationship between mean annual temperature (MAT) and length of the cold season, measured as the number of months with mean monthly temperature (Tm) below 5 °C. Each circle represents a different oak species. Data are mean values of at least 40 locations within the distribution range of each species. Geographical distribution coordinates and climatic characteristics for each oak species were obtained from herbarium data and WorldClimV2.0 database, respectively, following the methodology described in [16••]. *** means a p value < 0.001

Functional Implications of Sclerophylly

Sclerophylly as a Key Factor Modulating Oak Photosynthesis in the Context of the Leaf Economics Spectrum

LMA—the most widespread quantitative proxy for sclerophylly—is one of the major traits in the leaf economics spectrum (LES), which globally describes the coordinated variations in leaf structural, chemical, and photosynthetic characteristics [6]. The negative association between LMA and A_{mass} at a global scale has been linked to a higher investment in non-photosynthetic structural tissues and/or a lower efficiency of the photosynthetically active mesophyll [148].

However, the relationships among area-based net CO_2 assimilation (A_{area}), area-based nitrogen (N_{area}), and LMA are less clear and could be modulated by the influence of different physiological and anatomical traits [38•]. Bearing in mind this general framework, these authors put in evidence the role of different mechanistic traits that could exert opposite effects on A_{area} associated with changes in LMA [38•]. Thus, Onoda et al. [38•] concluded that, globally, diffusive and biochemical limitations to photosynthesis associated with thicker cell walls (i.e., lower mesophyll conductance to CO_2 and lower N allocation to Rubisco, respectively) can be complemented by greater N_{area} in species with large LMA, thereby largely equalizing A_{area} across species with contrasting LMA.

Besides global variation across all species, meaningful variations among species in foliage traits have also been reported within a single genus. This is the case of *Quercus*, which exhibits a strong intra- [149] and interspecific [16••] variability in LMA. In this regard, the meta-analysis performed by Peguero-Pina et al. [150] demonstrated that the genus *Quercus* shows a broad range of variation in several major traits in LES (i.e., LMA, N_{mass} and A_{mass}), covering a high span within the full LES. In this section, we have extended this meta-analysis up to 71 oak species with the dataset available from the TRY Plant Trait Database ([40]; see Supplementary Material for details), which also included additional traits such as N_{area}, A_{area}, and stomatal conductance for H_2O (g_s).

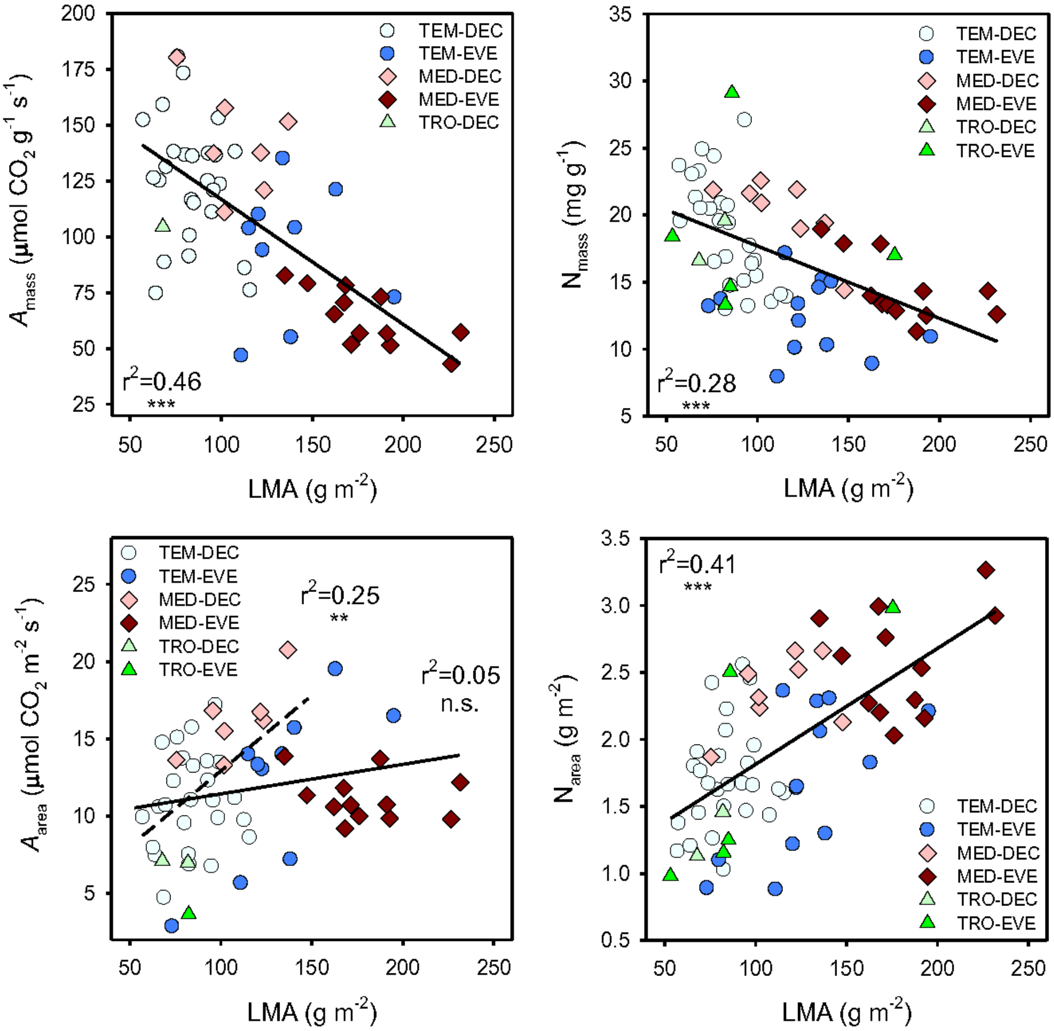

Fig. 8 Relationships between leaf dry mass per unit area (LMA) and (i) photosynthetic capacity per leaf dry mass (A_{mass}) (upper left panel), (ii) photosynthetic capacity per leaf area (A_{area}) (down left panel), (iii) nitrogen content per dry mass (N_{mass}) (upper right panel), and (iv) nitrogen content per leaf area (N_{area}) (lower right panel) for different *Quercus* species. Data obtained from literature (see Supplementary Material). Symbols as in Fig. 2

Overall, the results obtained confirm that the genus *Quercus* follows the general trends described in the LES, as LMA was negatively related with N_{mass} and A_{mass} ($p < 0.0001$, Fig. 8). However, this pattern was not observed when considering area-based relationships. Thus, the relationship between LMA and A_{area} was not statistically significant ($p = 0.095$, Fig. 8), although it was positive when considering only DEC species ($p = 0.002$, Fig. 8). Moreover, LMA and N_{area} showed a strong positive relationship ($p < 0.0001$, Fig. 8), which is in line with the results found by Yang et al. [151], for 483 species at 48 sites across China. Taken together, our findings could indicate the existence of additional factors modulating these relationships in oak species. Thus, an increase in mesophyll thickness concomitant to higher foliage robustness [41, 42] would explain the increase in N_{area}, probably because of overall greater number of cell layers and greater leaf volume [149, 150, 152]. In consequence, this phenomenon counteracts the negative influence of higher LMA values on photosynthetic capacity of oaks. By contrast, we have not found any significant relationship between LMA and g_s, mostly due to the occurrence of species with the lower g_s values in the groups with lower and higher LMA values (TEM-DEC and MED-EVE, respectively) (see in supplementary material figure 2). Otherwise, MED-DEC species with relatively low LMA values had the higher g_s values among all the oak species analyzed (see in supplementary material figure 2). This reflects the fact that transpiration rate should be independent of the amount of photosynthetic machinery per unit area [16••].

It is interesting that DEC and EVE oak species were grouped, respectively, towards the high and low return end, irrespective of their climatic origin. In fact, LMA is one of the most important functional traits that clearly separates EVE and DEC [16••, 83]. That is, within *Quercus*, these relationships seem to be more influenced by the leaf habit than by the climatic/ecological conditions of each species, indicating the coexistence of multiple trait values in the same climate [153]. Otherwise, Yang et al. [151] suggested that climate influences trait variation in part by selection for different life forms and families. In spite of this, these authors found a positive correlation between LMA and N_{area} and photosynthetic capacities after removal of climate effects, and concluded that it is still necessary to collect more systematic trait data across all climates and biomes. For instance, available data about tropical *Quercus* species are very limited, in spite of the great number of oak species occurring under these climatic conditions [58].

Both higher g_s and N_{area} yield higher A_{area} values in oaks, and the best fit for these correlations was a curve describing an exponential rise to a maximum assimilation rate of 17.5 and 19.1 µmol CO_2 m^{-2} s^{-1}, respectively (Fig. 9). In other words, there was a decreasing sensitivity of maximum CO_2 uptake per leaf area when g_s and N_{area} reached threshold values, which indicated that other factors could also constrain A_{area}, especially in species with higher g_s (i.e., MED-DEC) and N_{area} (i.e., MED-EVE and MED-DEC). In this regard, the diffusion of CO_2 from the sub-stomatal cavities to the sites of carboxylation (i.e., the mesophyll conductance, g_m) plays a key role in the photosynthetic process of *Quercus* species, [150]. This seems to be the case of MED-EVE from Europe and California [154•], but also for MED-DEC species such as *Q. faginea* [155]. It should be noted that several studies have revealed that g_m is negatively related to LMA [38•, 156, 157]. Specifically, the fact that g_m is limited by large LMA values has been mostly related to several leaf anatomical traits (i.e., mesophyll and cell wall thickness), and has been demonstrated for different species [148, 158–160] including MED oaks [154•]. Besides the increases in N_{area} associated to higher LMA values, Peguero-Pina et al. [161] demonstrated the existence of other anatomical adaptations at the cell level (increased mesophyll and chloroplast surface area exposed to intercellular air space) that allow MED-EVE to reach A_N

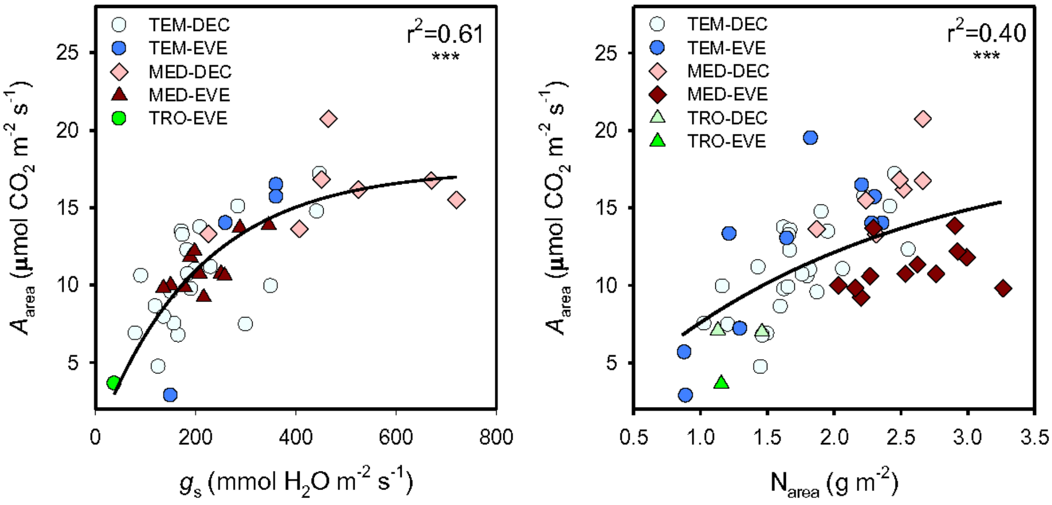

Fig. 9 Relationships between photosynthetic capacity per leaf area (A_{area}) and (i) stomatal conductance (g_s) (left panel) and (ii) nitrogen content per leaf area (N_{area}) (right panel) for different *Quercus* species. Data obtained from literature (see Supplementary Material). Symbols as in Fig. 2

values comparable to congeneric deciduous species despite their higher leaf thickness and LMA.

Functional Mechanisms Associating Sclerophylly and Drought Tolerance

The predominance of sclerophyllous oaks in habitats prone to induce water limitations, such as those under Mediterranean climate, has not been adequately explained in a functional way, so the role of a hard leaf for withstanding water shortage remains unclear. In this sense, different ecophysiological studies have revealed that Mediterranean sclerophyllous woody species show different, and even contrasted, strategies to cope with water stress [93, 162, 163] which question the existence of a single role of sclerophylly in terms of water relation [83].

A leaf can reach a high LMA through an increase in thickness (LT), a higher leaf density (LD) tissues, or the combination of both factors [5, 18, 41, 42], with very different functional consequences. Does an increase in LMA through a higher LD have a role in water relations? Oertli [164] proposed that leaves can withstand negative turgor pressure without being subjected to cell collapse, or cytorrhysis, if their tissue are constituted by small cells with thick walls. The extreme buckling of mesophyll cells at low water potentials (see [165]) will induce physical and irreversible damages in the plasmalemma. Oertli et al. [166] proposed that this mechanism of avoiding cytorrhysis by developing thick cell walls is especially important for plants living in dry habitats but with access to water along the year, as is the case of Mediterranean plants. However, this ability was not able to cope with extreme water deficit. The seminal idea of Oertli has been recently revisited [167], but further research is needed to accept this mechanism as an explanation for the abundance of sclerophyllous species in dry areas, such those under Mediterranean-type climates.

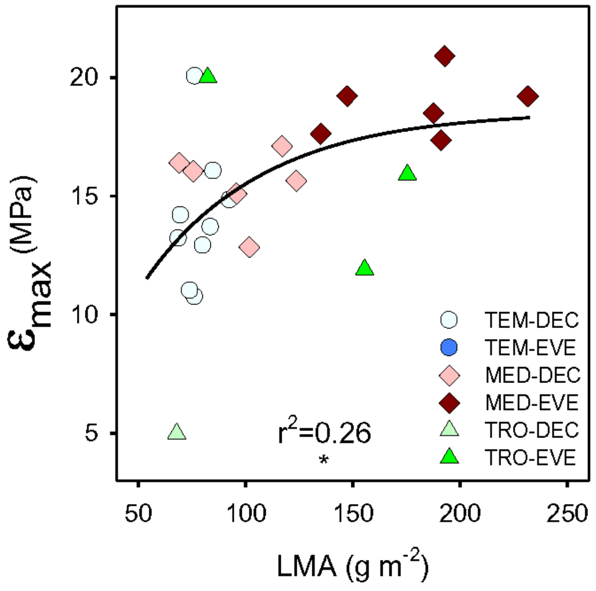

Fig. 10 Relationship between leaf dry mass per unit area (LMA) and the maximum bulk modulus of elasticity ($\varepsilon_{\max}$) for different *Quercus* species. Symbols as in Fig. 2

Closely related to this ability for avoiding cell collapse by thicker cell walls, the relation between LD and the cell rigidity ($\varepsilon_{\max}$) has been considered for explaining sclerophylly in water-limited habitats [5, 90], due to the role of a higher $\varepsilon_{\max}$ in plant water relations (see above). However, few authors have reported a positive correlation between LMA and $\varepsilon_{\max}$ [45, 168–170], whereas others suggested a lack of correlation between both traits [93, 162]. We have found a non-linear positive relationship between LMA and $\varepsilon_{\max}$ from the data provided by Corcuera et al. [83] for different *Quercus* species growing in a common garden (Fig. 10). We obtained a similar result when this meta-analysis was extended up to 25 oak species, including the dataset available from the TRY Plant Trait Database [40] (data not shown). A caution is needed when relating LMA and $\varepsilon_{\max}$, as the former is dependent on all the dry matter accumulated per area while the rigidity of the mesophyll cell walls should explain the latter [45, 93]. So, only the establishment of a closer relation between the morphological properties of the cell walls (thickness and rigidity) of these cells and their contribution to the overall LD could satisfy the idea that a higher LMA by denser leaf tissues could constitute an adaptive mechanism to cope with water deficit. Salleo et al. [168] proposed another adaptive advantage of sclerophyll in terms of water deficit, when he suggested that the water contained in the mechanical tissues of some sclerophyllous woody plant species would positively contributes to the recovery of the water deficit and its consequences [168]. Further studies should confirm this interesting idea.

Another way for increasing LD without a further increase in LT may be the increment in vein density, due to the anatomy of the vascular bundles and, eventually, of the bundle sheath extensions. A functional advantage, in terms of water limitations, of a higher vein density was proposed by Scoffoni et al. [171] and confirmed by Nardini et al. [172], when found that the higher the density of major vein—first, second, and third order—the higher the resistance to water stress-induced xylem cavitation. Further evidence of an increase of major vein density in oaks with increasing the aridity of their distribution range was found by Peguero-Pina et al. [82].

It is well known that drought-stressed plants close their stomata to minimize the water loss, and under such conditions, diffusion across the cuticle is the only route of water loss. So, the cuticular water permeability (*p*) has been used as an indicator of the efficiency of the cuticular transpiration barrier. The genus *Quercus*, presenting both deciduous and evergreen species, is ideal for testing whether sclerophylly can be considered as a functional adaptation to withstand drought at the leaf cuticular level. To address this question, we compiled cuticular water permeability and leaf mass per area (LMA) values in the literature and checked how these traits were related. The cuticular conductance ($g_{\min}$) represents the lowest conductance a leaf can reach when stomata are maximally closed as a consequence of desiccation stress [137]. Therefore, it is a proxy for cuticular permeability [169, 173, 174]. Both $g_{\min}$ and p were included in this analysis under the assumption that the influence of leaky stomata is very small or absent [169, 173, 174] and, therefore, they play the same ecological role. To the best of our knowledge, only seven publications reported concomitantly values of cuticular permeability and LMA of *Quercus* species (Supplementary Table S1). Cuticular permeability given in mmol m^{-2} s^{-1} were converted to m s^{-1} according to Kerstiens [175], where 0.41 mmol m^{-2} s^{-1} equals 1×10^{-5} m s^{-1}. In total, 21 individual data for permeability and 19 for LMA, including in some cases multiple data for a single plant species, were evaluated. LMA and permeability data ranged from 33.0 g m^{-2} (*Q. petraea*) to 245.7 g m^{-2} (*Q. coccifera*), and in the alternative dimension from 2.1×10^{-5} m s^{-1} (*Q. agrifolia*) to 17.9 × 10^{-5} m s^{-1} in (*Q. faginea*), respectively. Both plant traits had normal distributed data, and therefore, *t* tests could be used to investigate differences among group of species.

Pairs of LMA and cuticular water permeability were found for eight evergreen and five deciduous species of oaks. In contrast to the common notion, the barrier properties of the leaf cuticle are not correlated to LMA (see in supplementary material figure 3). This agrees with previous studies on xerophytic plant species [176, 177]. Therefore, LMA is not a predictor for cuticular transpiration.

Data for eight deciduous and six evergreen oak species were subjected to further analyses. LMA of deciduous oaks (77.4 ± 18.7 g m^{-2}) was over 2-fold lower than that found for evergreen oaks (179 ± 29.3 g m^{-2}; Fig. 11a). In contrast to

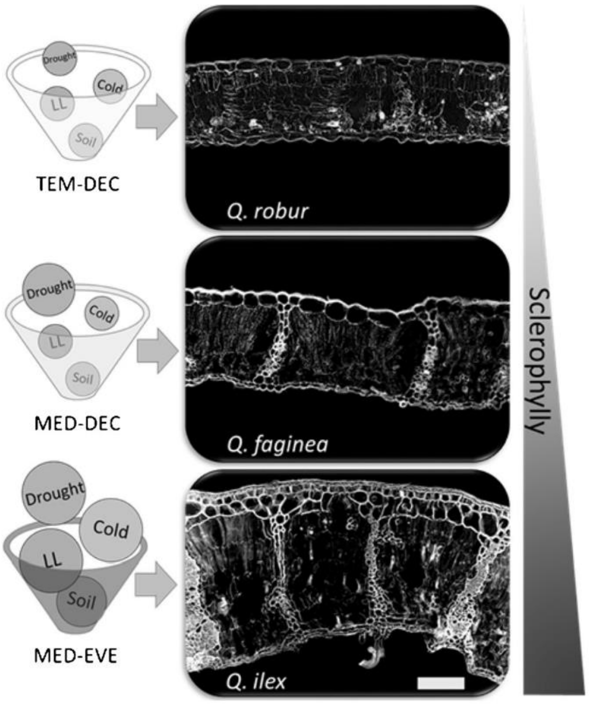

Fig. 12 A summary of the key factors promoting sclerophylly (circles) and the transverse section of the mesophyll leaf of *Quercus robur*, Q. faginea, and *Q. ilex* subsp. rotundifolia ranked from less to more sclerophyllous. Mesophyll bright areas mainly coincide with thick cell walls in epidermal structures, vascular bundles and bundle sheath extensions. Scale bar 100 µm. A high factor's circle size indicates a high influence of the factor on the specie

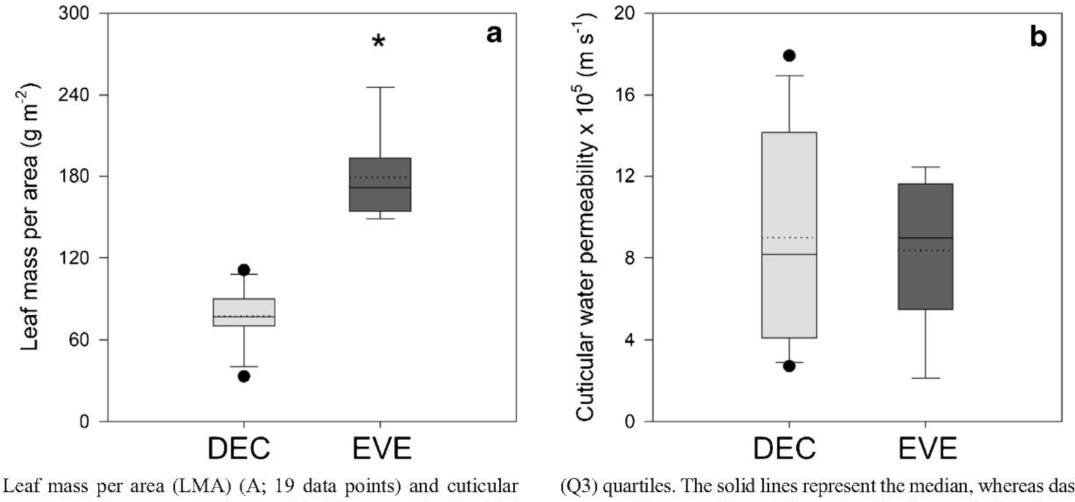

Fig. 11 Leaf mass per area (LMA) (A; 19 data points) and cuticular permeances or minimum conductances (A; 21 data points) of 14 *Quercus* species collected from the literature (Supplementary Table S1). Data represent eight deciduous oak species (DEC, 11 and 12 data points for LMA and permeability, respectively) and six evergreen oak species (EVE, 8 and 9 data points). The boxes stand for the lower (Q1) and upper (Q3) quartiles. The solid lines represent the median, whereas dashed lines represent the mean. The whiskers stand for the smallest and largest values that are not more than 1.5 interquartile ranges away from the box. The potential outliers falling outside the Q1–Q3 range are represented by dots. The asterisk indicates a significant difference of LMA between DEC and EVE oaks (*t* test, $p < 0.05$)

LMA, the cuticular permeability of deciduous oaks (9.0 ± 5.0×10^{-5} m s^{-1} to 21.93 ± 12.18 mmol m^{-2} s^{-1}; mean ± standard deviation) and evergreen oaks (8.4 ± 3.4 × 10^{-5} m s^{-1} or 20.40 ± 8.41 mmol m^{-2} s^{-1}) was very similar. Thus, no statistically significant difference was found between deciduous and evergreen oaks (Fig. 11b).

These findings demonstrate that high LMA does not affect the efficacy of the cuticular transpiration barrier and, therefore, does not support the hypothesis that the stiff leaves of *Quercus* would be a functional adaptation to cope with the summer drought through a decrease in cuticular transpiration. It has been widely accepted that waxes mainly make up the plant cuticular barrier against passive water loss [178]. However, this relation is not yet well understood. Cuticular waxes are complex mixtures of up to 150 components distributed into compound classes with different properties. Previous studies on several plant species have shown that cuticular permeability correlates neither with the cuticular wax loads nor with cuticle thickness [176, 177, 179, 180]. Recently, Bueno et al. [181] showed that *Q. coccifera* plants produce different leaf wax loads when grown either under arid or humid conditions, but that the cuticular transpiration barrier properties were not affected by the growing conditions. While the plants under arid conditions had a higher wax load, the relative compositions of the waxes were identical for both habitats.

Conclusion

Our critical review of the different proposed explanations for sclerophylly indicates that there is "no single way to Rome." The wide variability found for this trait within the genus *Quercus* cannot be explained alone by any of the ecological factors considered (water, nutrients, leaf longevity, herbivory, temperature), neither any of them could be fully discarded. Noteworthy, our study also suggests that these constraints may have a synergistic effect. Hence, habitats combining several ecological limitations are the ones where sclerophylly is more prevalent, as exemplified by the case of Mediterranean evergreens (see Fig. 12). Mediterranean habitats are unique in combining summer drought and a relatively cold winter, splitting the growing season in two isolated periods (spring and autumn). This has generally promoted leaf longevity, as a mechanism to take advantage of spring and autumn optimal conditions. As discussed above, these long-living leaves must be also hard enough to overcome accumulated (both abiotic and biotic) stressors. We could also identify a substantial knowledge gap regarding oak species in tropical biomes, despite being a major biodiversity hotspot for the genus. The few studies available so far suggest that evergreen oaks in tropical dry highlands may show converging physiological adaptations with Mediterranean oaks, a point that deserves further assessment. From a functional point of view, we can conclude that in oaks, leaf habit largely modulates the physiological implications of sclerophylly. We have highlighted that, among evergreens, sclerophylly has a cost not only in terms of carbon investment but also limits carbon uptake capacity, mainly through diffusional limitations. Conversely, the increase in LMA among deciduous oaks is associated with larger photosynthetic capacity, reflecting the strategy of Mediterranean deciduous oaks to maximize carbon gain during optimal periods, as an adaptation to the splitting of the growing season.

Acknowledgments The study has been supported by the TRY initiative on plant traits (http://www.try-db.org). The TRY initiative and database is hosted, developed, and maintained by J. Kattge and G. Boenisch (Max Planck Institute for Biogeochemistry, Jena, Germany). TRY is currently supported by Future Earth/bioDISCOVERY and the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig.

Funding Information This research was funded by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) grant number RTA2015—00054-C02-01 and by Gobierno de Aragón H09_20R research group. Work of D. A. F. is supported by a FPI-INIA contract BES-2017-081208. Research of D. S. K. is supported by a DOC INIA-CCAA contract co-funded by INIA and European Social Fund.

Compliance with Ethical Standards

Conflict of Interest David Alonso-Forn, Domingo Sancho-Knapik, Juan pedro Ferrio, José Javier Peguero-Pina, Amauri Bueno, Yusuke Onoda, Jeannine Cavender-Bares, Ülo Niinemets, Steven Jansen, Markus Riederer, Johannes H.C. Cornelissen, Yongfu Chai and Eustaqui Gil-Pelegrín declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- •• Of major importance

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Supplementary Material

Revisiting the functional basis of sclerophylly along the wide leaf economic spectrum of oaks: different roads to Rome

By

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Supplementary Figures

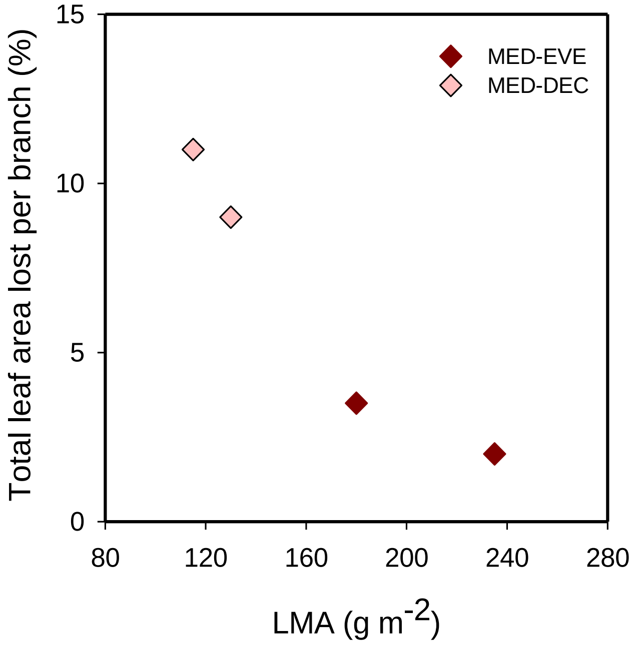

Fig. 1 Relation between leaf mass per area (LMA) and total leaf area lost per branch due to insect herbivory in four co-occurring Mediterranean *Quercus* species. Data redrawn from [133]

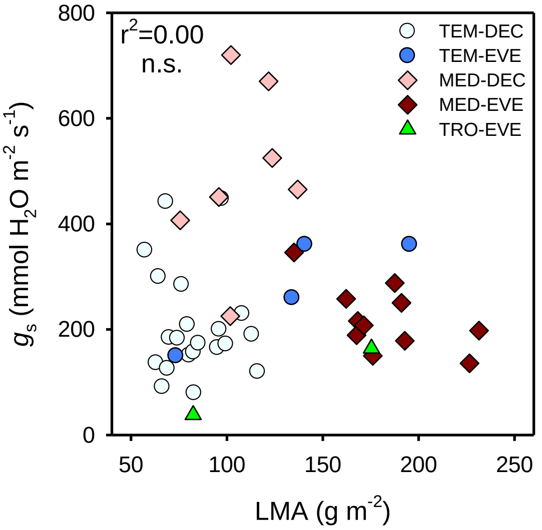

Fig. 2 Relationship between leaf dry mass per unit area (LMA) and stomatal conductance (g_s) for different *Quercus* species. Data obtained from literature (see Supplementary Material). Symbols as in Figure 2.

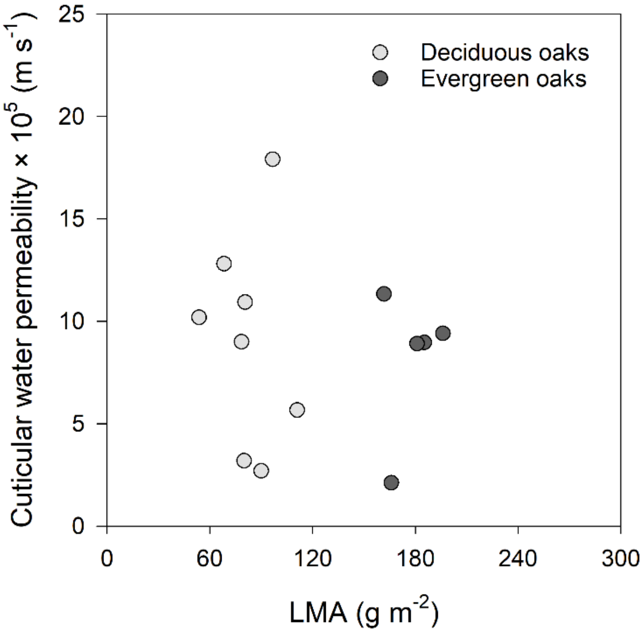

Fig. 3 Plot of leaf cuticular water permeability versus leaf mass per area (LMA) of 13 *Quercus* species (eight deciduous, five evergreen). Data were compiled from the literature (see Supplementary material)

Extended Methods

Compilation of plant functional traits

Data on leaf mass per area (LMA), drought-tolerance traits (PLC50, PLC88, bulk modulus of elasticity - ε_{max}, cuticular conductance - g_{min}), leaf life-span (LL), and photosynthetic traits (nitrogen content per dry mass - N_{mass} and per area - N_{area}, photosynthetic rate per mass - A_{mass} and per area - A_{area}, stomatal conductance - gs) were compiled from the literature, and eventually expanded with data retrieved from the TRY – global database on plant traits [1], retrieving relevant data for 98 oak species / subspecies worldwide (see Table S1).

Relationship between LMA and site conditions of oaks across the USA

Following Rueda et al. [2] we extracted information on oak distribution from FIA database (http://www.fia.fs.fed.us/; accessed march 2019); including all subplots and microplots with presence of oak species, but excluding observations indentified only to the genus level (*Quercus* sp.). Species codes and symbols were used to identify the species at the subplot and microplot level, respectively, according to the information provided in FIA reference tables (for further details about the organization of the FIA database see [3] and [4]). Soil information for plots with presence of oaks was also retrieved from FIA database [3]. Information for separate soil horizons was not available in all cases, therefore mean soil nutrient values were used, combining data from all soil layers. In the case of nitrogen, data was also available for the forest floor, and analysed separately. We finally combined vegetation and soil data at the plot level, identified as unique combinations of latitude and longitude coordinates. This resulted in 126296 sites, including soil information for 55 different oak species. Combining the two datasets, we ended up with 36 oak species with LMA data, among those included in the FIA dataset, covering 124687 sites across the USA (Table S2). Most sites (68%) had at least two different species (up to 12 species per site, with an average of 2.5 species per site), while the average number of sites per species was 8755, ranging from 191 in *Q. lobata* Née to 47320 in *Q. alba* L. Finally, we attributed to each species a typical value of LMA (based on the database and literature survey above) and then correlated this with the average site conditions across all the plots with presence of the species.

TABLE S1. Species included in the literature and dataset survey and references. The prevailing biome was determined according to the species distribution, following [5]. For simplicity, we considered as evergreen all the species having the potential to retain their leaves for at least eleven months, therefore including genuine evergreen, sub-evergreen, semi-evergreen and facultative drought-deciduous species.

Species	**Biome**	**Leaf habit**	**LMA**	**Drought Tolerance**	**Leaf Lifespan**	**Photosynthetic traits**
Q. acuta Thunb.	TEM	EVE	[6][7][8]		[6][9][7]	[10]
Q. acutissima Carruth.	TEM	DEC	[11][12][13][14][8]			[15][13][16][17][12] [18][19][20]
Q. afares Pomel	MED	DEC	[5]	[5]	[21]	
Q. agrifolia Née	MED	EVE	[22][23][24][25][26][6][27]	[28][29][30]		[27][25][17][6] [24][22][23]
Q. alba L.	TEM	DEC	[31][29][32][24][11][33][6]	[34][29][35]		[17][6][24][33][36][37] [31][29][38][39][32]
Q. aliena Blume	TEM	DEC	[11][12]	[29]		[40][12][15][41]
Q. argentata Korth.	TRO	EVE	[42]			[42]
Q. arizonica Sarg.	ARID	EVE	[43]			[11]
Q. austrina Small	TEM	DEC	[44][6][45]		[6]	[45][44]
Q. baronii Skan	TRO	EVE	[12]			[12]
Q. berberidifolia Liebm.	MED	EVE	[29][11]	[28][46][47][48][49][29]		[46][50]
Q. canariensis Willd.	MED	DEC	[11][51][52][26]		[53]	[51][54]
Q. castaneifolia C.A.Mey.	MED	DEC	[55]			
Q. cerrioides (x) Willk. & Costa	MED	DEC	[51]			
Q. cerris L.	MED	DEC	[56][55][57][58][59] [60][26][6]	[35][5]	[61]	[57][60][59][58][55] [56][26][17][62]
Q. chapmanii Sarg.	TEM	DEC	[44][6][45]		[45]	[45][44][63]
Q. chenii Nakai	TEM	EVE	[12]			[12]
Q. chrysolepis Liebm.	MED	EVE	[27]	[35][5]		[27][17]

Species	**Biome**	**Leaf habit**	**LMA**	**Drought Tolerance**	**Leaf Lifespan**	**Photosynthetic traits**
Q. coccifera L.	MED	EVE	[58][64][59][11][65][60][26][6][27]	[66][29][5][67][68]	[61][69]	[27][59][65][17][57][6][64][26][70][71][29][72]
Q. coccinea Münchh.	TEM	DEC	[31][6][14]		[6]	[17][6][73][37][74][75][65][19]
Q. copeyensis C.H.Mull.	TEM	EVE	[65]			[65][17]
Q. costaricensis Liebm.	TEM	EVE	[76][65]			[65][17][76]
Q. dentata Thunb.	TEM	DEC	[8]			
Q. douglasii Hook. & Arn.	MED	DEC	[23][25][26][6][77]			[25][17][77][6][26][23]
Q. dumosa Nutt.	MED	EVE	[23][25][6]			[25][6][23][78]
Q. ellipsoidalis E.J.Hill.	TEM	DEC	[31][24][65][60][79][6]		[6]	[60][65][79][24][26][31][74][75]
Q. emoryi Torr.	ARID	EVE	[43]			
Q. fabri Hance	TEM	DEC	[12][37]			[16][17][80][40][12][37]
Q. faginea Lam.	MED	DEC	[57][11][65][51][60][26][6]	[81][35][82][5]	[61][69][83][21][6]	[65][17][57][51][73][6][37][26][84][70][82][54]
Q. falcata Michx.	TEM	DEC	[29][44][13][6][14][45]	[34][29]	[45][6]	[44][13][17][29][45]
Q. floribunda Lindl.	TRO	EVE	[85]	[86]		
Q. frainetto Ten.	MED	DEC	[56]	[87][29][35]		
Q. fusiformis Small	TEM	EVE	[88]	[89][29][88]		
Q. gambelii Nutt.	TEM	DEC	[90][91][26][43]	[92][93][94][29]		[95][96][91][26]
Q. garryana Douglas ex Hook.	MED	DEC	[97]	[97]		
Q. gemelliflora Blume	TRO	EVE	[98]	[98]		
Q. geminata Small	TEM	EVE	[44][6][45]		[6][45]	[44][6][45]
Q. georgiana M.A.Curtis	TEM	DEC	[55]			
Q. gilva Blume	TEM	EVE	[12][7][8]		[7]	[17][12][37][99]
Q. glauca Thunb.	TRO	EVE	[12][37][100][7][8][12][37]	[98][101]	[100][7]	[99][16][17][80][12][37]
Q. grisea Liebm.	ARID	EVE	[43]			

Species	**Biome**	**Leaf habit**	**LMA**	**Drought Tolerance**	**Leaf Lifespan**	**Photosynthetic traits**
Q. hemisphaerica Bartram	TEM	EVE	[44][6][45]		[45]	[45][44]
Q. hypoleucoides A.Camus	ARID	EVE	[43]			
Q. ilex subsp. *ilex* L.	MED	EVE	[31][22][29][56][102][55][103][57][24][59][11][104][65][51][105][52][60][26][6][27]	[106][107][108][109][110][111][112][113][114][29][115][35][116][117]	[69][69][69][21][61]	[65][17][104][103][102][73][6][24][57][60][103][59][22][29][26][31][118][119][120][71][121][122][123][117][54][72]
Q. ilex subsp. *rotundifolia* Lam.	MED	EVE	[57][11][60][26][6][27]	[106][124][35][117][5]	[61][83][6]	[17][6][37][84][70][117]
Q. ilicifolia Wangenh.	TEM	DEC	[37]			[73][37][125]
Q. incana Bartram	TEM	DEC	[44][17][6][45]		[45]	[17][45][126]
Q. kelloggii Newb.	MED	DEC	[25][26][6]			
Q. laevis Walter	TEM	DEC	[31][44][11][17][45]	[81][29]	[45][6][6]	[44][17][45][74][75][126]
Q. lanata Sm. in A.Rees	TRO	EVE	[85][127]	[128]		
Q. lancifolia Schldtl. & Cham.	TRO	EVE	[129]	[129]		
Q. laurifolia Michx.	TEM	DEC	[44][11][45]	[35]	[45][6]	[44][17][45]
Q. leucotrichophora A.Camus	TRO	EVE	[85]	[86]		
Q. liaotungensis Koidz	TRO	DEC	[130]	[131][132]		[15]
Q. lobata Née	MED	DEC	[23][24][25][26][6]			[25][17][6][24][26][23][133]
Q. lusitanica Lam.	MED	DEC	[6]			[6]
Q. macrocarpa Michx.	TEM	DEC	[31][24][11][65][60][79][26][6]	[134]	[6]	[65][79][26][17][74][75][135][31]
Q. margaretta (Ashe) Small	TEM	DEC	[44][17][6][45]		[45]	[17][45][44][126]
Q. marilandica (L.)Münchh.	TEN	DEC	[136]	[136]		
Q. michauxii Nutt.	TEM	DEC	[31][44][32][24][11][65][33][6][45]	[29][65]	[45][6]	[44][17][73][65][6][24][37][31][11][45][32]
Q. minima (Sarg.)Small	TEM	EVE	[44][6][45]		[45][6]	[44][45]

Species	**Biome**	**Leaf habit**	**LMA**	**Drought Tolerance**	**Leaf Lifespan**	**Photosynthetic traits**
Q. mongolica Fisch. ex Ledeb.	TRO	DEC	[11][65][12][26][6][8]		[137][6]	[80][40][65][138][139][140][141][12][26][6][142][143]
Q. muehlenbergii Engelm.	TEM	DEC	[26]	[5][134]		
Q. myrsinaefolia Blume	TEM	EVE	[31][11][33][37][8]			[33][37][144][40]
Q. myrtifolia Willd.	TEM	EVE	[44][6][45]		[45]	[45][44]
Q. nigra L.	TEM	DEC	[29][44][13][6][45]	[34][29][35]	[45]	[44][55][13][17][29][45]
Q. oleoides Schltdl. & Cham.	TEM	EVE	[145][146][147]	[148][29]		[146][145][147]
Q. palustris Münchh.	TEM	DEC	[32][14]	[115]		
Q. petraea (Matt.)Liebl.	TEM	DEC	[55][103][57][58][24][59][104][149][150][65][51][151][60][26][6]	[152][111][114][153][29][115][35][154][155][5]		[60][59][65][156][157][104][24][149][57][51][55][151][150][26][37][158][159]
Q. phellos L.	TEM	DEC	[29][14]	[34][29]		[29]
Q. prinus L.	TEM	DEC	[11]	[160]		[161][162]
Q. pubescens Willd.1805	MED	DEC	[57][64][59][105][60][26][6]	[152][111][163][113][114][29][116]		[71][164][72][17][60][59][58][29][118][119][120][6][64][105][26][37][165][166]
Q. pumila Walter	TEM	DEC	[6][45]		[45]	[45]
Q. pyrenaica Willd.	MED	DEC	[59][11][65][51][52][60][26][6]	[35][154]	[61][69][69][69][83][6]	[65][60][59][51][52][17][37][6][26][84][70][54]
Q. robur L.	TEM	DEC	[31][56][103][57][167][24][59][11][150][65][168][17][60][26][6]	[152][152][169][114][153][49][29][115][35][82][5]	[61][6]	[170][171][172][156][82][57][60][103][59][65][104][103][168][17][55][6][73][24][57][150][56][26][31][167]
Q. robur subsp. *brutia* (Ten.)O.Schwarz	TEM	DEC	[60]			
Q. rubra L.	TEM	DEC	[31][29][55][57][32][24][59][11][104][65][17][33][173][174][60][36][26][6][14]	[152][175][34][114][29][115][35][65][173]	[6]	[57][60][59][161][176][162][177][178][179][171][172][104][17][174][173][55][73]

Species	**Biome**	**Leaf habit**	**LMA**	**Drought Tolerance**	**Leaf Lifespan**	**Photosynthetic traits**
						[6][24][29][33][57][36][37][29][26][31][32]
Q. rugosa Née	TRO	EVE	[43]			
Q. salicina Blume	TRO	EVE	[7][8][180]		[9][7]	
Q. sebifera Trel.	MED	EVE	[29][11]	[46][29]		[29][46][50]
Q. semecarpifolia Sm. in A.Rees	TRO	EVE	[127][85]	[181]		
Q. serrata Thunb	TEM	DEC	[100][7][8][12]	[182][183][101]	[100][7]	[40][55][12]
Q. sessilifolia Blume	TEM	EVE	[8]			
Q. shumardii Buckley	TEM	DEC	[11][45]		[45][6]	[45]
Q. stellata Wangehn.	TEM	DEC	[29][11][13][6][45]	[34][29][136]	[45]	[45][13][29][73]
Q. stewardiana A.Camus	TRO	EVE	[12]			[12]
Q. suber L.	MED	EVE	[22][56][102][57][59][11][65][51][105][52][60][26][6][27]	[124][114][184][29][115][35][5]	[61][69][83][21][6]	[60][59][58][65][17][102][73][6][22][84][70][54][27]
Q. subpyrenaica Villar	MED	DEC	[185]	[186]		
Q. subsericea A.Camus	TRO	EVE	[98]	[98]		
Q. texana Buckley	TEM	DEC	[14]			
Q. turbinella Greene	MED	DEC	[31][22][24][33][26][6]	[160][29]	[6]	[6][22]
Q. vaccinifolia Kellogg	MED	EVE	[5]	[5]		
Q. variabilis Blume	TEM	DEC	[26][100][8]		[100]	
Q. velutina Lam.	TEM	DEC	[31][32][24][65][6]	[35][65]		[187][17][73][65][31][6][24][26][32]
Q. virginiana Mill.	TEM	EVE	[31][22][44][145][24][6][14][45]		[45]	[44][145][22][45][74][75]
Q. wislizeni A.DC.	MED	EVE	[27]	[169][29]		[27]
Q.wutaishanica Mayr	TRO	DEC	[11]			[11][80][40]
Q. xalapensis Bonpl.	TRO	DEC	[6]		[6]	

Table S2. Species included in the study of geographic variation in leaf mass per area (LMA) across the USA. For each species, we include the species code (SPCD) and symbol used in the Forest Inventory, main biome within the USA, typical leaf habit, number of sites, and mean and standard deviation of Latitude, Longitude, Elevation and LMA. LMA values based on compiled data (source: see Table S1).

Species	**SPCD**	**SYMBOL**	**Main Biome**	**Leaf habit**	**N. of sites**	**Latitude**	**Longitude**	**Elevation (m)**	**LMA (g m^{-2})**
Q. agrifolia	801	QUAG	MED	EVE	488	36.7 ±1.8	-121.3 ±1.7	423 ±302	164 ±51 (7)
Q. alba	802	QUAL	TEM	DEC	47320	36.3 ±3.3	-85.3 ±5.6	243 ±192	82 ±13 (7)
Q. arizonica	803	QUAR	ARID	EVE	1435	33.4 ±0.9	-109.6 ±1.8	1,871 ±256	168 ±20 (1)
Q. chrysolepis	805	QUCH2	MED	EVE	2045	39.8 ±2.3	-122.0 ±1.9	953 ±433	188 ±17 (1)
Q. coccinea	806	QUCO2	TEM	DEC	15688	36.5 ±2.0	-82.8 ±4.3	341 ±245	133 ±76 (3)
Q. douglasii	807	QUDO	MED	DEC	1741	34.8 ±3.6	-102.2 ±19.0	268 ±294	137 ±14 (5)
Q. ellipsoidalis	809	QUEL	TEM	DEC	2632	44.7 ±1.5	-89.1 ±3.6	313 ±73	72 ±20 (6)
Q. emoryi	810	QUEM	ARID	EVE	784	33.1 ±1.1	-110.2 ±1.3	1,710 ±199	168 ±26 (1)
Q. falcata	812	QUFA	TEM	DEC	31302	33.8 ±2.0	-86.7 ±5.5	141 ±115	109 ±24 (6)
Q. gambelii	814	QUGA	TEM	DEC	5036	36.7 ±2.2	-108.8 ±2.5	2,282 ±261	83 ±14 (4)
Q. garryana	815	QUGA4	MED	DEC	918	42.4 ±2.4	-122.6 ±1.0	636 ±392	118 ±7 (1)
Q. grisea	846	QUGR3	ARID	EVE	393	33.2 ±1.2	-107.8 ±1.9	2,011 ±282	151 ±37 (1)
Q. hypoleucoides	843	QUHY	ARID	EVE	234	32.6 ±0.6	-109.5 ±1.1	1,997 ±252	220 ±22 (1)
Q. ilicifolia	816	QUIL	TEM	DEC	397	39.4 ±2.3	-76.7 ±4.3	311 ±288	82 ±9 (1)
Q. incana	842	QUIN	TEM	DEC	869	31.8 ±1.5	-84.7 ±4.2	72 ±51	105 ±12 (4)
Q. kelloggii	818	QUKE	MED	DEC	2033	39.9 ±1.9	-121.8 ±1.5	942 ±437	108 ±16 (3)
Q. laevis	819	QULA2	TEM	DEC	2621	31.9 ±2.0	-83.1 ±2.9	61 ±46	113 ±9 (5)
Q. laurifolia	820	QULA3	TEM	DEC	14681	31.8 ±1.8	-84.1 ±3.9	59 ±58	98 ±19 (3)
Q. lobata	821	QULO	MED	DEC	191	38.3 ±1.7	-121.8 ±1.4	561 ±365	122 ±20 (5)
Q. macrocarpa	823	QUMA2	TEM	DEC	4324	44.2 ±3.3	-92.9 ±4.4	371 ±216	68 ±15 (8)
Q. michauxii	825	QUMI	TEM	DEC	3600	33.6 ±2.3	-85.2 ±6.1	66 ±72	87 ±26 (9)
Q. minima	841	QUMI2	TEM	EVE	666	29.8 ±1.2	-83.1 ±2.3	28 ±70	162 ±19 (3)
Q. muehlenbergii	826	QUMU	TEM	DEC	4612	36.9 ±1.9	-88.6 ±4.3	262 ±140	51 ±0 (1)
Q. nigra	827	QUNI	TEM	DEC	36969	32.7 ±1.7	-86.2 ±5.2	86 ±70	96 ±30 (5)
Q. palustris	830	QUPA2	TEM	DEC	1288	38.8 ±2.5	-86.6 ±6.1	193 ±101	108 ±16 (2)

Q. phellos	831	QUPH	TEM	DEC	13076	33.6 ±1.9	-86.3 ±6.0	91 ±73	146 ±118 (2)
Q. prinus	832	QUPR2	TEM	DEC	13524	37.0 ±2.1	-82.1 ±3.2	460 ±265	93 ±13 (2)
Q. rubra	833	QURU	TEM	DEC	35492	39.2 ±4.0	-84.2 ±6.1	344 ±221	73 ±16 (19)
Q. rugosa	847	QURU4	ARID	EVE	361	33.2 ±1.0	-109.8 ±1.2	1,838 ±259	188 ±14 (1)
Q. shumardii	834	QUSH	TEM	DEC	2583	34.5 ±2.3	-89.5 ±4.8	164 ±116	99 ±7 (2)
Q. stellata	835	QUST	TEM	DEC	27730	34.2 ±2.1	-88.5 ±5.6	175 ±125	115 ±42 (5)
Q. texana	828	QUTE	TEM	DEC	1481	32.6 ±1.7	-91.3 ±1.8	49 ±49	122 ±24 (1)
Q. turbinella	8459	QUTU2	MED	DEC	689	34.7 ±1.3	-111.4 ±2.4	1,606 ±257	146 ±11 (6)
Q. velutina	837	QUVE	TEM	DEC	31325	36.9 ±2.8	-85.4 ±5.6	281 ±195	73 ±22 (5)
Q. virginiana	838	QUVI	TEM	EVE	5924	30.4 ±1.6	-86.6 ±7.0	116 ±194	164 ±58 (8)
Q. wislizeni	839	QUWI2	MED	EVE	715	38.1 ±1.8	-120.9 ±1.6	663 ±419	162 ±8 (1)

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Capítulo III

Deciduous and evergreen oaks show contrasting adaptative responses in leaf mass per area across environments

New Phytologist

Research

Deciduous and evergreen oaks show contrasting adaptive responses in leaf mass per area across environments

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Received: *30 July 2020*
Accepted: *11 December 2020*

New Phytologist (2021)
doi: 10.1111/nph.17151

Key words: climate, deciduous, evergreen, leaf density, leaf mass per area, leaf thickness, *Quercus*.

Summary

- Increases in leaf mass per area (LMA) are commonly observed in response to environmental stresses and are achieved through increases in leaf thickness and/or leaf density. Here, we investigated how the two underlying components of LMA differ in relation to species native climates and phylogeny, across deciduous and evergreen species.
- Using a phylogenetic approach, we quantified anatomical, compositional and climatic variables from 40 deciduous and 45 evergreen *Quercus* species from across the Northern Hemisphere growing in a common garden.
- Deciduous species from shorter growing seasons tended to have leaves with lower LMA and leaf thickness than those from longer growing seasons, while the opposite pattern was found for evergreens. For both habits, LMA and thickness increased in arid environments. However, this shift was associated with increased leaf density in evergreens but reduced density in deciduous species.
- Deciduous and evergreen oaks showed fundamental leaf morphological differences that revealed a diverse adaptive response. While LMA in deciduous species may have diversified in tight coordination with thickness mainly modulated by aridity, diversification of LMA within evergreens appears to be dependent on the infrageneric group, with diversification in leaf thickness modulated by both aridity and cold, while diversification in leaf density is only modulated by aridity.

Introduction

Sclerophyllous leaves are tough and frequently hard and stiff (Grubb, 1986; Turner, 1994; Read & Sanson, 2003). To obtain a quantitative proxy value for this leaf trait, most ecophysiological studies calculate either the specific leaf area or its inverse, the leaf mass per unit area (LMA) (e.g. Witkowski & Lamont, 1991; Groom & Lamont, 1999; Osone *et al.*, 2008; Poorter *et al.*, 2012; Gil-Pelegrín *et al.*, 2017). Although the ecological and functional significance of LMA is still under debate (Kikuzawa *et al.*, 2013; Alonso-Forn *et al.*, 2020), it is widely accepted that an increase in LMA is a general response to environmental stress (Onoda *et al.*, 2011; Alonso-Forn *et al.*, 2020). Thus, many studies have reported a higher LMA under conditions of drought stress (Turner, 1994; Reich *et al.*, 1999; Jordan *et al.*, 2005), low winter temperatures (Ogaya & Peñuelas, 2007; González-Zurdo *et al.*, 2016; Niinemets, 2016), or under conditions of low nutrient availability (Loveless, 1962; Niinemets *et al.*, 2009; Hassiotou *et al.*, 2010). A high LMA has also been traditionally interpreted as a trait that increases the structural resistance of leaves, as it may protect the leaf against herbivory or mechanical damage (Turner, 1994; Reich *et al.*, 1999; Sack *et al.*, 2013) and so expecting to achieve longer lifespans (Coley *et al.*, 1985; Reich *et al.*, 1999). However, Alonso-Forn *et al.*, (2020) recently revisited the role of these four factors (drought, cold, nutrient availability and leaf longevity) on sclerophylly within the genus *Quercus* L. and they concluded that no sole factor could explain all the variation observed in LMA, suggesting that these constraints may have a synergistic effect. Additionally, the mechanisms driving higher LMA at lower temperatures across species are still unclear (Alonso-Forn *et al.*, 2020), urging the consideration that both severity of cold and length of the cold season be considered together in ecological studies. This segregation between the duration (length of time during which stress occurs) and intensity (extreme value or accumulated value of a climatic stress factor) has been previously incorporated in studies when analysing drought stress (e.g. Granier *et al.*, 1999), but rarely when considering cold stress.

LMA, defined above as the ratio between leaf mass and leaf area, is also related to leaf thickness (LT, μm) and leaf density (LD, mg cm^{-3}, i.e. dry mass per hydrated volume), given that LMA (g m^{-2}) = LT × LD (Witkowski & Lamont, 1991; Poorter *et al.*, 2009; John *et al.*, 2017). These structural variables can also depend on other parameters associated with leaf anatomy and chemical composition. While variation in LT is mainly attributed to changes in mesophyll thickness, LD is dependent (amongst other things) on the chemistry of cell walls and vein traits (Villar *et al.*, 2013; John *et al.*, 2017; Peguero-Pina *et al.*, 2017a). In addition, variation in LMA related to changes in LT or LD has been suggested to have two major and potentially contrasting consequences for leaf function, linked to leaf economic spectrum theory (Wright *et al.*, 2004; Shipley *et al.*, 2006). First, as performing photosynthesis is the main function of the mesophyll, we might expect that the increase in LMA would be associated with an increase in mesophyll thickness, to lead to a greater capacity for C assimilation per unit leaf area due to a higher amount of photosynthetic tissue per unit area (Niinemets, 1999; Niinemets, 2001; Peguero-Pina *et al.*, 2017a). Moreover, a higher instantaneous photosynthetic rate per unit leaf area would compensate for a shorter favourable season caused by a higher duration of stressful seasonally climatic events such as aridity or cold (Kikuzawa *et al.*, 2013; Peguero-Pina *et al.*, 2016a; Berdugo *et al.*, 2020). However, an increase in mesophyll thickness may increase the nitrogen content per unit area (Onoda *et al.*, 2017) and, therefore, increase the risk for herbivory (Meloni *et al.*, 2012). Thus, if changes in LMA are associated with changes in photosynthetically competent tissues, thicker leaves that result in high LMA would achieve higher instantaneous assimilation rates per unit area compensating for a longer duration of climate stress events, but at the expense of a lower protection given by a stronger herbivore pressure (Scenario 1, Fig. 1). Second, changes in LMA associated with LD imply variation in the relative amount of cell types or variation in biomass allocation (Niinemets, 2001; Hassiotou *et al.*, 2010; Griffith *et al.*, 2016). For instance, a higher cell wall fraction with respect to cytoplasm should lead to an increase in LD (Poorter *et al.*, 2009) giving more structural resistance to the cell (Wyka & Oleksyn, 2014) needed under more intense climate stress events (Oertli *et al.*, 1990; Scholz *et al.*, 2012; Ding *et al.*, 2014; Alonso-Forn *et al.*, 2020), but inevitably reducing the efficiency of photosynthesis due to a reduction in cell wall permeability to CO$_2$ and N allocation in photosynthetic proteins (Onoda *et al.*, 2017). Similarly, higher vein density, which can also contribute to increasing LD (Alonso-Forn *et al.*, 2020), has been observed to be greater in more arid biomes (Sack & Scoffoni, 2013), to the potential detriment of reducing the amount of photosynthetic tissues. Therefore, higher LMA associated with an increase in LD, and thus of 'protective' traits against intense climatic stresses, could trigger an increase in leaf lifespan, compensating for the lower instantaneous C assimilation rate caused by an increase in the proportion of nonphotosynthetic tissues (Kikuzawa, 1995; Niinemets, 2001; Kikuzawa & Lechowicz, 2011; Scenario 2, Fig. 1).

In general, LMA and leaf functioning differ between deciduous and evergreen species. Deciduous and evergreen species indeed have been shown to differ in: (1) LMA values, with lower values in deciduous species (e.g. Castro-Díez *et al.*, 2000; Poorter *et al.*, 2009), (2) the leaf structural traits behind increases in LMA (de la Riva *et al.*, 2016), (3) biomass allocation (Puglielli

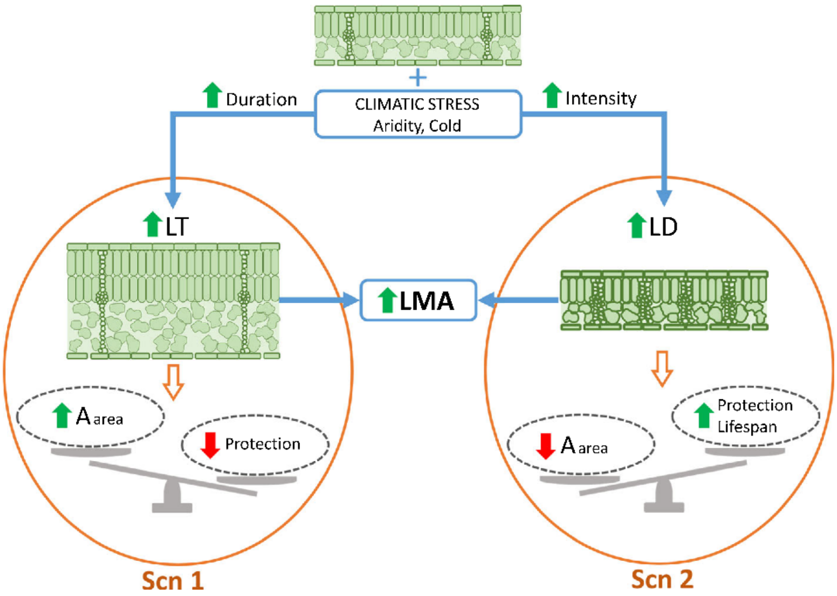

Fig. 1 Schematic representation of the two leaf mass per area (LMA) diversification scenarios. Scenario 1 (Scn 1): leaf thickness (LT) increases LMA to increase A_{area} in order to compensate for a shorter favourable season. Scenario 2 (Scn 2): leaf density (LD) increases LMA as a protective mechanism against severe climatic factors. A_{area}, carbon assimilation per area.

et al., 2020), and (4) leaf longevity (Kikuzawa, 1991; Wright *et al.*, 2005; Kikuzawa *et al.*, 2013). For instance, a recent meta-analysis highlighted that, although variations in LMA were associated with LT and LD within both leaf habits, the variation across deciduous oak species was mainly due to variation in LT, while variation across evergreen oaks was better explained by variation in LD (Escudero *et al.*, 2017). In this sense, assuming that species from both leaf habits can fit into the two LMA diversification scenarios, the relative importance of Scenario 1 (LT increases LMA in order to compensate photosynthetically for a shorter favourable season) and Scenario 2 (LD increases LMA as a protective mechanism against severe climatic factors) within each leaf habit is also expected to be different. While variation of LMA within deciduous species could be more strongly associated with Scenario 1, variation within evergreen species might be more strongly associated with Scenario 2. Furthermore, it is imperative to investigate these scenarios within a phylogenetic framework, to help understand why plants have evolved certain leaf traits rather than others, which is a central question in evolutionary biology (Pigliucci, 2007).

In this study, we investigated the two LMA diversification scenarios in terms of several leaf traits enabling variation of LMA across deciduous and evergreen *Quercus* species. We quantified 14 leaf anatomical, morphological and chemical traits in a set of 40 deciduous and 45 evergreen *Quercus* species growing in a common garden in northern Spain. On the one hand, the genus *Quercus* is an excellent system to perform this study, as it not only minimises phylogenetic variation (compared with studies performed across diverse species), but also displays strong variation in LMA across species adapted to a large variety of climatic conditions, from those in nemoral–temperate areas to those in Mediterranean or tropical environments (Gil-Pelegrín *et al.*, 2017). Therefore, the genus *Quercus* constitutes a unique living laboratory to understand global adaptive patterns along the leaf economic spectrum in forest trees (Alonso-Forn *et al.*, 2020). On the other hand, by allowing the contribution of environmental variation to be ruled out as a factor in phenotypic variation, common gardens allow interspecific comparisons of traits that result from long-term adaptation to the environmental conditions where different species have evolved. LMA could therefore be analysed in relation to the species' native climate.

Four main objectives were addressed: (1) to quantify and contrast the diversification in leaf morphology, anatomy and chemical composition between deciduous and evergreen oaks; (2) to elucidate within each leaf habit which of the two components, LT or LD, was more relevant in the evolution of LMA in *Quercus*; (3) to identify for each leaf habit which anatomical and compositional traits are associated with increases in LT and LD; and (4) to investigate the role of climate in the differential diversification of LMA within deciduous and evergreen oaks. We hypothesised that diversification in LT was the main driver behind the diversification in LMA within deciduous oaks, whereas the diversification in LD would be more important within evergreen oaks. We also hypothesised that the duration of climatic stress would have a greater impact than stress intensity within deciduous species on LMA, but would have similar or less importance within evergreens.

Materials and Methods

Plant material

To perform this study, 85 oak species from across the Northern Hemisphere (Supporting Information Table S1) were sampled from living collections in the Jardín Botánico de Iturrarán (43°13′N, 02°01′W, 70 m asl, Gipuzkoa, Spain, www.iturraran.org). This common garden features temperate climatic conditions with a mean annual temperature of 14.5°C and a total annual precipitation of 1631 mm (Peguero-Pina *et al.*, 2016b). Current year, fully developed leaves were collected from southern-exposed branches of three trees per species during the early morning (07:00-09:00 h, solar time). All trees sampled were mature trees (15–25 yr old) to ensure leaf traits were measured at similar ontogenic changes (Puglielli *et al.*, 2020). Leaves were sealed in plastic bags and carried to the laboratory in portable coolers. As LT and LD variations can be explored through a series of anatomical and chemical traits (Villar *et al.*, 2013; John *et al.*, 2017), we used one set of 10 leaves per species (three or four leaves per tree) to measure leaf area, leaf mass and leaf fibre concentration, and another set of five leaves per species (one or two leaves per tree) to measure diverse anatomical parameters (details to be described later). Oak species were classified according to their leaf habit as evergreen (45 species), if they retained their leaves during the whole year and leaf lifespan was at least 12 months, and deciduous (40 species), if they lost all leaves during the winter season (Table S1). This classification was carried out with personal observations on the specimens of the common garden. We did not observe any species that retained their leaves during the whole year with a leaf lifespan lower than 12 months. Finally, climatic information based on the natural distribution range of each oak species was obtained to study the leaf traits in relation to climatic variables (to be described later).

Leaf mass per unit area and fibre concentration

Leaf area was measured by digitising 10 leaves per species and using ImageJ analysis software (http://rsb.info.nih.gov/nih-image/). Afterwards, leaves were oven dried for 3 d at 70°C and weighed using an analytical balance (Sartorius AG Model BP221S; Sartorius AG, Goettingen, Germany). LMA was then calculated as the ratio of the foliage dry mass to foliage area. Later, leaves were ground with a bead mill (TissueLyser II; Qiagen, Hilden, Germany) for fibre analysis. Hemicellulose, cellulose and lignin + cutin concentration were obtained by quantifying neutral detergent fibre and acid detergent lignin following the method of Goering & Van Soest (1970). Fibre concentration, obtained from a bulk of 10 leaves, was expressed as milligrams of fibre per gram of leaf dry mass. Abbreviations and units of these traits are shown in Table 1(a). The mean values (±SE) of leaf area and LMA, and the bulk values of fibre concentration are available in Table S1.

Table 1 List, units, abbreviations and association with the scenarios for (a) leaf traits and (b) climatic variables.

	Units	Abbreviation	Scenario
(a) Leaf traits			
Leaf mass per unit area	g m^{-2}	LMA	1, 2
Leaf thickness	µm	LT	1
Leaf tissue density	mg cm^{-3}	LD	2
Leaf area	cm^{2}	LA	1, 2
Hemicellulose concentration	mg g^{-1}	HC	2
Cellulose concentration	mg g^{-1}	CC	2
Lignin + cutin concentration	mg g^{-1}	LCC	2
Palisade mesophyll thickness	µm	PMT	1
Spongy mesophyll thickness	µm	SMT	1
Palisade mesophyll cell rows	count	PCR	1
Palisade cell length	µm	PCL	1, 2
Palisade cell width	µm	PCW	1, 2
Interveinal distance	µm	IVD	2
Bundle sheath extension width	µm	BSEW	2
(b) Climatic variables			
Mean monthly temperature	°C	t_m	1, 2
Monthly precipitation	mm	p_m	1, 2
Growing season. Number of months with $t_m > 5$°C	Month	GS	1
Period of full plant growth. Number of months with $t_m > 10$°C	Month	FPG	1
Arid period. Number of months with $t_m > 10$°C and $2\ t_m > p_m$	Month	AP	1
Annual mean temperature	°C	T	1, 2
Minimum temperature of coldest month	°C	TMIN	2
Mean temperature of driest quarter	°C	TDRY	2
Annual precipitation	mm	P	1, 2
Arid intensity. Sum of $(2\ t_m - p_m)$ for months with $t_m > 10$°C and $2\ t_m > p_m$		AI	2

Leaf cross-sectional anatomy

A transverse section (*c.* 3 × 2 mm) was cut between the secondary veins of the middle part of each leaf. Sections were fixed in paraffin and embedded in paraffin blocks (Ruzin, 1999). Leaf cross-sections (20 µm in thickness) were obtained from the blocks using a rotary microtome (HM 350 S; Microm International GmbH, Walldorf, Germany). Cross-sections were deparaffinised and stained following the procedure of Johansen's safranin and fast green (Johansen, 1940; Ruzin, 1999). Afterwards, images of the cross-sections were taken using a digital camera (Optikam Pro 5; Optika Microscopes, Ponteranica, Italy) coupled to a light microscope (Optika B-600TiFL; Optika Microscopes, Ponteranica, Italy) (Fig. 2). Anatomical parameters including LT, palisade and spongy mesophyll thickness, number of palisade cell row layers, palisade cell size, interveinal distance (IVD) and bundle sheath extension width (BSEW), were then measured from the images using IMAGEJ software (Fig. 2). We measured one value per cross-section for each anatomical parameter, except for IVD and BSEW, for which we measured two values, and for palisade cell size for which we measured palisade cell length and width of 20 cells per cross-section. Finally, for each species, the LD was calculated as the ratio between LMA and LT (Witkowski & Lamont, 1991; Niinemets, 1999). Abbreviations of leaf anatomy traits, their units and their association with the scenarios are shown in Table 1(a). The mean values (±SE) of these anatomical traits for each species are available in Table S1.

Climatic variables

Climatic information (bioclimatic variables; mean monthly temperature, t_m; and monthly precipitation, p_m) of species natural distributions was obtained from the WorldClim database (http://www.worldclim.org/) using geographical distribution coordinates obtained from herbarium data (Gil-Pelegrín *et al.*, 2017). From this climatic information, we selected six climatic variables related to the duration and intensity of the seasonal stress, including cold and arid-related variables. Indeed, we used two climatic variables related to cold stress duration: the growing season and the period of full plant growth, defined as the number of months when monthly temperatures were higher than 5°C and higher than 10°C, respectively (Wypych *et al.*, 2017). We further selected one climatic variable related to arid stress duration: the aridity period, which we based off climatic diagrams (Breckle, 2002) that define aridity as the number of months where 2 × monthly temperature (t_m) > monthly precipitation (p_m), considering only those months with mean temperatures higher than 10°C. We selected one climatic variable that characterises cold stress intensity, the minimum temperature of the coldest month. We finally selected two climatic variables characterising aridity stress intensity: the aridity intensity, defined as the sum of $(2t_m - p_m)$ for months with $2t_m > p_m$ and $t_m > 10$°C, and the temperature of the driest quarter (TDRY) (Table 1b). For a better understanding of TDRY, we identified the season of the year with the driest quarter by analysing the monthly precipitation of the localities for each species. This analysis showed that most of the species included in this study had their driest quarter either during summer (June, July, August) or during winter (December, January, February) (Fig. S1). Additionally, we include in the analyses the mean annual temperature and the mean annual precipitation as standard variables for comparative purposes with other studies. Abbreviations of climatic variables, their units and their association with each scenario are shown in Table 1(b). The mean values (±SE) of the eight climatic variables used in this study for each species are available in Table S2.

These climatic variables from all species were previously analysed using principal components analysis in order to identify the species within the eight climatic variables considered (Fig. S2). This previous analysis also showed that thermal-related variables (including the growing season and the period of full plant growth) weighed more in the first component, while arid variables weighed more in the second component.

Data analysis

First, Student's *t*-tests were used to compare the leaf trait values between deciduous and evergreen species in order to identify

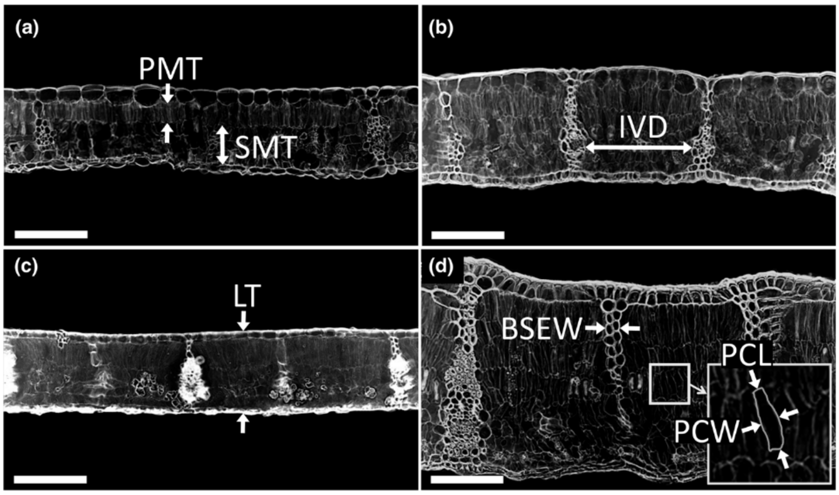

Fig. 2 Leaf mesophyll cross-sections of two deciduous (a, b) and two evergreen (c, d) *Quercus* species differing in leaf mass per area (LMA). (a) *Quercus robur* (LMA = 86 g m^{-2}). (b) *Quercus marilandica* (108 g m^{-2}). (c) *Quercus insignis* (90 g m^{-2}). (d) *Quercus miquihuanensis* (198 g m^{-2}). BSEW, bundle sheath extension width; IVD, interveinal distance; LT, leaf thickness; PCL, palisade cell length; PCW, palisade cell width; PMT, palisade mesophyll thickness; SMT, spongy mesophyll thickness. Bars, 100 μm.

their differences in terms of LMA, anatomy and chemical composition.

Second, evolutionary dynamics of traits following the approach of previous studies (Chatelet *et al.*, 2013; Scoffoni *et al.*, 2016) were examined with the aim of analysing within each leaf habit which of the two components, LT or LD, was more associated to the diversification of LMA in oaks. A phylogenetic tree was inferred from Hermida-Carrera *et al.* (2017), pruned to match species measured in this study. The phylogenetic tree was constructed using rbcL sequences from 85 *Quercus* species and *Fagus crenata* as a root species (see Table S3 for GenBank accession numbers), resulting in a tree similar to that shown by Hipp *et al.* (2020) and consistent in the assignment of species to subgenera (subgenus *Cerris* and subgenus *Quercus*) and sections (Denk *et al.*, 2017). Then, to visualise how increases in either LT and/or LD related to increases in LMA, we calculated the divergence of LT and LD from LMA across the study's oak species by first standardising the species means for all three traits to values between 0 and 100. Then, we calculated the absolute value of the difference between the standardised LMA and the standardised LT and LD values respectively for each species. As such, a divergence value close to 0 between LT and LMA across species would mean that both traits co-evolved very tightly. We also calculated the divergence of the two first climatic principal components (Fig. S2) from LMA, LT and LD in order to elucidate whether climate was tightly coordinated with these leaf traits across species.

Third, we compared the fit of three different models of trait evolution: a Brownian motion (BM) model, an Ornstein–Uhlenbeck (OU) model with a single global optimum (OU1) and an OU model with two optima either evergreen or deciduous species (OU2). The best fit model for each variable was chosen using the computed Akaike Information Criterion corrected for small sample size, and the corresponding variable was transformed appropriately. Then, relationships between leaf traits and climatic variables across different species within each leaf habit were explored with regression and correlation analyses (SPSS statistical package; SPSS Inc., Chicago, IL, USA). Analyses were performed using phylogenetically independent contrasts (PICS) (Harvey & Pagel, 1992). Interspecific differences in leaf traits were tested by one-way ANOVA (SPSS).

All analyses were performed in R (v.3.6.1) using the packages Analysis of Phylogenetics and Evolution (APE; Paradis *et al.*, 2004), Ornstein–Uhlenbeck models for phylogenetic Comparative Hypotheses (OUCH; Butler & King, 2004), and Analysis of Evolutionary Diversification (GEIGER; Harmon *et al.*, 2008).

Results

Variation in anatomical and chemical composition across species and leaf habits

All leaf traits analysed exhibited strong interspecific variability, with leaf area showing the highest coefficient of variation (Table 2). LMA, palisade and spongy mesophyll thickness, and IVD showed intermediate coefficients of variation, whereas LD showed fewer differences between the different species. Hemicellulose and cellulose concentration and palisade cell size also showed relatively low variability (Table 2).

The coefficients of variation for LMA, LT, LD, leaf area, hemicellulose content, spongy mesophyll thickness and palisade cell length were 1.2–1.9-fold higher for evergreen than for deciduous species (Table 2). Evergreen and deciduous leaf habits also showed significant differences in the mean values of most leaf traits ($P<0.05$; Table 2). Evergreen oaks had 1.4-fold higher LMA and LT than deciduous species, but 2.3-fold smaller leaf area. Cellulose concentration and lignin + cutin concentration were 1.3-fold and 1.1-fold higher,

Table 2 Mean value, maximum (max) and minimum (min) values, and coefficient of variation (CV) of leaf traits for deciduous (DEC) and evergreen (EVE) *Quercus* species.

	LMA	LT	LD	LA	HC	CC	LCC	PMT	SMT	PCR	PCL	PCW	IVD	BSEW
DEC (mean)	92 ± 2 a	135 ± 5 a	695 ± 19 a	42 ± 5 a	180 ± 5 a	150 ± 4 a	129 ± 6 a	62 ± 3 a	44 ± 2 a	1.7 ± 0.1 a	38 ± 2 a	7.4 ± 0.2 a	301 ± 17 a	12.6 ± 0.5 a
EVE (mean)	130 ± 6 b	191 ± 7 b	694 ± 27 a	19 ± 2 b	169 ± 6 a	193 ± 4 b	148 ± 6 b	88 ± 4 b	74 ± 3 b	2.2 ± 0.1 b	40 ± 1 a	8.1 ± 0.2 b	198 ± 7 b	15.7 ± 0.6 b
DEC (max)	129	232	1058	141	252	210	260	137	69	3	65	11	584	21.4
DEC (min)	53	87	487	3	119	104	74	24	28	1	23	6	173	5.4
EVE (max)	223	337	1205	75	270	290	264	161	135	3	56	11	346	25.1
EVE (min)	62	106	435	2	78	128	76	36	35	1	23	6	118	9.6
DEC (CV)	17	22	17	75	18	16	32	32	25	27	28	13	35	27
EVE (CV)	31	26	26	88	24	16	26	31	30	25	17	13	25	25
All species (CV)	32	30	22	91	22	20	29	36	39	29	22	14	39	28

Mean values are mean ± SE. $n_{DEC} = 40$. $n_{EVE} = 45$. Leaf traits notation as in Table 1. Different letters indicate statistically significant differences ($P < 0.05$) between deciduous and evergreen species. CV for all species is also shown.

respectively, in evergreen than in deciduous species. The larger thickness of evergreen species was due to the thicker mesophyll, mainly due to the 1.7-fold larger spongy mesophyll contribution (Table 2). There were also differences in the vascular bundle traits. Evergreen oaks showed a significant 1.5-fold lower IVD and, therefore, a higher vein density, and a significant 1.3-fold higher value of BSEW than deciduous oaks (Table 2). By contrast, LD, hemicellulose concentration and palisade cell length did not show significant differences between the two leaf habits ($P < 0.05$; Table 2).

Dynamics and models of trait evolution

Across species, LT and LD evolved in coordination with shifts in LMA, such that the direction and magnitude of evolutionary shifts in LT or LD corresponded to shifts in LMA (Fig. S3). However, LMA appears to have evolved in tighter coordination with LT than it did with LD; 83% of all deciduous oak species and 66% of the evergreen species belonging to subgenus *Quercus* showed a tighter coordination between LT and LMA (expressed as lower divergence between the traits; Fig. 3). By contrast, LD showed tighter coordination with LMA in *c.* 70% of the evergreen oaks within the subgenus *Cerris* (Fig. 3). Notably, high values of LMA could be found in any clade, but were preferably associated with evergreen species (Fig. 3).

Concerning the coordination between LMA and climate, 71% of the species showed a divergence value below 15, with at least one of the climatic principal components, that is LMA showed a very tight coordination with climate in most species analysed (Fig. 3). Across species from subgenus *Cerris*, LMA, LT and LD showed a slight tighter coordination with PC1 (the thermal component; Fig. S2) than they did with PC2 (the arid component; Figs 3,S2,S4). Within subgenus *Quercus*, LMA and LT also showed a slight tighter coordination with PC1, while LD showed a tighter coordination with PC2 (Figs 3,S4).

Comparing the fit of the three different models of trait evolution (BM, OU1 and OU2), the model that best fitted most of our trait variables (e.g. LMA, LT) was the OU2 model with two trait optimum (for deciduous and evergreen species) (Table S4). However, for some variables (e.g. LD) the fit of OU2 was similar or slightly lower than OU1 (single trait optimum).

Correlations in anatomical and chemical composition within leaf habits

Within deciduous species, LMA showed a positive association with LT (including its thickness components palisade and spongy mesophyll thickness and number of palisade cell rows), LD, palisade cell length, cellulose concentration and lignin + cutin concentration ($R = 0.436$–0.643, $P < 0.01$), and a negative association with IVD ($R = -0.596$, $P < 0.001$; Table 3a; Fig. 4). LT was positively associated with the thickness of the different tissue layers, with palisade cell rows, BSEW, cellulose and lignin + cutin concentration ($R = 0.316$–0.737, $P < 0.05$; Table 3a). LT was also negatively associated with LD and leaf area ($R = -0.408$ and -0.457, $P < 0.01$; Table 3a). LD showed

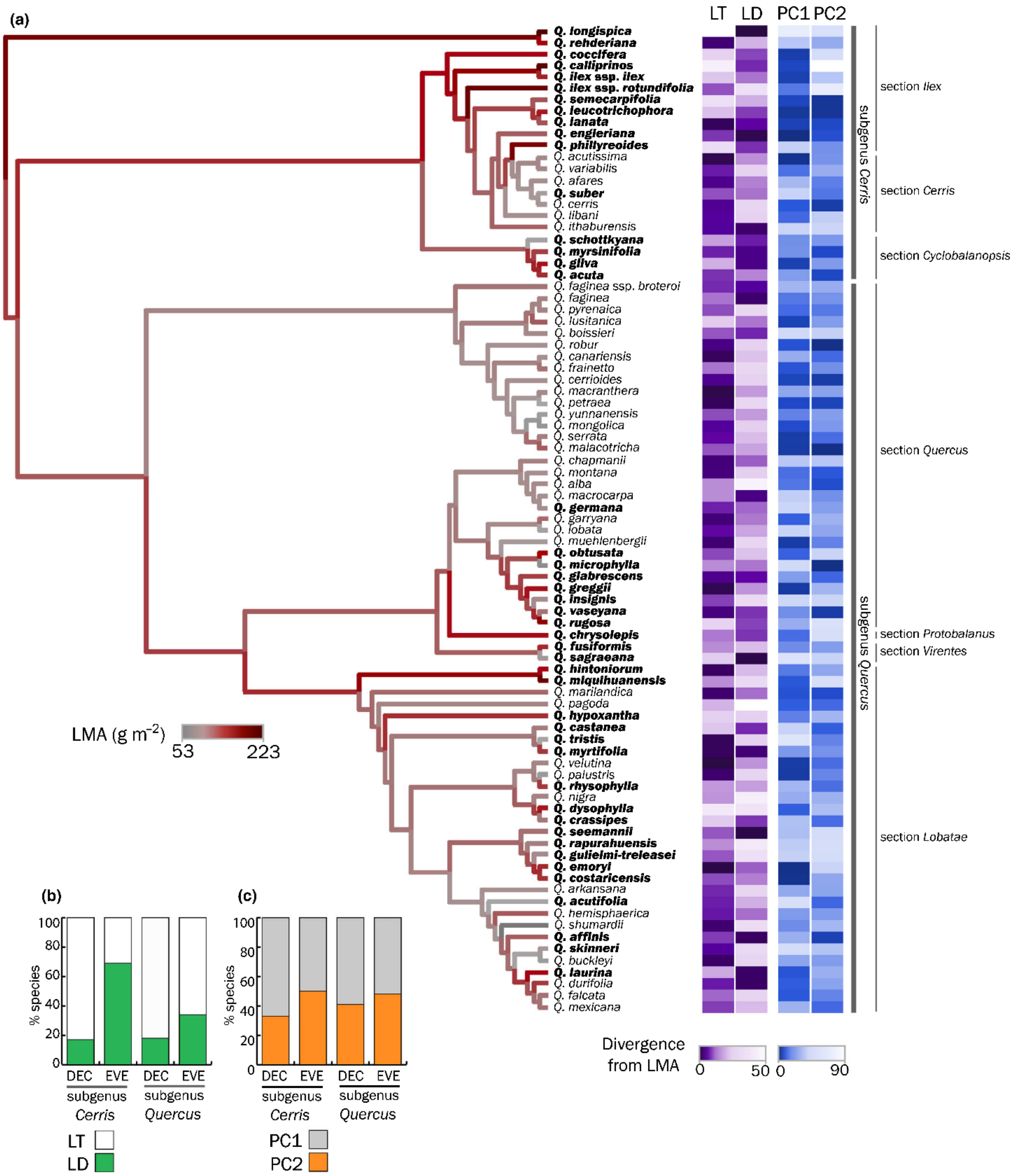

Fig. 3 (a) *Quercus* phylogenetic tree based on rbcL showing the evolution of leaf mass per area (LMA) in coordination with leaf thickness (LT), leaf density (LD) and climate. Darker colour for LMA indicates a higher value. Darker colours for LT, LD and the two first climatic principal components (PC1, PC2, see Supporting Information Fig. S2) indicate tight coordinated evolution with LMA (= low divergence) across our study's species. Bolded and nonbolded species names indicate evergreen and deciduous species, respectively. (b, c) Percentage of species showing a higher coordination (= lower divergence) between LMA and LT (white), LMA and LD (green), LMA and PC1 (grey), and LMA and PC2 (orange) for both leaf habits and both subgenera.

Table 3 Correlation coefficients between leaf traits for (a) deciduous (DEC) and (b) evergreen (EVE) *Quercus* species using phylogenetically independent contrasts (PICS).

(a) DEC	LMA	LT	LD	LA	(b) EVE	LMA	LT	LD	LA
LT	0.567***				LT	0.686***			
LD	0.449**	−0.408**			LD	0.405**	−0.354*		
LA		−0.457**			LA		−0.444**		
HC				0.572***	HC				
CC	0.525***	0.546***			CC	0.577***	0.474**		
LCC	0.465**	0.372*			LCC	0.317*			
PMT	0.575***	0.605***			PMT	0.817***	0.909***		−0.350*
SMT	0.590***	0.737***	−0.369*	−0.712***	SMT	0.528***	0.729***	−0.499*	−0.364*
PCR	0.436**	0.318*			PCR	0.774***	0.715***		
PCL	0.643***			−0.459**	PCL	0.536***	0.468**	−0.355*	−0.537***
PCW					PCW			−0.382*	
IVD	−0.596***		−0.489**	0.326*	IVD				
BSEW		0.316*	−0.475**	−0.513***	BSEW	0.584***	0.732***		−0.304*

Leaf traits notation as in Table 1. Significance levels: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

negative correlations with spongy mesophyll thickness, IVD and with BSEW ($R=-0.369$ to -0.489, $P<0.05$; Table 3a).

Within the evergreen species – as in the deciduous species – LMA showed positive associations with LT (including palisade and spongy mesophyll thickness and number of palisade cell rows), LD, palisade cell length, cellulose and lignin + cutin concentration ($R=0.317$–0.817, $P<0.05$), being also associated with BSEW ($R=0.584$, $P<0.001$; Table 3b; Fig. 4). LT was positively related to the thickness of the different mesophyll tissue layers, palisade cell rows and cell length, BSEW and to cellulose concentration ($R=0.468$–0.909, $P<0.01$) and negatively related to LD and leaf area ($R = -0.354$ and -0.444, $P<0.05$; Table 3b), similar to the deciduous species. Within the evergreen species, LD also showed a negative correlation with spongy mesophyll thickness and palisade cell size, including palisade cell length and width ($R=-0.366$ to -0.499, $P<0.05$; Table 3b).

Relationship of climate and leaf anatomy within leaf habits

Within deciduous species, LMA was positively correlated with the growing season ($R=0.376$, $P<0.05$) and to mean temperature of driest quarter (TDRY; $R=0.345$, $P<0.04$; Table 4a). Leaf area was also related to TDRY, but the trend was the opposite of that found for LMA (Table 4a). Additionally, leaf area also showed a negative correlation coefficient with minimum temperature of the coldest month (TMIN; $R=-0.393$, $P<0.05$; Table 4a). Surprisingly, LT within deciduous species was correlated with all climatic variables considered in this study, being positively associated with growing season, full plant growth period, aridity period, aridity intensity, mean annual temperature, TMIN and TDRY ($R=0.341$–0.521, $P<0.05$), and negatively associated with annual precipitation ($R=-0.483$, $P<0.01$; Table S4). LD was positively correlated with annual precipitation ($R=0.523$, $P<0.001$) and negatively correlated with aridity period and aridity intensity ($R=-0.465$ and -0.537, $P<0.01$; Table 4a).

For the evergreen species, LMA and LT were negatively correlated with the period of full plant growth ($R=-0.350$ and -0.432, $P<0.05$), a trend that was opposite to that found in the deciduous species between these leaf traits and the growing season (Table 4a). LMA and LT were also negatively correlated with annual mean temperature, minimum temperature of coldest month and annual precipitation ($R=-0.471$ to -0.663, $P<0.01$), and positively correlated with aridity period ($R=0.381$–0.581; $P<0.05$; Table 4b). LMA was also positively correlated with aridity intensity ($R=0.401$, $P<0.01$; Table 4b). By contrast, leaf area showed positive correlations with minimum temperature of coldest month and annual precipitation ($R=0.645$ and 0.742, $P<0.001$) and negative correlations with aridity period and aridity intensity ($R=-0.420$ and -0.344, $P<0.05$), trends that were opposite to those found for LMA (Table 4b). Finally, LD was positively correlated with mean temperature of driest quarter, aridity period and aridity intensity ($R=0.316$–0.494, $P<0.05$; Table 4b).

Discussion

In this study, we analysed leaves from 85 *Quercus* species from across the Northern Hemisphere but living in a common garden. Our results showed that the evolution of leaf traits follows the OU2 model, which clearly segregates oaks in two main functional groups, deciduous and evergreen. Irrespective of the phylogeny or the species' natural habitats, these two groups differed in anatomical leaf attributes such as LMA, LT or leaf area (Table 2), as previously reported by other studies (Corcuera *et al.*, 2002; Escudero *et al.*, 2017). By contrast, they did not differ in LD despite their significant differences in leaf traits related to density, such as cellulose concentration, IVD and bundle sheet extension width (Table 2). This fact could be due to the thicker spongy mesophyll of evergreens that may promote a larger amount of intercellular airspaces, counteracting the effect of these other leaf traits on LD (Escudero *et al.*, 2017).

Taking into account the segregation given by the OU2 model, we evaluated variation in LMA related to changes in LT or LD within each leaf habit according to two scenarios (Fig. 1) linked to the leaf economic spectrum theory (Wright *et al.*, 2004).

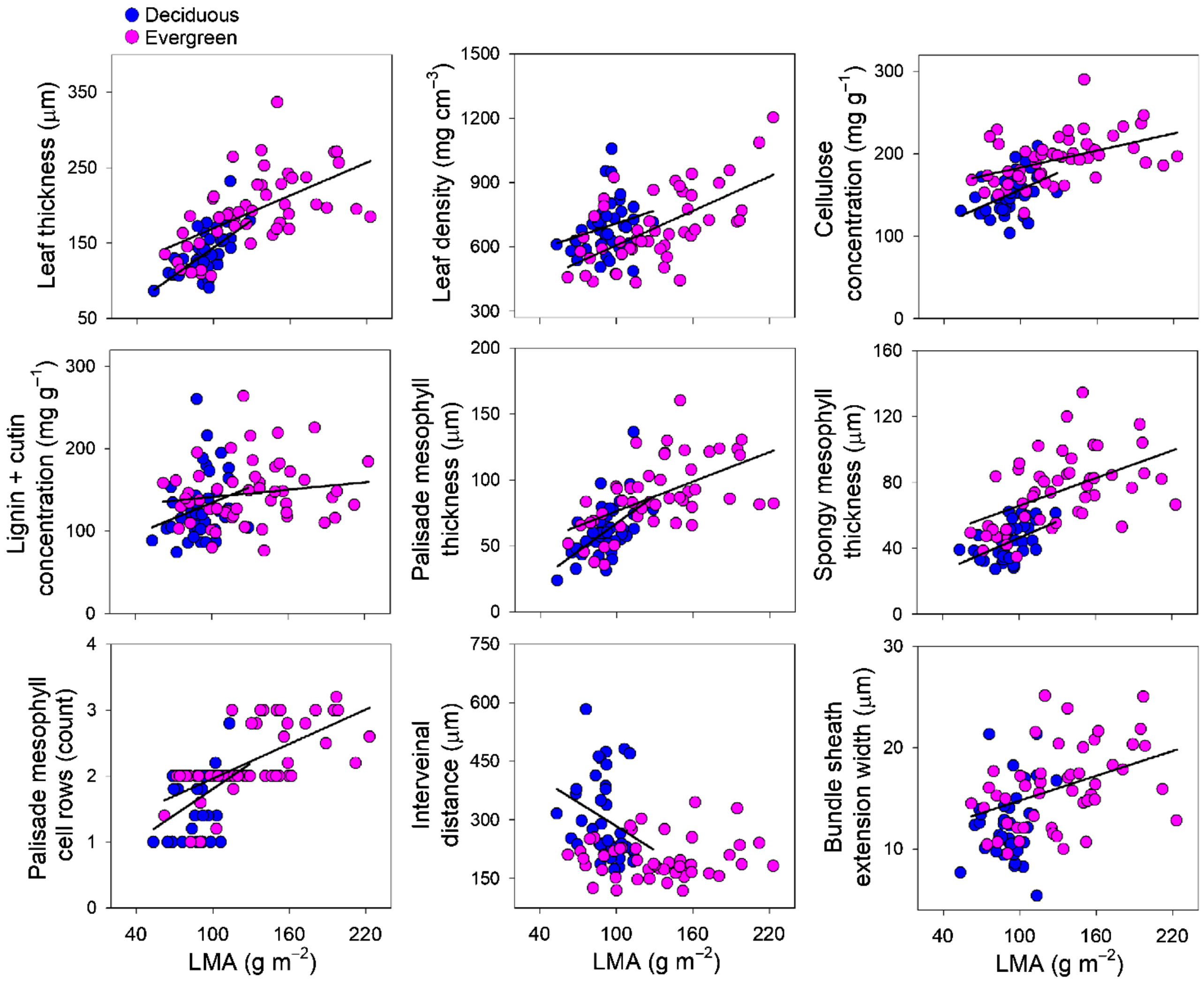

Fig. 4 Relationships between leaf mass per area (LMA) and key leaf traits for deciduous (blue) and evergreen (pink) *Quercus* species. Regression coefficients for the phylogenetic independent contrasts (PICS) are shown in Table 3. Each circle is the mean value of one particular species.

Scenario 1: LMA associated with photosynthetically competent tissues to compensate a shorter favourable season

As leaves are essentially organs utilised for photosynthesis (Turner, 1994), Scenario 1 contemplated that an increase in LMA would be achieved by thicker leaves in order to increase the instantaneous carbon assimilation rates per unit area to compensate for shorter favourable seasons in stressful climatic environments (Fig. 1). When this scenario was analysed within evergreen oak species, our results effectively showed that evergreen oaks living in environments with lower periods of full plant growth (i.e. longer cold periods) and/or with higher aridity period, increased LMA by increasing LT (Table 4; Fig. 5). This suggested that evergreen oaks inhabiting higher altitudes (e.g. the subtropicals *Q. rehderiana* and *Q. rugosa*), with longer dry periods (e.g. the subtropical *Q. obtusata*) or at higher latitudes under more continental climatic conditions and longer dry summers (e.g. the Mediterranean oaks *Q. chrysolepis* and *Q. ilex* ssp. *rotundifolia*), tended to exhibit a higher LMA due to a higher LT than those evergreen oaks inhabiting lower altitudes, lower latitudes and with a minimal hot dry season (e.g. the subtropicals *Q. sagraeana, Q. seemannii* or *Q. insignis*; Fig. S2). By contrast, when analysing Scenario 1 within deciduous species, our results showed that the deciduous oaks with higher LMA due to a higher LT inhabited environments with shorter cold periods (which was opposite to our prediction in Scenario 1) but with longer and more intense aridity periods. This opposite pattern related to the length of the growing season found for deciduous species compared with the evergreens seemed to be consistent with global patterns (Wright *et al.*, 2005) and prediction based on a cost–benefit model (Kikuzawa *et al.*, 2013). However, for deciduous species, a longer aridity period may counteract the effect of a shorter cold period by shortening the favourable season (Peguero-Pina *et al.*, 2016a).

Table 4 Correlation coefficients between leaf traits and climatic variables for (a) deciduous (DEC) and (b) evergreen (EVE) *Quercus* species using phylogenetically independent contrasts (PICS).

(a) DEC	LMA	LT	LD	LA	(b) EVE	LMA	LT	LD	LA
GS	0.376*	0.427**			GS				
FPG		0.341*			FPG	−0.350*	−0.432**		
AP		0.521***	−0.465**		AP	0.581***	0.381*	0.327*	−0.420**
T		0.370*			T	−0.598***	−0.638***		
TMIN		0.325*		−0.393*	TMIN	−0.471**	−0.611***		0.645***
TDRY	0.345*	0.502**		−0.348*	TDRY			0.494***	
P		−0.483**	0.523***		P	−0.495***	−0.663***		0.742***
AI		0.445**	−0.537***		AI	0.401**		0.316*	−0.344*

Leaf traits and climatic variables notation as in Table 1. Significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

This counteracted effect, together with the positive association between aridity intensity and LT, pointed to aridity stress as the main driver increasing LT within deciduous species. In this sense, a higher LT not only would increase the instantaneous carbon assimilation rates per unit area when water is more readily available, but would also improve water use efficiency under drought conditions (Wright *et al.*, 2001; Gil-Pelegrín *et al.*, 2017). Examples of such deciduous species inhabiting arid environments could be the subtropical *Q. mexicana* or the European and North American Mediterranean-type oaks *Q. ithaburensis* and *Q. garryana* (Fig. S2).

The increase in LT that promoted an increase in LMA was given by increasing the mesophyll thickness mainly through increasing the number of cell layers, both within deciduous and evergreen (Table 3; Fig 4), which has been previously observed across diverse species (de la Riva *et al.*, 2016; John *et al.*, 2017). This increase in mesophyll thickness has the potential to increase photosynthetic rates per unit leaf area (A_{area}) (Niinemets *et al.*, 1999). However, this assumption should be made carefully as there are other leaf traits that could modify A_{area} (Peguero-Pina *et al.*, 2017b). For example, increases in cell wall thickness that promote higher cellulose concentration (Terashima *et al.*, 2006) may result in a reduction in A_{area} (Peguero-Pina *et al.*, 2017a,c). Therefore, the positive correlation found in this study between palisade mesophyll thickness and cellulose concentration, both in deciduous ($R = 0.333$, $P < 0.05$) and evergreen ($R = 0.607$, $P < 0.001$; data not shown) species, could counteract the presumed positive effect of a higher mesophyll thickness on A_{area}. Additionally, the presumed increase in A_{area} could also be negated by an increase in nonphotosynthetic tissues. Our results showed that a higher LT is coordinated with a higher BSEW in both leaf habits, and reduces the amount of photosynthetic tissues (Table 3; Fig. 5). This relationship suggests that thicker leaves might have higher mesophyll structural reinforcement, which may prevent thickness shrinkage during dehydration processes (Sancho-Knapik *et al.*, 2011). In these two cases (a higher cell wall thickness and thicker bundle sheath extensions), LT and density could be considered coupled rather than being a trade-off relationship (Onoda *et al.*, 2017). Finally, the assumed higher A_{area} of oak species with higher LT may also be diminished at the leaf level by the reduction of leaf area (Tables 3, 4). Given that reduction in leaf size is a key modification that allows plants to withstand water deficit (Baldocchi & Xu, 2007), oaks inhabiting arid environments could increase LT to increase A_{area} in order to compensate for their smaller leaf area (Peguero-Pina *et al.*, 2016a).

Scenario 2: LMA as a protective mechanism against severe climatic factors

Protection will require further investment that would promote tougher, harder and stiffer leaves that might increase the leaf lifespan of a particular species (Turner, 1994; Read & Sanson, 2003). Therefore, our Scenario 2 considered that an increase in LMA would be associated with denser leaves, in order to increase their resistance under stressed climatic conditions, and therefore to increase their lifespan (Fig. 1). When analysing Scenario 2 within evergreen oaks, our results showed that those species living in environments with higher aridity intensity and higher temperature of the driest quarter (TDRY), increased LMA by increasing LD (Table 4b; Fig. 5). As TDRY can mostly occur during winter or during summer (Fig. S1), it seems that those evergreens with denser leaves are those inhabiting environments with drier summers (e.g. the Mediterranean *Q. calliprinos* and *Q. coccifera*, Fig. S2). By contrast with evergreens and contrary to our Scenario 2, an increase in aridity intensity within deciduous oaks was directly associated with an increase in LMA through increases in LT, as mention above, but with decreases in LD (Table 4a). This result suggested that, within deciduous oaks, higher aridity promotes thicker and less dense leaves (e.g. in the Mediterranean-type *Q. garryana* or *Q. lusitanica*), which may improve A_{area} by increasing CO_2 mesophyll conductance (Niinemets *et al.*, 2009).

The positive relationship found between LMA and LD within each leaf habit, as reported before by other authors (Groom & Lamont, 1997; Poorter *et al.*, 2009; Read *et al.*, 2016), was negatively influenced by the increase in spongy mesophyll thickness, both in evergreen and deciduous oaks. In other words, a higher spongy thickness might promote a larger volume of intercellular air spaces that could decrease the overall LD and, therefore, weaken other relationships. However, we found that a higher LD was associated with a reduction in cell size within evergreen species, and with a lower IVD (i.e. higher vein density) through a decrease in leaf area within deciduous species, anatomical

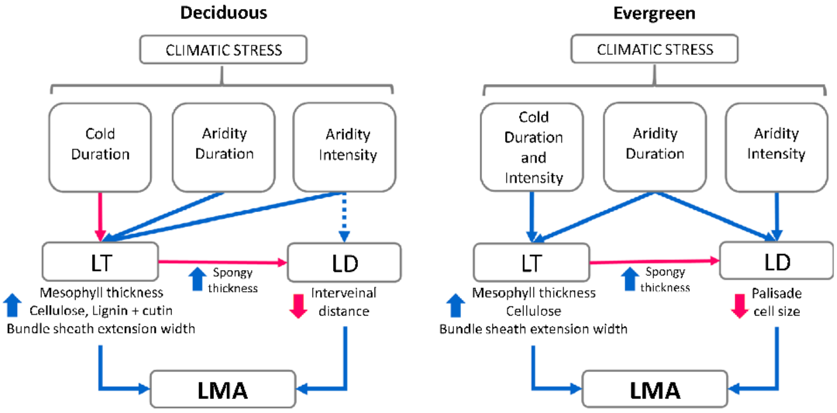

Fig. 5 Schematic representation of the main associations found for anatomical (Table 3) and climatic variables (Table 4) for deciduous and evergreen *Quercus* species. A blue arrow indicates a positive association, while a pink arrow indicates a negative association between two traits. The dashed line indicates an indirect association. LD, leaf density; LMA, leaf mass per area; LT, leaf thickness.

modifications that have been reported as protective adaptations to withstand water stress (Oertli *et al.*, 1990; Scoffoni *et al.*, 2011; Ding *et al.*, 2014; Nardini *et al.*, 2014). Furthermore, we can also highlight other protection mechanisms within evergreen oaks found in this study related to LT. As abundant intercellular air spaces provide room to accommodate extracellular ice (Wyka & Oleksyn, 2014), the higher LT due to a higher spongy mesophyll thickness found in evergreen species inhabiting colder environments (Tables 3, 4; Fig. 5), may protect evergreen species against frost damage. Additionally, as low winter temperatures may cause damage by absorption of excess light under limiting conditions for photosynthesis, being thicker may also reduce the probability for photodamage (Peguero-Pina *et al.*, 2009). To our knowledge, there has been no previous report of cold intensity as a determinant factor of interspecific variation in LMA, although Wright *et al.* (2005) observed similar effects of mean annual temperature in evergreen species. However, other authors observed intraspecific trends in the LMA of evergreen species also related to gradients in winter temperatures (Ogaya & Peñuelas, 2007; Mediavilla *et al.*, 2012; González-Zurdo *et al.*, 2016; Niinemets, 2016). Moreover, minimum temperature of the coldest month in our study was also positively related to the length of the growing season ($R = 0.8$, $P < 0.001$; Fig. S5), suggesting that environments with colder winters are also sites with shorter growing seasons, and therefore sites with longer winters (van Ommen Kloeke *et al.*, 2012). Taking into account that our results showed that an increase in LMA may constitute an adaptation against both cold duration and cold intensity in evergreen oaks (Fig. 5), further research is needed to partition these two effects and pinpoint whether a higher LMA is a direct response to one and/or the other.

Diversity in evolutionary trends

Our results showed that across most of the oaks included in this study, LMA evolved in greater coordination with LT than with LD (Fig. 3). This trend was found in deciduous oaks and in the evergreen species from the subgenus *Quercus*, mainly belonging to the *Lobatae* and *Quercus* sections (Denk *et al*, 2017). By contrast, in the evergreen oaks from the subgenus *Cerris* – mainly belonging to sections *Ilex* and *Cyclobalanopsis* – LMA converged more with LD (Fig. 3). This discrepancy within evergreen oaks, could be due to the ancestral origin of each group. While the ancestors of sections *Lobatae* and *Quercus* are inferred to have occurred at higher latitudes (Hipp *et al.*, 2018; Cavender-Bares, 2019), the ancestors of *Ilex* and *Cyclobalanopsis* may have inhabited lower latitudes (Barrón *et al.*, 2017; Jiang *et al.*, 2019). Consequently, ancestors of *Lobatae* and *Quercus* could have increased LMA by increasing LT in order to achieve higher carbon assimilation rates to compensate for a shorter favourable season typical of higher latitude environments where daylength varies considerably from summer to winter solstice. By contrast, ancestors of *Ilex* and *Cyclobalanopsis* could have increased LMA by increasing LD in order to protect their leaves against stress factors (e.g. mechanical damage) and, therefore, to increase their leaf lifespan under environments with longer growing seasons, typical of lower latitudes. The possibility that different oaks ancestors could depend primarily on the length of the growing season could help to explain why the thermal climatic component– which also includes the growing-season length variables – was slightly more important in explaining variation in LMA than the arid component. Further research is needed to fully test these new hypotheses. However, our results clearly showed that LMA was tightly coordinated with climate, independently of leaf habit or the climatic component considered. This reinforces climate as an important driver of leaf functioning in oaks; this was recently pointed out by Ramírez-Valiente *et al.* (2020) and supported by work in oaks across altitudinal gradients (Fallon & Cavender-Bares, 2018).

Finally, our data also revealed that within *Lobatae* oaks, LMA changed according to the latitude of the species distribution (Fig. S6). All *Lobatae* oaks inhabiting higher latitudes (*c.* 40°) are deciduous, and tend to have lower LMA values than *Lobate* oaks found at middle latitudes (*c.* 25°). This variation within deciduous *Lobatae* oaks is tightly coordinated with changes in the length of the growing season and temperature. At middle latitudes (*c.* 25°), there is a shift in leaf habit in our set of species, from deciduous to evergreen, all having higher LMA than those living at lower latitudes (*c.* 10–15°). According to our data, the variation in LMA within evergreen *Lobatae* oaks is associated with changes in temperature and precipitation of species climatic niches (Fig. S6). The association of LMA with latitude within *Lobatae* oaks could be a consequence of the radiation from a high-latitude ancestor (Hipp *et al.*, 2018) towards lower latitudes across North and Central America (Cavender-Bares, 2019). However, these outcomes within *Lobatae* oaks should be considered carefully, as not all *Lobatae* oaks were included in our study. Further research is needed to confirm this result and to elucidate the main drivers of leaf trait evolution within the others infrageneric groups.

Acknowledgements

Financial support from Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) grant no. RTA2015-00054-C02-01, from Ministerio de Ciencia e Innovación grant number PID2019-106701RR-I00 and from Gobierno de Aragón H09_20R research group. The work of DAF was supported by a FPI-INIA contract BES-2017-081208. The work of CS was supported by a National Science Foundation (NSF)-CAREER grant no. 231715. We thank Elena Martí Beltrán for her valuable help searching the species distribution and obtaining the climatic data. We thank Ruth Cuevas Calvo for helping in the anatomy cross-section process. We thank Francisco Garin García for his meaningful support in the recollection of oak leaves and identification of the species leaf habit. We also thank Jardín Botánico de Iturrarán for allowing us to collect oak leaf samples from their garden. Finally, we would like to thank the anonymous reviewers for their careful reading of our manuscript and their insightful and valuable suggestions.

Author contributions

DS-K, TGA-A, JJP-P and EG-P planned and designed the research. DS-K, DA-F and JJP-P performed the measurements. DS-K, AE, SM, JZ and AM generated phylogenetic data. DS-K, AE, SM, CS, JZ, JC-B, DA-F, JPF, JJP-P and EG-P analysed climatic and phylogenetic data. DS-K, AE and CS drafted the manuscript. All authors edited the manuscript with valuable inputs.

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Data availability

Data that support the findings of this study are openly available in TRY at http://doi.org/10.17871/TRY.64.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Season location of the driest quarter of the year.

Fig. S2 Principal components analysis of the climatic variables.

Fig. S3 Oak phylogenetic trees showing the evolution of leaf mass per area, leaf thickness and leaf density.

Fig. S4 Divergence of the climatic principal components from leaf thickness and density.

Fig. S5 Relationship between minimum temperature and length of the favourable season.

Fig. S6 Relationships within section *Lobatae* between leaf mas per area, latitude and climatic variables.

Table S1 Leaf habit and leaf traits mean values of the study's species.

Table S2 Climatic variables mean values of the species' climatic origins.

Table S3 *Quercus* GenBank accession numbers for rbcL gen.

Table S4 Model fitting using Brownian motion and the Ornstein–Uhlenbeck models.

New Phytologist Supporting Information

Article title: **Deciduous and evergreen oaks show contrasting adaptive responses in leaf mass per area across environments**

Authors: Domingo Sancho-Knapik, Alfonso Escudero, Sonia Mediavilla, Christine Scoffoni, Joseph Zailaa, Jeannine Cavender-Bares, Tomas Gómez Ávarez-Arenas, Arántzazu Molins, David Alonso-Forn, Juan Pedro Ferrio, José Javier Peguero-Pina, Eustaquio Gil-Pelegrín.

Article acceptance date: 11 December 2020

The following Supporting Information is available for this article:

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Fig. S1 Season location of the driest quarter of the year for deciduous (DEC) and evergreen (EVE) *Quercus* species considered in this study. Data are mean ± SE.

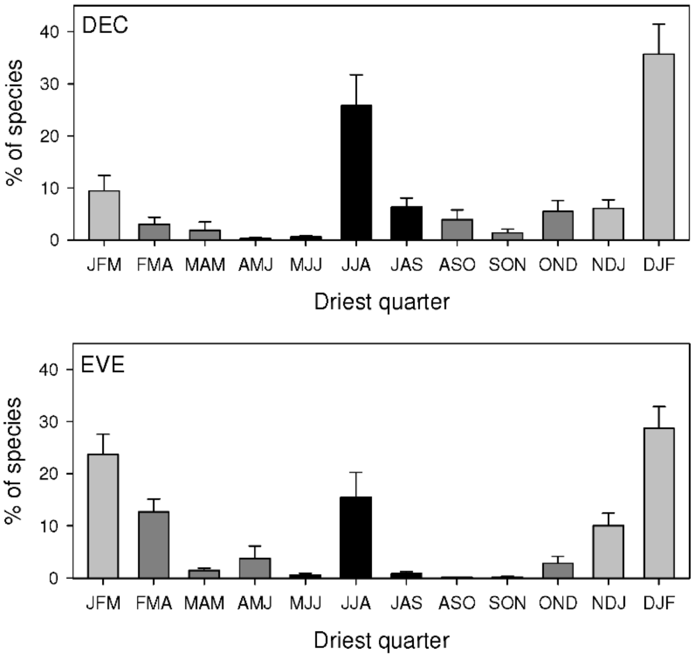


Fig. S2 Principal components analysis of the climatic variables taking into account all *Quercus* species. Climatic notation as in Table 1b. Roman numerals indicate an estimated position within the climatic variables of several main Zonobiomes (Zb) (Breckle, 2002). II, Zb of the Humido-arid Tropical Summer Rain Region; III, Zb of Subtropical Arid Climates; IV, Zb of the Arido-Humid Winter Rain (Mediterranean) Region; V, Zb of the Warm-Temperate Humid Climate; VI, Zb of the Temperate Nemoral Climate; VII, Zb of the Arid-Temperature Climate.

Breckle SW. 2002. *Walter's vegetation of the Earth. The ecological systems of the Geo-Biosphere. 4th-edition*. Berlin, DE: Springer

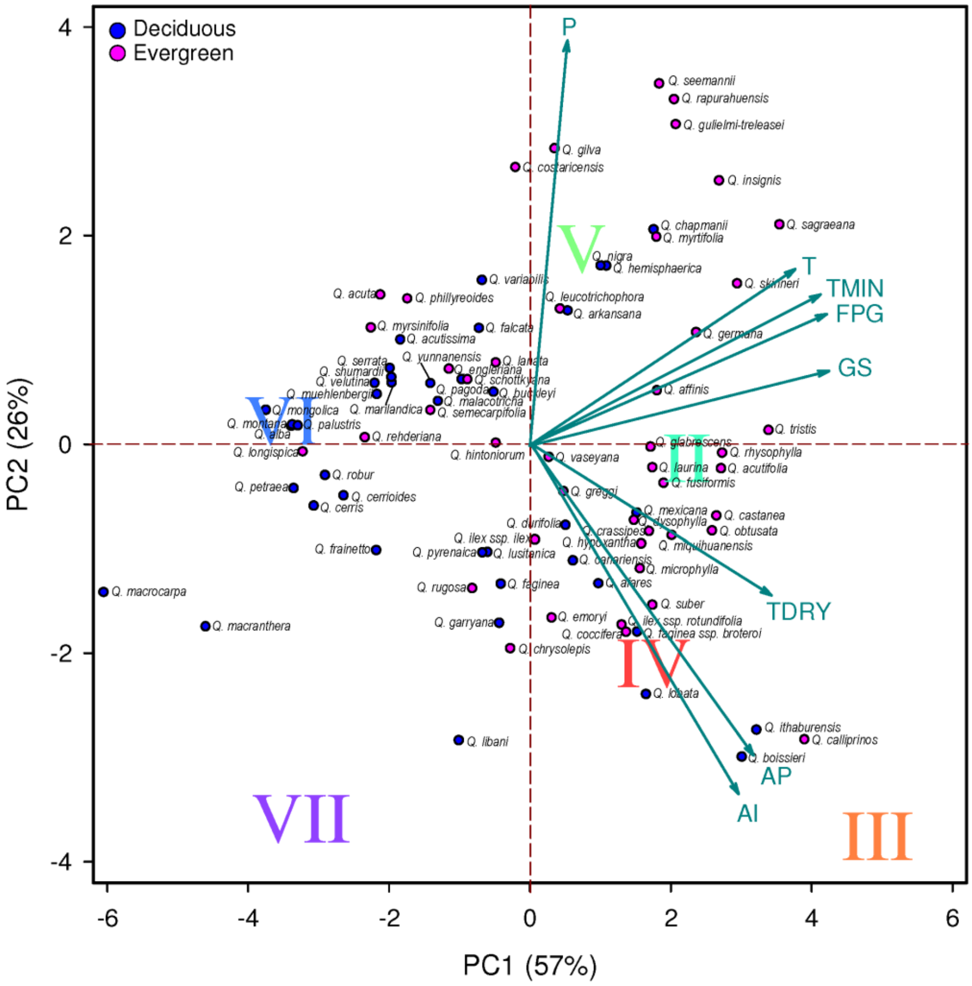
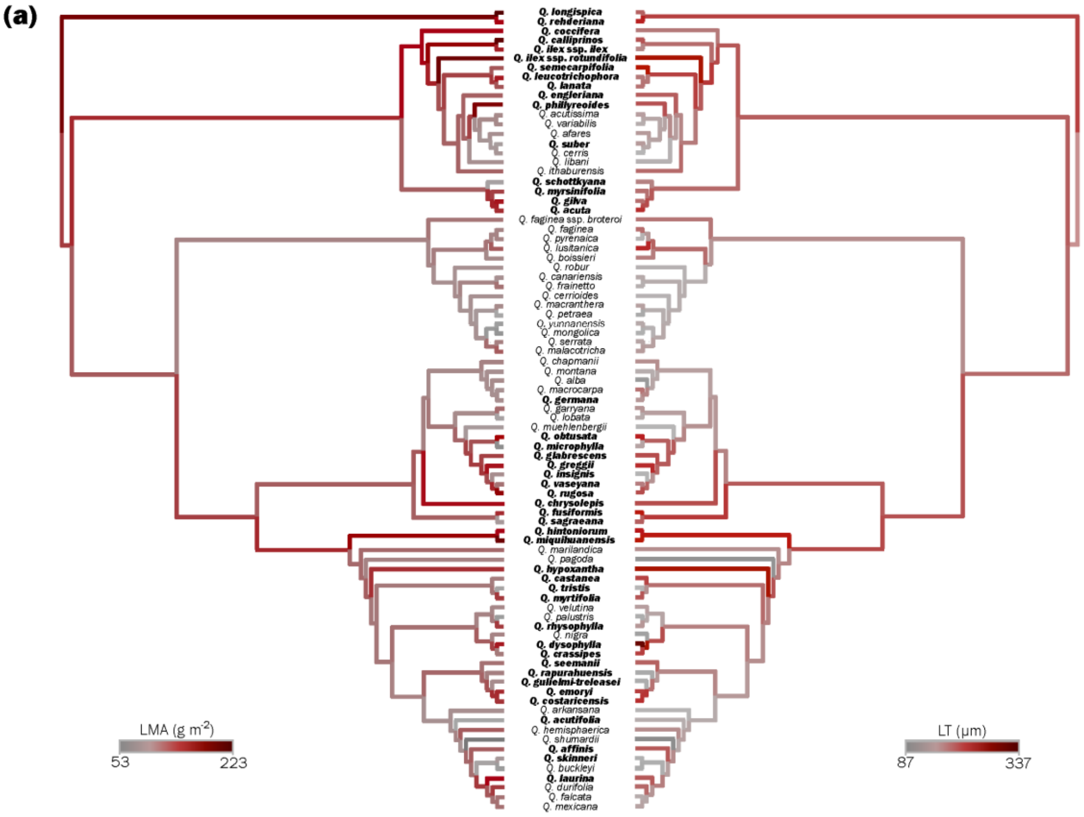


Fig. S3 Oak phylogenetic trees showing the evolution of (a, b) leaf mass per area (LMA), (a) leaf thickness (LT) and (b) leaf density (LD) across the study's species. Bolded and non-bolded species names indicate evergreen and deciduous species respectively.



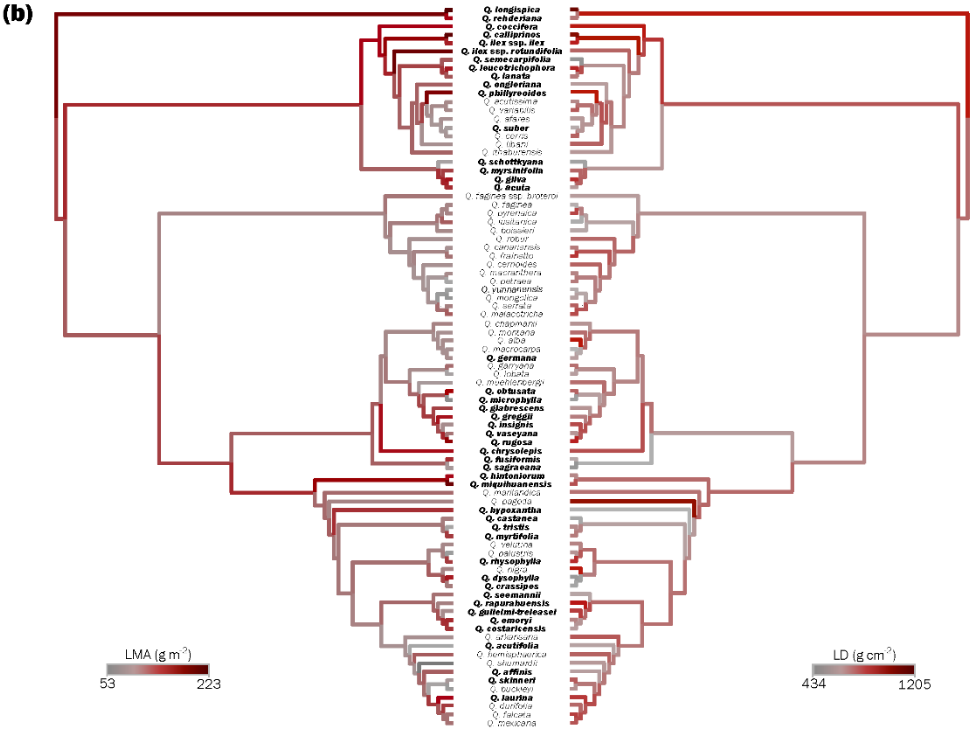

(b)
Q. longispica
Q. rehderiana
Q. coccifera
Q. calliprinos
Q. ilex ssp. ilex
Q. ilex ssp. rotundifolia
Q. semecarpifolia
Q. leucotrichophora
Q. lanata
Q. engleriana
Q. phillyreoides
Q. acutissima
Q. variabilis
Q. afares
Q. suber
Q. cerris
Q. libani
Q. ithaburensis
Q. schottkyana
Q. myrsinifolia
Q. gilva
Q. acuta
Q. faginea ssp. broteroi
Q. faginea
Q. pyrenaica
Q. lusitanica
Q. boissieri
Q. robur
Q. canariensis
Q. frainetto
Q. cerrioides
Q. macranthera
Q. petraea
Q. yunnanensis
Q. mongolica
Q. serrata
Q. malacotricha
Q. chapmanii
Q. montana
Q. alba
Q. macrocarpa
Q. germana
Q. garryana
Q. lobata
Q. muehlenbergii
Q. obtusata
Q. microphylla
Q. glabrescens
Q. greggii
Q. insignis
Q. vaseyana
Q. rugosa
Q. chrysolepis
Q. fusiformis
Q. sagraeana
Q. hintoniorum
Q. miquihuanensis
Q. marilandica
Q. pagoda
Q. hypoxantha
Q. castanea
Q. tristis
Q. myrtifolia
Q. velutina
Q. palustris
Q. rhysophylla
Q. nigra
Q. dysophylla
Q. crassipes
Q. seemannii
Q. rapurahuensis
Q. gulielmi-treleasei
Q. emoryi
Q. costaricensis
Q. arkansana
Q. acutifolia
Q. hemisphaerica
Q. shumardii
Q. affinis
Q. skinneri
Q. buckleyi
Q. laurina
Q. durifolia
Q. falcata
Q. mexicana
LMA (g m⁻²)
53
223
LD (g cm⁻³)
434
1205

Fig. S4 (a) Divergence of the two first climatic principal components (PC1, PC2) from leaf thickness (LT) and leaf density (LD). Bolded and non-bolded *Quercus* species names indicate evergreen (EVE) and deciduous (DEC) species respectively. (b, c) Percentage of *Quercus* species showing a lower divergence (i.e. higher coordination) between LT (b), LD (c) and the principal components (PC1 in grey and PC2 in orange) for both leaf habits and both subgenus.

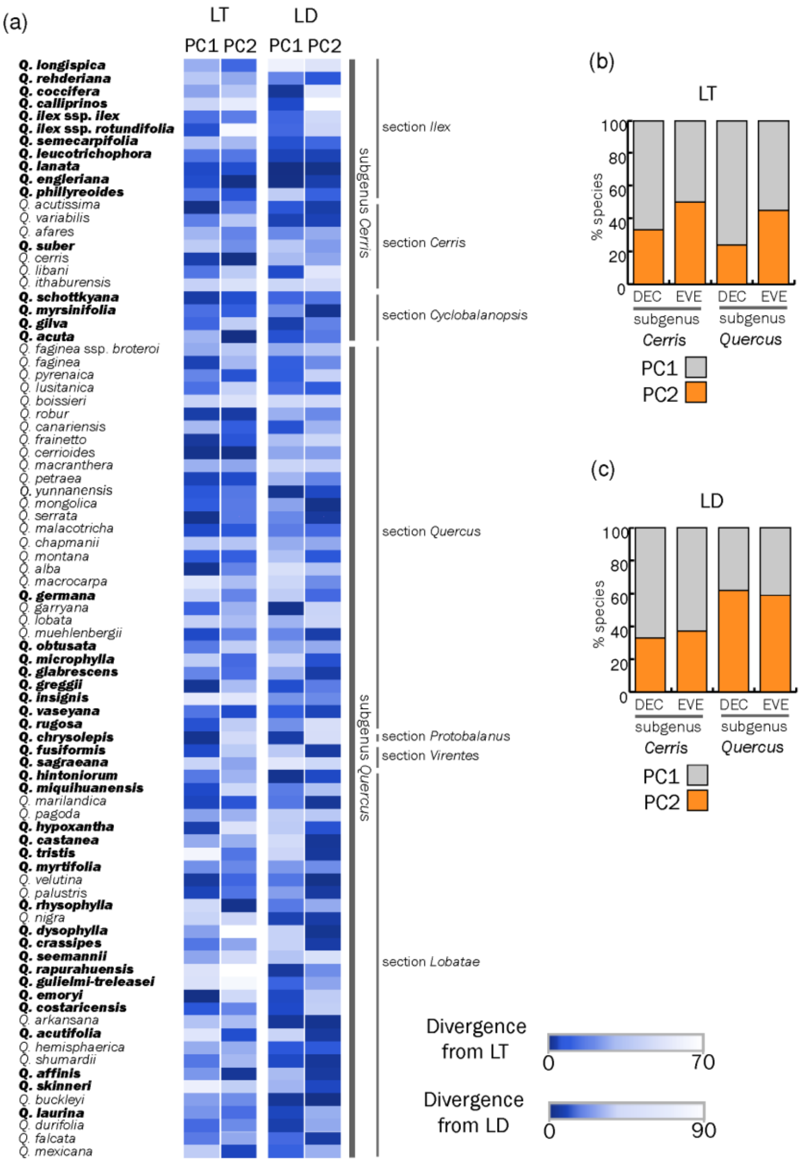


Fig. S5 Relationships between the minimum temperature of coldest month (TMIN) and the length (number of months) of the growing season (GS) and the period of full plant growth (FPG) for deciduous (DEC) and evergreen (EVE) *Quercus* species. Species with TMIN > 10ºC were not considered for the correlations. P<0.001.

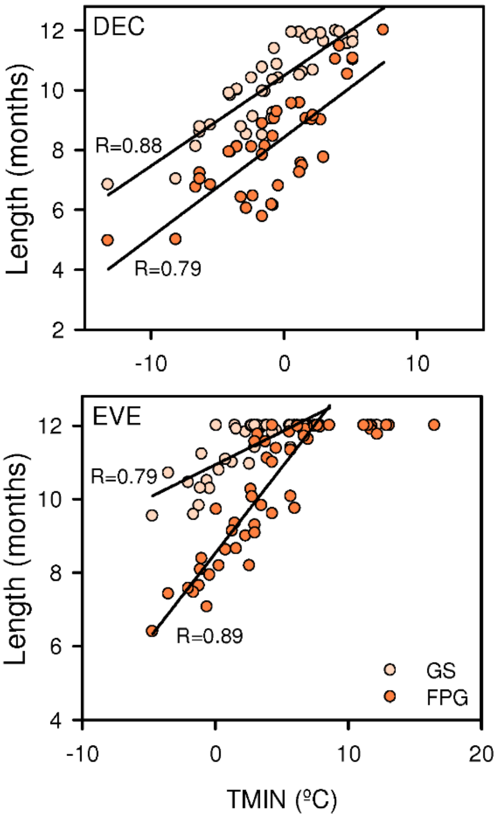


Fig. S6 Relationships within *Quercus* section *Lobatae* between leaf mas per area (LMA) and latitude, growing season (GS), minimum temperature of coldest month (TMIN), mean temperature of driest quarter (TDRY) and annual precipitation (P). DEC, deciduous; EVE, evergreen. Latitude was obtained from herbarium data. Each circle is the mean value of one particular species. Climatic variables not included in this figure showed non-significant correlations with LMA within *Quercus* section *Lobatae* for both leaf habits.

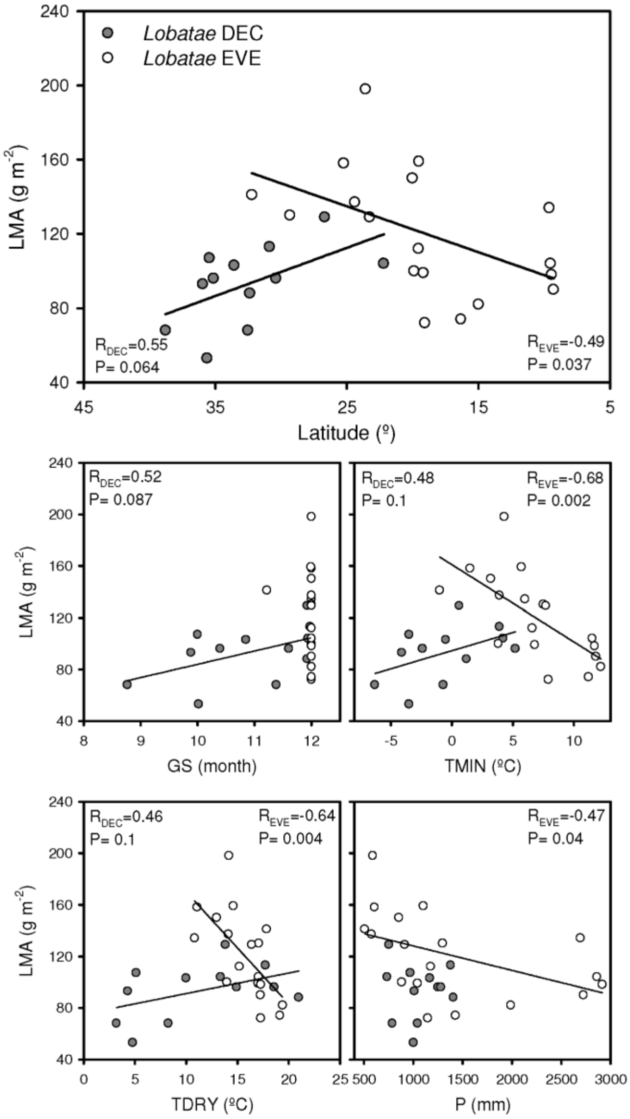


Table S1 Leaf habit and leaf traits mean values (± SE) of the study's oak species growing in a common garden in northern Spain (separate XLSX file). These data are openly available in TRY at http://doi.org/10.17871/TRY.64

Table S2 Climatic variables mean values (±se) of the oak species' climatic origins (separate XLSX file).

Table S3 *Quercus* GenBank accession numbers for rbcL gen. All sequences were obtained from Hermida-Carrera *et al*. (2017) except those in bold, that were newly analyzed for this study following the same procedure described in Hermida-Carrera *et al*. (2017), and that underlined that was directly obtained from the GenBank.

Hermida-Carrera C, Mario A, Fares MA, Fernández A, Gil-Pelegrín E, Kapralov MV, Mir A, Molins A, Peguero-Pina JJ, Rocha J *et al.* 2017. Positively selected amino acid replacements within the RuBisCO enzyme of oak trees are associated with ecological adaptations. *PLoS ONE* **12**: e0188984

Species	Accession number	Species	Accession number
Q. acuta	MF044967	*Q. leucotrichophora*	MF044850
Q. acutifolia	MF044890	*Q. libani*	MF044946
Q. acutissima	MF044941	*Q. lobata*	MF044851
Q. afares	MF044942	*Q. longispica*	MF044957
Q. affinis	MF044891	*Q. lusitanica*	MF044852
Q. alba	MF044819	*Q. macranthera*	MF044853
Q. arkansana	MF044893	*Q. macrocarpa*	MF044854
Q. boissieri	MF044825	*Q. malacotricha*	MF044855
Q. buckleyi	MF044895	*Q. marilandica*	MF044920
Q. calliprinos	MF044953	*Q. mexicana*	MF044921
Q. canariensis	MF044827	*Q. microphylla*	MF044859
Q. castanea	MF044898	*Q. miquihuanensis*	**MT646902**
Q. cerrioides	MF044828	*Q. mongolica*	MF044861
Q. cerris	MF044943	*Q. montana*	MF044862
Q. chapmanii	MF044829	*Q. muehlenbergii*	MF044863
Q. chrysolepis	MF044964	*Q. myrsinifolia*	MF044972
Q. coccifera	MF044953	*Q. myrtifolia*	MF044922
Q. costaricensis	MF044900	*Q. nigra*	MF044923
Q. crassipes	MF044902	*Q. obtusata*	MF044865
Q. durifolia	MF044907	*Q. pagoda*	MF044924

Q. dysophylla	MF044908
Q. emoryi	MF044909
Q. engleriana	MF044833
Q. faginea	MF044835
Q. faginea ssp. broteroi	MF044826
Q. falcata	MF044911
Q. frainetto	MF044836
Q. fusiformis	LM652999
Q. garryana	MF044838
Q. germana	MF044840
Q. gilva	MF044969
Q. glabrescens	MF044841
Q. greggii	MF044842
Q. gulielmi-treleasei	MF044913
Q. hemisphaerica	MF044914
Q. hintoniorum	**MT646901**
Q. hypoxantha	**MT646903**
Q. ilex ssp. ilex	MF044956
Q. ilex ssp. rotundifolia	MF044962
Q. insignis	MF044847
Q. ithaburensis	MF044945
Q. lanata	MF044848
Q. laurina	MF044919
Q. palustris	MF044925
Q. petraea	MF044871
Q. phillyreoides	MF044959
Q. pyrenaica	MF044875
Q. rapurahuensis	MF044927
Q. rehderiana	MF044960
Q. rhysophylla	MF044928
Q. robur	MF044876
Q. rugosa	MF044877
Q. sagraeana	MF044830
Q. schottkyana	MF044974
Q. seemannii	MF044932
Q. semecarpifolia	MF044963
Q. serrata	MF044879
Q. shumardii	MF044933
Q. skinneri	MF044934
Q. suber	MF044949
Q. tristis	MF044936
Q. variabilis	MF044951
Q. vaseyana	MF044883
Q. velutina	MF044938
Q. yunnanensis	MF044887
Fagus crenata	MF044982

Table S4 Model fitting for *Quercus* species using the Brownian Motion (BM) and the Ornstein-Uhlenbeck models either with a single global optimum (OU1) or with two phenologically driven optimas (OU2). AICc: Akaike Information Criterion corrected. Traits notation as in Table 1.

	AICc				OU Parameters				
Trait	BM.AICc	OU1.AICc	OU2.AICc	Best Fit Model	OU1 alpha	OU1 optimum	OU2 alpha	OU2 optimum1	OU2 optimum2
LMA	1012	857	831	OU2	1203	112	1579	132	91
LT	1060	910	881	OU2	832	166	1428	194	135
LD	1221	1099	1104	OU1	467	692	402	698	692
LA	1072	807	794	OU2	58918	30	75610	19	41
HC	1099	862	863	OU1/OU2	16730	174	131789	169	181
CC	1079	847	812	OU2	49366	173	5814	194	152
LCC	1053	875	874	OU1/OU2	2247	140	2533	149	130
PMT	957	806	787	OU2	587	77	978	90	63
SMT	919	778	737	OU2	888	59	2144	73	43
PCR	298	149	129	OU2	575	2	710	2	2
PCL	761	613	617	OU1	609	39	1002	40	38
PCW	496	260	255	OU2	45087	8	90430	8	7
IVD	1154	1019	992	OU2	776	247	1316	196	308
BSEW	744	480	471	OU2	80246	14	51222	16	13
GS	425	285	261	OU2	614	11	992	11	10
FPG	547	368	342	OU2	1753	9	5772	10	8
AP	628	371	366	OU2	82032	2	19641	3	2
T	557	440	432	OU2	323	15	351	16	14
TMIN	739	527	504	OU2	56248	2	10790	4	-1
TDRY	720	567	568	OU1/OU2	1258	13	1662	14	12
P	1511	1320	1318	OU1/OU2	7128	1113	38548	1245	963
AI	1105	940	943	OU1	65825	49	94128	55	43

Capítulo IV

Disentangling leaf structural and material properties in relation to their anatomical and chemical compositional traits in oaks (*Quercus* L.)

Disentangling leaf structural and material properties in relation to their anatomical and chemical compositional traits in oaks (*Quercus* L.)

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Abstract

- *Background and Aims*: The existence of sclerophyllous plants has been considered an adaptive strategy against different environmental stresses. As it literally means "hard-leaved", it is essential to quantify the leaf mechanical properties to understand sclerophylly. However, the relative importance of each leaf trait on mechanical properties is not yet well established.
- *Methods*: Genus *Quercus* is an excellent system to shed light on this since it minimizes phylogenetic variation while having a wide variation in sclerophylly. Thus, leaf anatomical traits and cell wall composition were measured, analyzing their relationship with LMA and leaf mechanical properties in a set of 25 oak species.
- *Key results*: The upper epidermis outer wall had a strong and direct contribution to the leaf mechanical strength. Moreover, cellulose plays a critical role in increasing leaf strength and toughness. The PCA plot based on leaf trait values clearly separated *Quercus* species into two groups corresponding to evergreen and deciduous species.
- *Conclusions*: Sclerophyllous *Quercus* species are tougher and stronger due to their thicker epidermis outer wall and/or higher cellulose concentration. Furthermore, section *Ilex* species share common traits regardless of they occupy quite different climates. In addition, evergreen species living in Mediterranean-type climates share common leaf traits irrespective of their different phylogenetic origin.

Key words: Biomechanics, leaf anatomical traits, leaf mechanical resistance, leaf mass per area (LMA), oaks (*Quercus*), sclerophylly.

4.1. Introduction

Sclerophyllous leaves have been considered a universal adaptive strategy in response to different environmental stresses (Alonso-Forn *et al.*, 2020). Thus, stress factors such as water deficit (e.g., Schimper, 1903; Oertli *et al.*, 1990), nutrient shortage (Loveless, 1961, 1962; Beadle, 1966), low temperatures (Koppel and Heinsoo, 1994; Lamontagne *et al.*, 1998) and physical damage (Chabot and Hicks, 1982; Grubb, 1986) would have a synergistic effect that may explain the variation in sclerophylly. This trait is a characteristic recognized by botanists according to a physiognomic criterion. Thus, botanists have described sclerophyllous leaves as hard, tough, stiff and leathery (Schimper, 1903; Seddon, 1974; Turner, 1994). Given that the concept of sclerophylly is a perception, it is difficult to obtain an accurate measure or objective classification of sclerophylly (Edwards *et al.*, 2000). Moreover, as sclerophylly literally means "hard-leaved" it is essential to quantify the leaf mechanical properties to understand this trait.

Most ecophysiological studies use the leaf mass per unit area (LMA) as a quantitative proxy value for sclerophylly (e.g., Niinemets, 2001; Wright *et al.*, 2004). LMA is a combination of leaf thickness (LT) and leaf density (LD), which in turn depend on a great variety of anatomical and compositional leaf traits such as vein density, vein diameter, number of cell layers, cell size, air space fraction or fiber content (Mediavilla *et al.*, 2008; John *et al.*, 2017; Sancho-Knapik *et al.*, 2021). Sclerophylly has been well studied in the genus *Quercus*, and in this regard, Sancho-Knapik *et al*. (2021) recently found that LT influences the increase in LMA more than LD. While LMA provides a good proxy for leaf "hardness", it is not necessarily a measure of leaf mechanical resistance (Onoda *et al.*, 2011).

Direct approaches to quantify leaf sclerophylly require the measurement of leaf mechanical properties (Aranwela *et al.*, 1999; Read and Sanson, 2003; Onoda *et al.*, 2011). These include fracture-related properties, such as strength and toughness, which refer to the ability to resist an applied force and applied work, respectively (Cherrett, 1968; Williams, 1954; Coley, 1983; Choong *et al.*, 1992). A higher structural strength (i.e., maximum force to fracture), a higher structural toughness (i.e., work to fracture) and their thickness-normalized properties namely specific strength and specific toughness have been previously associated with a higher leaf sclerophylly (Edwards *et al.*, 2000; Wright and Cannon, 2001; Read and Sanson, 2003). Leaf mechanical properties can be analyzed in relation to their underlying components, which is analogous to the decomposition of LMA into LD and LT (Lucas *et al.*, 2000; Kitajima and Poorter, 2010; Lusk *et al.*, 2010). In the punch-and-die test, structural mechanical properties such as maximum force or work to fracture per unit fracture length (punch strength and punch toughness) can be decomposed into material properties (specific punch strength and specific punch toughness) and LT (Onoda *et al.*, 2008; Onoda *et al.*, 2011; Westbrook *et al.*, 2011). Material properties can be further analyzed in relation to tissue density or cell wall fiber content (percentage of hemicellulose, cellulose, and lignin per unit leaf dry mass or per unit volume).

The relative importance of each element of leaf tissue (e.g., cuticle, epidermis, palisade mesophyll, spongy mesophyll and vascular bundle extension) on the mechanical properties is not well established because their relative volume fraction have different influences on LMA and mechanical properties. Actually, several studies found that some anatomical variables strongly influenced leaf toughness through the reinforcement of certain structures with little effect on the amount of accumulated biomass per unit surface area (Edwards *et al.*, 2000; Westbrook *et al.*, 2011; Onoda *et al.*, 2012, 2015). Onoda *et al.* (2015) showed that some structural traits such as cuticle thickness have a significant impact on mechanical strength and toughness. The main objectives of the present study were to determine the leaf anatomical and compositional traits underlying a higher leaf strength and leaf toughness. The objective was achieved by measuring leaf anatomical traits and cell wall fibre composition and analyzing their relationship with both LMA and leaf mechanical properties.

The genus *Quercus* is an excellent system to perform this study, as it not only minimizes phylogenetic variation (compared to studies performed across diverse species), but also displays a wide variation in leaf sclerophylly across species (Gil-Pelegrín *et al.*, 2017). In particular, section *Ilex* species has been considered as pre-adapted to dry climates (He *et al.*, 2014). Although their ancestors occupied tropical and subtropical wet forests in the Himalaya-Hengduan mountains, some species exhibit xeromorphic-like traits (Het *et al.*, 2014; Jiang *et al.*, 2019). These traits would have allowed them to cope with hot-dry seasonal conditions that occur in Mediterranean-type climates (Martín-Sánchez et al. 2022).

The present study was performed on a set of 25 oak species (*Quercus* spp.) with different leaf habits (deciduous and evergreen) grown in a common garden in Northeastern Spain. We analysed 41 leaf structural, morphological, physicochemical and anatomical traits on these species (for species names see **Fig. 5)**. Specifically, we address the following hypotheses. (i) While structural mechanical properties are better correlated with leaf anatomical traits, leaf material properties are better correlated with cell wall composition. (ii) Sclerophyllous leaves are stronger and tougher due to their thicker epidermis outer wall and/or higher cellulose concentration.

4.2. Material and Methods

4.2.1. Plant Material

For this study, leaves were sampled from a living collection of 25 oak species, maintained in the experimental fields from CITA de Aragón (41°390N, 0°520W, 200 m a.s.l., Zaragoza, Spain). This common garden features Mediterranean climatic conditions with a mean annual temperature of 15.4 °C and total annual precipitation of 298 mm. Oak trees were *ca*. 20 years old; they were drop irrigated twice per week and pruned if it was necessary. Current year, fully developed, mature leaves were collected from south-exposed branches of one individual per species during the early morning. Leaves were stored in sealed plastic bags and carried to the laboratory in portable coolers. One set of 10 leaves was used for punch and die tests to measure the mechanical properties, leaf strength and toughness. A second set of nine leaves was aimed to phenolic compounds analysis. A third set of 10 leaves was utilized to obtain LMA. A fourth set of 10 leaves was used for leaf fibre content analysis, and a fifth set of 10 leaves was used to obtain the morphological and anatomical traits.

Table 1. List of leaf mechanical properties, anatomical and compositional traits with their abbreviations and units.

Abbr.	Parameter	Units	Abbr.	Parameter	Units
BSE_cp	Bundle sheath extension cover percentage	%	LT	Leaf thickness	μm
BSE_d	Bundle sheath extension density	mm mm^{-2}	N.LD	Nitrogen per unit volume	mg cm^{-3}
BSE_w	Bundle sheath extension width	μm	Ncw_Ntotal	Nitrogen in cell wall:Nitrogen	
C.LD	Carbon per unit volume	mg cm^{-3}	Ntotal	Nitrogen content	g 100^{-1} g^{-1}
CC	Cellulose content	mg g^{-1}	PM_cl	Palisade mesophyll cell length	μm
Ccw_Ctotal	Carbon in cell wall:Carbon		PM_cw	Palisade mesophyll cell width	μm
CD	Cellulose per unit volume	mg cm^{-3}	PM_nl	Palisade mesophyll number of layers	
Ctotal	Carbon content	g 100^{-1} g^{-1}	PMT	Palisade mesophyll thickness	μm
Ctotal_Ntotal	Ratio carbon:nitrogen		PS	Punch strength	kN m^{-1}
HC	Hemicellulose content	mg g^{-1}	SM_p	Spongy mesophyll porosity	%
HCD	Hemicellulose per unit volume	mg cm^{-3}	SMT	Spongy mesophyll thickness	μm
LCC	Lignin and cutin content	mg g^{-1}	SPS	Specific Punch Strength	MN m^{-2}
LCD	Lignin and cutin per unit volume	mg cm^{-3}	SWF	Specific Work to Fracture	kJ m^{-2}
LD	Leaf density	mg cm^{-3}	UE_latw	Upper epidermis lateral wall	μm
LE_latw	Lower epidermis lateral wall	μm	UE_lu	Upper epidermis lumen cell	μm^2
LE_lu	Lower epidermis lumen cell	μm^2	UE_lul	Upper epidermis lumen length	μm
LE_lul	Lower epidermis lumen length	μm	UE_luw	Upper epidermis lumen width	μm
LE_luw	Lower epidermis lumen width	μm	UE_ow	Upper epidermis outer wall	μm
LE_ow	Lower epidermis outer wall	μm	UET	Upper epidermis thickness	μm
LET	Lower epidermis thickness	μm	WF	Work of fracture	J m^{-1}
LMA	Leaf mass per unit area	g m^{-2}			

4.2.2. Mechanical properties: punch and die test

Punch and die tests consisted of punching a hole through the leaf lamina. As the punch contacts the leaf surface, the tip applies pressure until it overcomes the tensile strength of the leaf, causing a fracture. As a result, a leaf hole is produced and the compressive forces on the punch are released. A flat-ended and sharp-edged cylindrical punch made of steel of 2 mm diameter with a clearance of 0.05 mm was built and mounted onto the moving head of the mechanical tester Mach-1 V500C MA001 system (Biomomentum, Inc., Québec, Canada). Similar to Read and Sanson (2003) a die designed to fit the punch was located in the threaded base of the machine. A typical trial allowed the punch to penetrate the die to a depth of 2 mm at a speed of 30 mm min^{-1}. Data for all punch tests were collected at a rate of 100 Hz and were used to generate force-displacement curves (Fig. **S1a**). Before every set of measurements, a blank test was performed as a calibration in order to account for measuring the load forces due to the background friction caused by the proximity of the die walls to the punch tip. Leaf thickness (LT) was also measured with a micrometer (GT-H10L, Keyence, Osaka, Japan) attached to an amplifier unit (GT-75AP, Keyence, Osaka, Japan) just before each punch and die test.

To minimize variation in results due to differences between leaves of the same species, the leaf tissue tested was standardized: major veins were avoided and all trials were made halfway between the secondary veins delimiting the upper and lower borders of the intercostal panel. When the size of the species under study was big enough, four tests were conducted on each of the five leaves selected. Otherwise, two tests were performed and ten leaves were selected (Fig. **S1b**). All the mechanical tests were taken at room temperature with leaves fully hydrated.

The analysis of the curves allowed to obtain the following parameters: maximum force (F_{max}) defined as the highest load value, punch strength (PS) calculated as F_{max} divided by the circumference of the punch, and the leaf toughness or work of fracture (WF), calculated as the area below the curve between the initial contact of the punch with the leaf and F_{max} (Fig. **S1a**). The starting point of the curve was counted from 10% of F_{max}, to avoid the effect of the leaf's three-dimensional structure. Afterwards, the specific punch strength (SPS) and the specific work of fracture (SWF) were expressed per unit area of fracture (see all abbreviations and units in **Table 1**).

4.2.3. Leaf mass per area, compositional content, lignification and arid intensity

To calculate LMA, one disc (12.6 mm^2 in area) per leaf was obtained between the secondary veins of 10 leaves per species. Discs were oven-dried for 3 days at 70 °C and afterwards, they were weighed to obtain their dry mass. LMA was then calculated as the ratio between the dry mass and the disc area. Additionally, leaf density (LD) was calculated as the ratio between LMA and LT (Niinemets, 1999; Sancho-Knapik *et al.*, 2021).

For fibre content calculation, 10 fresh leaves were oven-dried for 3 days at 70 °C. Then, the petiole and mid-rib were removed. The rest of the plant material was ground and values of hemicellulose content (HC), cellulose content (CC) and lignin + cutin content (LCC) were obtained by quantifying neutral detergent fibre (NDF) and acid detergent lignin (ADL) following the method of Goering and Van Soest (1970). The amount of total foliar nitrogen and carbon (Ntotal and Ctotal, respectively) in dry leaves was measured using an organic element analyzer (Flash EA 112, Thermo Fisher Scientific Inc., Waltham, MA, USA). The cellular composition was obtained after the neutral detergent fibre (NDF) procedure according to the method of Goering and Van Soest (1970). The nitrogen content of the cell wall fraction was further estimated using the elemental analyzer described above.

To detect the presence of lignified anatomical structures, 15-20 µm cross-sections were cut with a microtome (HM 350 S, MICROM GmbH, Walldorf, Germany). Then, cross-sections were stained with a drop of phloroglucinol-HCl solution or Wiesner stain, prepared as a mixture of 2:1 of 3 % phloroglucinol in absolute ethanol and concentrated HCl (Pradhan and Loqué, 2014). Sections were observed under a light microscope (OPTIKA B-600TiFL, Optika Microscopes, Ponteranica, Italy) where lignified tissues appeared as fuchsia in colour. A few drops of Safranin and AstraBlue 0.1% double stain were added to confirm tissue lignification with a second method. After 30 seconds, the samples were rinsed with distilled water and observed under light and epifluorescence microscopy (OPTIKA B-600TiFL, Optika Microscopes, Ponteranica, Italy) using a green filter.

We selected a climatic variable to characterise aridity stress intensity: the arid intensity, defined as the sum of ($2t_\mathrm{m} - p_\mathrm{m}$) for months with $2t_\mathrm{m} > p_m$ and $t_\mathrm{m} >$ 10°C. Being t_m, the mean monthly temperature and p_m, the mean monthly precipitation. Data was obtained from Sancho-Knapik *et al.* (2019).

4.2.4. Anatomical traits

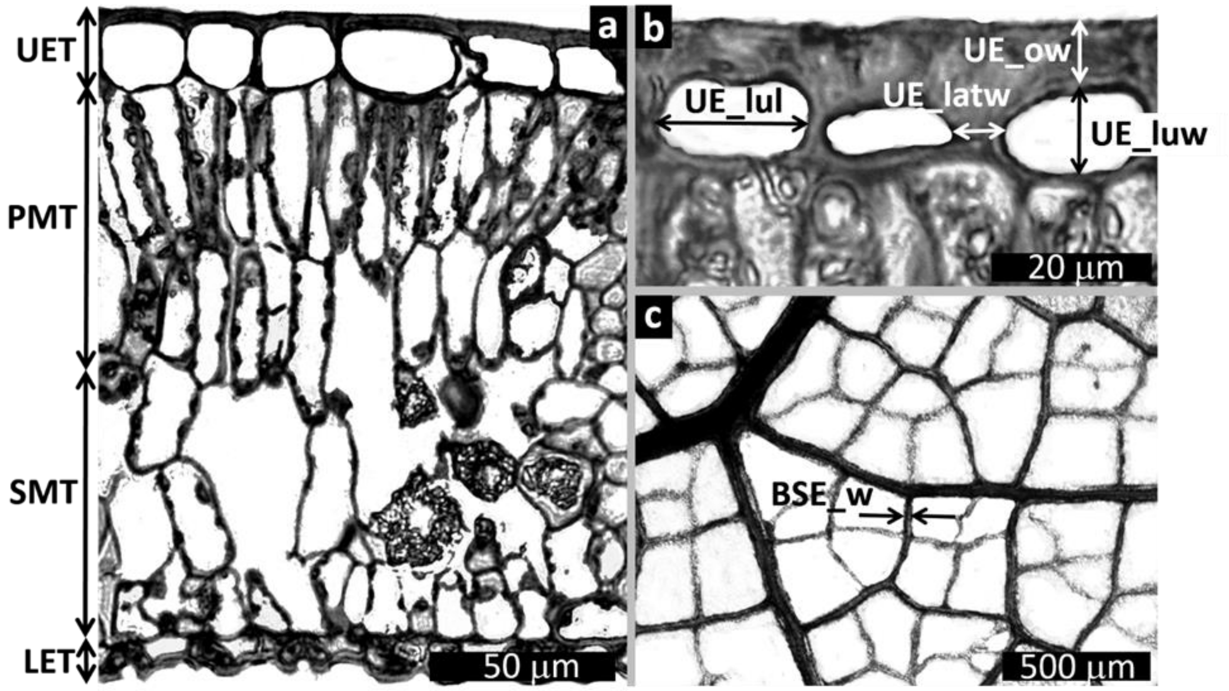

Fig. 1 Leaf mesophyll cross section of *Quercus myrsinifolia* (a), detail of the upper epidermis of *Q. chrysolepis* (b), and detail of leaf venation of *Q. shumardii* (c), showing diverse anatomical leaf traits measured in this study. Trait notation as in **Table 1**.

Vein morphological parameters were determined in a set of five mature leaves per species following the method described in Scoffoni *et al*. (2011) with some modifications. Leaf sections obtained between secondary veins were chemically cleared with 5% NaOH in an aqueous solution, washed with a bleach solution, dehydrated in an ethanol dilution series (70, 90, 95 and 100 %) and stained with safranin. Afterwards, one image (40x) per sample was taken using a camera (Canon EOS M100) coupled to a microscopy (OPTIKA B-600TiFL, Optika Microscopes, Ponteranica, Italy) and venation-related traits were measured in three fields per leaf using the ImageJ software. Bundle sheath extension density (BSE_d) was calculated as the ratio between the sum of all bundle sheath extension lengths and sampled area. The cover percentage of the leaf surface occupied by bundle sheath extension (BSE_cp) and the mean width of the bundle sheath extension (BSE_w; Fig. **1c**) were obtained using the Image J software.

Finally, anatomical sections of five leaves per species were obtained by gradual dehydration with ethanol, propylene oxide as a transition agent and inclusion in Araldite. One mesophyll image (200x) per leaf section was taken using a camera (Canon EOS M100) coupled to a microscope (OPTIKA B-600TiFL, Optika Microscopes, Ponteranica, Italy) and the following parameters were measured in five fields of each image: palisade and spongy mesophyll thickness (PMT and SMT, respectively; Fig. **1a**), number of cell layers in the palisade mesophyll (PM_nl), palisade mesophyll cell width and length (PM_cw and PM_cl, respectively), spongy mesophyll porosity (SM_p), upper and lower epidermis thickness (UET and LET, respectively; Fig. **1a**), upper and lower epidermis outer wall (UE_ow and LE_ow, respectively; Fig. **1b**), upper and lower epidermis lumen width and length (UE_luw, UE_lul, LE_luw and LE_lul, respectively; Fig. **1b**), upper and lower epidermis lateral wall (UE_latw and LE_latw, respectively; Fig. **1b**) and cell lumen size (UE_lu and LE_lu, respectively). See all parameters, abbreviations and units in **Table 1.**

4.2.5. Structural equation models (SEM)

The correlation analyses identified the independent variables most correlated to punch strength and specific punch strength (Fig. **S2**). With this information in mind, we proposed two mechanistic models including the most representative variables to estimate the network of correlations between traits related to leaf mechanical properties, which were assessed with structural equation models (SEM). In the first model, the leaf structural properties (i.e., leaf thickness, palisade mesophyll thickness, spongy mesophyll thickness, upper - lower epidermis thickness and upper - lower epidermis outer wall thickness) were related to punch strength (Fig. **3a**). In the second model, the leaf material properties (leaf density, cellulose content, hemicellulose content, and lignin and cutin content) were related to the specific punch strength (Fig. **3b**).

4.2.6. Leaf construction cost

Leaf samples from the seven studied species were oven dried at 70 ºC for 3 d until constant mass, ground and homogenized. Total leaf N concentration was determined with an Organic Elemental Analyzer (Flash EA 112, Thermo Fisher Scientific Inc., MA, USA). Ash concentration was determined gravimetrically after the combustion of duplicated samples for at least 4 h at 500 ºC. The heat of combustion was determined in triplicate samples of 18-24 mg with an adiabatic bomb calorimeter (Phillipson Gentry Instruments, Inc., USA) with correction for ignition wire melting (Villar and Merino, 2001), following the procedure of Phillipson (1964). Leaf construction cost (CC) (g glucose g^{-1}) was calculated according to Williams *et al.* (1987) as:

$$CC = \frac{\left(0.06968H_c - 0.065\right)\left(1-[A]\right) + \frac{7.5kN}{14.0067}}{Eg} \tag{10}$$

where H_c is the ash-free heat of combustion (kJ g^{-1}), $[A]$ is the ash concentration (g ash g^{-1} dry mass), N is the tissue nitrogen concentration (g g^{-1} dry mass), *E.g.*, is the growth efficiency (0.89 for woody leaves; Williams *et al.*, 1987) and k is the oxidation state of the nitrogen source (+5 for nitrate or –3 for ammonium). In well-aerated Mediterranean soils, nitrate is the main source of nitrogen and thus, k = +5. However, given that nitrate reduction in leaves can occur at the expense of reductive equivalents generated at a light with no apparent cost, we note that the nitrogen contribution to the construction cost also depends on the share of nitrate reduction above- and belowground (Niinemets, 1997; Niinemets, 1999).

4.2.7. Statistical analysis

Data passed Shapiro–Wilk and Bartlett tests for normality and equality of variances, respectively. Interspecific differences in leaf traits were evaluated by one-way ANOVA. All analyses were performed in R (R Core Team, 2021). To summarize the multivariate relationships among anatomical traits and *Quercus* species, a principal components analysis (PCA) with two components was carried out. This PCA was conducted using the FactoMineR: PCA package (Josse and Husson, 2008). To find correlations between all studied parameters, a Pearson's correlation matrix was performed using 'corrplot' package (Wei and Simko, 2021). SEM were implemented using 'lavaan' package (Rosseel, 2012). Standardized major axis (SMA) regressions (Warton *et al.*, 2006) were fitted to summarise the "scaling" relationship between two variables. To ensure our results did not artificially arise from a lack of phylogenetic independence, we additionally performed phylogenetic generalized least squares analysis assuming that trait evolution mimics Brownian motion and using the phylogeny from Hipp *et al*. (2020).

4.3. Results

4.3.1. Leaf mass per area and leaf traits behind mechanical properties

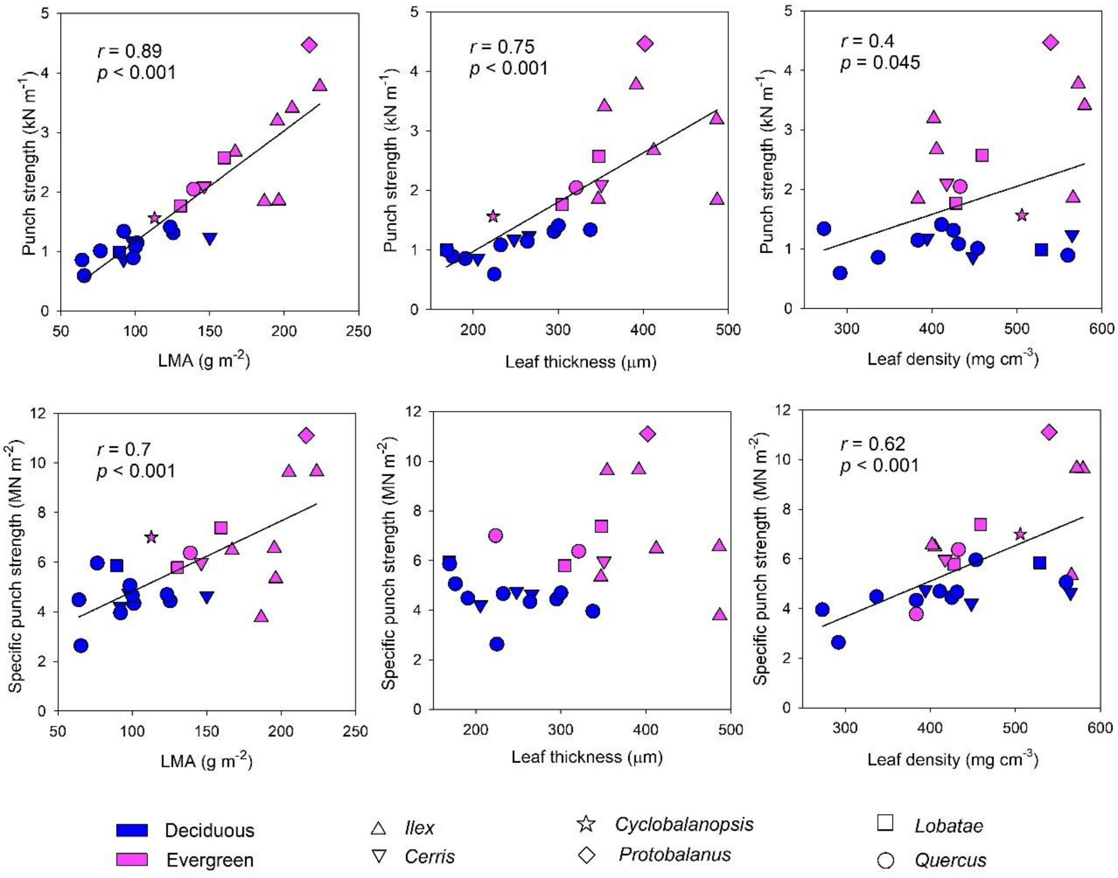

Fig. 2 Main relationships between physical parameters (punch strength and specific punch strength) and leaf mass per area, leaf thickness, and leaf density, for deciduous (DEC; blue) and evergreen (EVE; pink) *Quercus* species. Each point belongs to a *Quercus* species and represents its mean value. Black continuous line is the correlation considering all species when correlation was significative ($p < 0.05$).

Leaf mass per area (LMA), which is a combination of leaf thickness (LT) and leaf density (LD), ranged from 64.2 to 223.9 g m^{-2}. LT ranged from 169 to 487 μm and showed a good correlation ($r = 0.84$, $p < 0.001$) with LMA. However, LD showed less variation from 273 to 579 mg cm^{-3} and had a moderate correlation ($r = 0.52$, $p = 0.008$) with LMA. Oak species in this study showed a wide range in the values of the leaf mechanical properties measured. Punch strength (PS) and specific punch strength (SPS) ranged from 0.59 to 4.47 kN m^{-1} and from 2.63 to 11.11 MN m^{-2}, respectively (Fig. **2**), work of fracture (WF) from 0.07 to 1.31 J m^{-1}, specific work of fracture (SWF) from 0.33 to 3.26 kJ m^{-2}. The species that showed the highest values of these mechanical parameters were *Quercus chrysolepis*, *Q. phillyreoides*, *Q. coccifera* and *Q. ilex* subsp. *rotundifolia*. In contrast, the species with the overall lowest values were *Q. lobata*, *Q. robur* and *Q. cerris*. In general, measured mechanical properties showed a good correlation ($r > 0.7$, $p < 0.001$) with LMA (Fig. **2**) even when phylogeny was considered (r > 0.73, p < 0.01; Fig. **S4**). PS showed a strong positive correlation ($r = 0.75$, $p < 0.001$) with LT, but a weak correlation ($r = 0.4$, $p = 0.045$) with LD. Moreover, PS can be mathematically expressed as the product of SPS, and LT with LT having a relative contribution of 0.51 to PS. SPS was better correlated ($r = 0.62$, $p < 0.001$) with LD than with LT ($r = 0.38$, $P > 0.05$; Fig. **2**). Furthermore, leaves showed higher PS and WF at a given LMA as the scaling coefficients of the standardized major axis slopes were significantly steeper than 1 (1.47 for PS and 2.06 for WF; see Fig. **S3**). As has been seen in LMA and leaf mechanical resistance measurements, a wide range of variation was found in the compositional and anatomical variables (see table **S1** and TRY dataset).

Both material and mechanical properties were positively correlated with cellulose concentration (CC; $r > 0.72$, $p < 0.001$), palisade and spongy mesophyll thicknesses (PMT and SMT, respectively; $r = 0.50$ and 0.86, repectively $p < 0.05$) including the components palisade mesophyll cell width and number of layers (PM_ct and PM_nl, respectively; $r = 0.43$ to 0.79, $p < 0.05$), upper epidermis and lower epidermis outer wall and lateral wall (UE_ow, UE_latw and LE_ow, LE_latw; $r = 0.59$ to 0.86, $p < 0.01$) and bundle sheet extension density, width and cover percentage (BSE_d, BSE_w and BSE_cp, respectively; $r = 0.42$ to 0.79, $p < 0.05$; Fig. **S2**). Upper epidermis lumen cell (UE_lul) were negatively correlated with mechanical properties ($r < -0.61$). The rest of the anatomical and compositional traits showed less significant correlation coefficients ($r < 0.5$) or did not show significant correlations with the mechanical properties (Fig. **S2**). The presence of lignified anatomical structures was detected in all species, specifically in the bundle sheath. However, clearly lignified epidermis only appeared in the most sclerophyllous species (eg. *Q. chrysolepis*, *Q. phyllireoides* and *Q. coccifera*).

Leaf carbon concentration and nitrogen concentration in cell wall (Ncw_Ctotal and Ncw_Ntotal) were not related to leaf mechanical traits. However, leaf carbon concentration and C/N ratio did have a moderate correlation with PS (C.LD and C_N; $r = 0.40$, $p = 0.049$ and $r = 0.41$, $p = 0.044$, respectively).

4.3.3 Structural equation models (SEM)

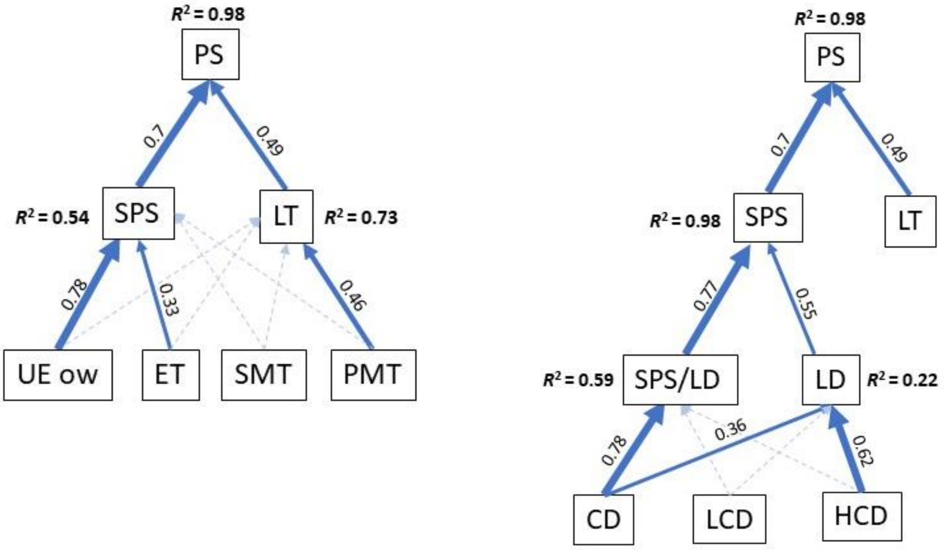

Fig. 3 Structural equation models (SEM) for the contribution of (a) anatomical and (b) compositional variables to leaf strength. punch strength (PS) and b) specific punch strength (SPS). The figure shows the full model, with significant correlations ($p < 0.05$) highlighted by solid arrows. PS, punch strength; SPS, specific punch strength; LT, leaf thickness; LD, leaf density; PMT and SMT, palisade and spongy mesophyll thickness, respectively; ET, summatory of upper and lower epidermis thickness; UE_ow, upper epidermis outer wall; CD, cellulose content per unit volume; HCD, hemicellulose content per unit volume; LCD, lignin and cutin content per unit volume. Numbers denote standardized path coefficients.

In the designed paths for SEMs, leaf traits were divided, on the one hand, into anatomical traits (thickness of the different tissue layers) related to LT and SPS (Fig. **3a**), and, on the other hand, into compositional traits (cell wall components) related to LD and SPS/LD (Fig. **3b**). In the anatomical traits model, both LT and SPS showed a high contribution to PS ($r = 0.49$ and $r = 0.7$, respectively). Regarding anatomical traits, LT was highly explained by PMT ($r = 0.46$) while SMT and ET showed a non-significant association with LT (Fig. **3a)**. On the other hand, SPS was strongly associated with the upper epidermis outer wall thickness (UE_ow) ($r = 0.78$) while it did not influence LT. Furthermore, the sum of upper and lower epidermis thickness (ET) had a moderate influence on

SPS ($r = 0.33$). UE_ow and ET together explained much of the variation in SPS ($R^2 = 0.54$).

In the compositional traits model, SPS was strongly associated with both LD ($r = 0.55$) and SPS/LD ($r = 0.77$). As expected, LD was explained by a combination of compositional traits, especially hemicellulose content per unit volume (HCD, $r = 0.62$) and cellulose content per unit volume (CD, $r = 0.36$). SPS/LD was explained by a strong ($r = 0.78$) direct influence of CD (Fig. **3b**).

4.3.4. Principal component analysis and phylogeny

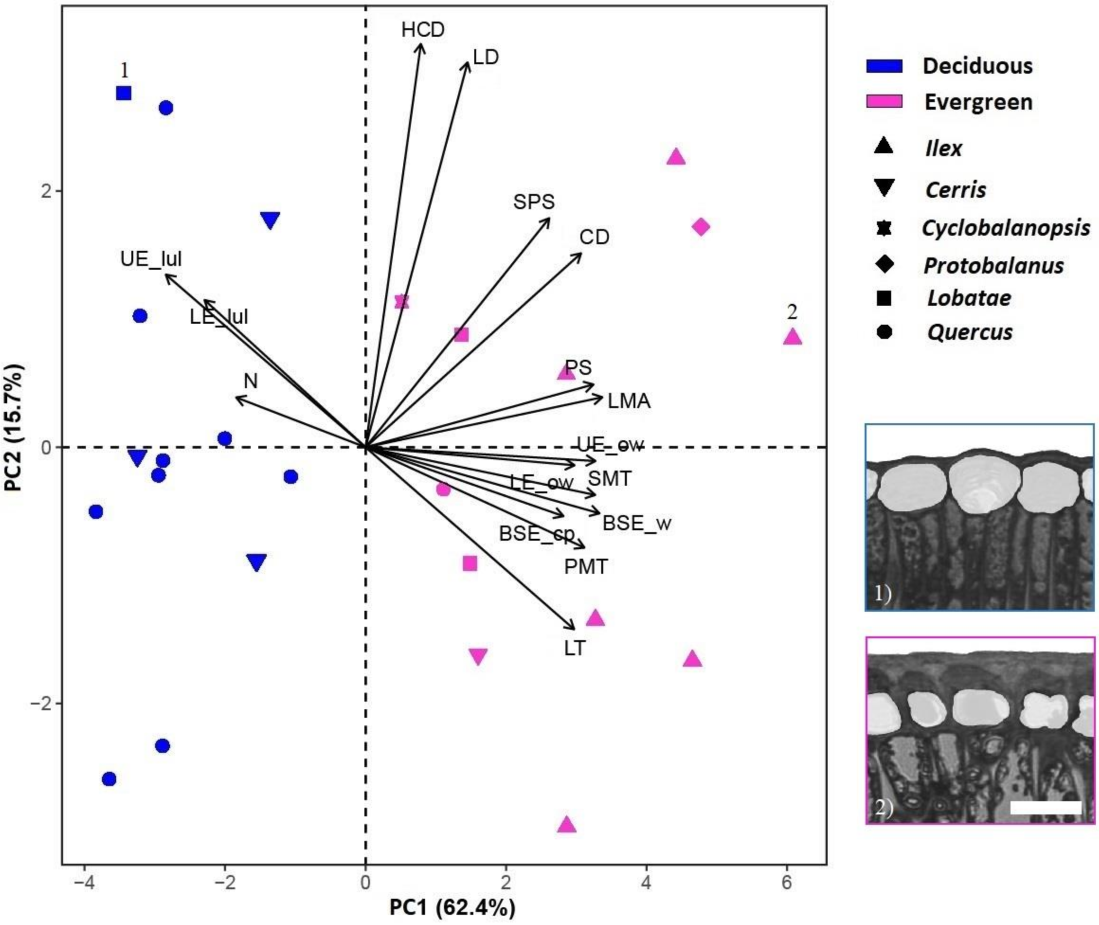

Fig. 4 Principal component analysis (PCA) of leaf variables (black lines) in 25 *Quercus* species (dots). Each symbol corresponds to a genus *Quercus* section. Variables: PS, punch strength; SPS, specific punch strength; LMA, leaf dry mass per unit area; LT, leaf thickness; LD, leaf density; CD, cellulose content per unit volume; HCD, hemicellulose content per unit volume; LCD, lignin and cutin content per unit volume; PMT, palisade mesophyll thickness; SMT, spongy mesophyll thickness; UET, upper epidermis thickness; LET, lower epidermis thickness; UE_ow, upper epidermis outer wall; LE_ow, lower epidermis outer wall; BSE_cp, bundle sheath extension cover

percentage; BSE_w, bundle sheath extension width; N, Nitrogen content; UE_lu, upper epidermis cell lumen size; LE_lu, lower epidermis cell lumen size. Images of the epidermis of (1) *Q. shumardii* and (2) *Q. phillyreoides*. Scale bar, 20 μm.

A principal component analysis (PCA) based on a selection of the strongest correlations between variables in the correlation matrix (Fig. **S2**) and considering the SEM, was performed. This PCA showed that the first main component (explaining the 62.4% variability) grouped PS, LMA, CD, PMT, SMT, UE_ow and BSE_w, whereas the second main component (explaining the 15.7% variability), grouped LD and HCD (Fig. **4**). The scores of the studied *Quercus* species in the PCA biplot indicated that the mechanical, compositional and anatomical traits analyzed clear differentiated deciduous and evergreen species although could not differentiate the species according to the climate (Fig. **4**).

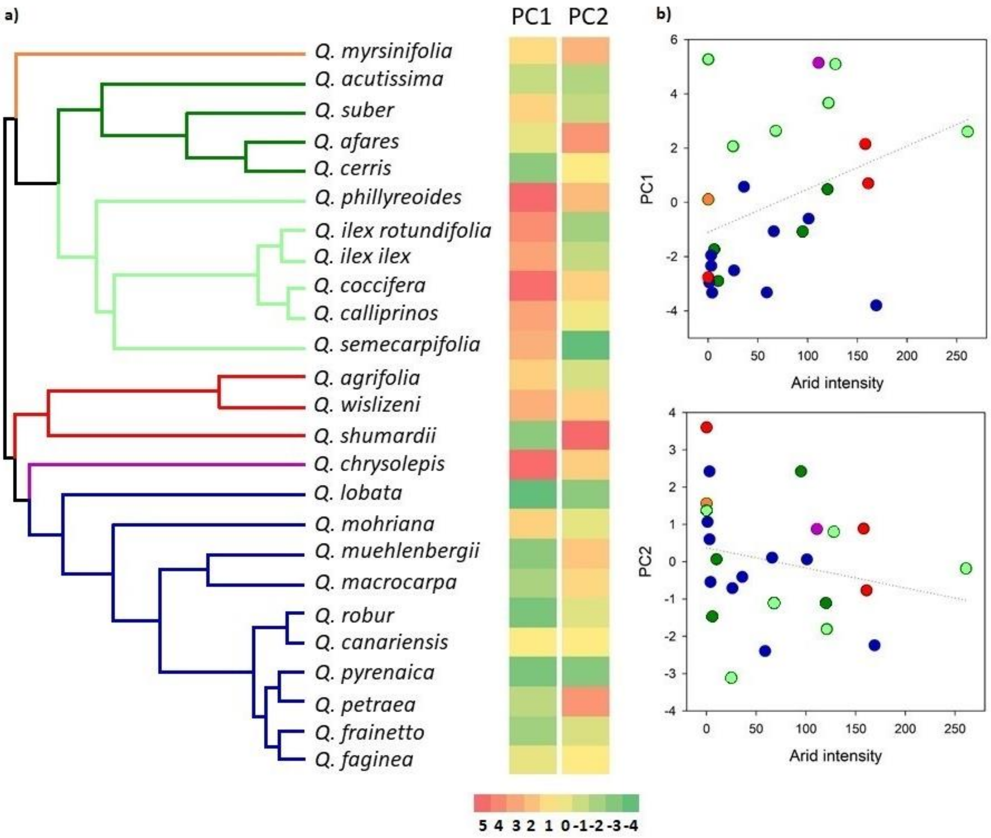

Fig. 5 a) *Quercus* phylogenetic tree based on Hipp *et al*. (2020). Colors in the tree represent *Quercus* sections; in orange, section *Cyclobalanopsis*; dark green, section *Cerris*; light green, section *Ilex*; red, section *Lobatae*; fuchsia, section *Protobalanus*; blue, section *Quercus*. PC1 and PC2. b) Relationship between PC1 and PC2 and Arid intensity (AI). The colors of the circles refer to the phylogenetic tree sections. It should be noted that the lower the AI value, the higher the degree of aridity.

Regarding the phylogeny, all the species of the *Quercus* section, except *Q. mohriana*, which is evergreen, tend to have low LMA, PS, cellulose content, thin mesophylls and UE_ow (Fig. **5a**). In contrast, all species of the *Ilex* sect. had a strong coordination with those traits related to sclerophylly as high LMA, PS, cellulose content, thick mesophyll and UE_ow, although they inhabit very different climates. *Q. chrysolepis* (sect. *Protobalanus*), presented sclerophyllous traits. Among the studied sect. *Lobatae* species, those evergreens (*Q. agrifolia* and *Q. wislizeni*) had a strong coordination with sclerophyllous traits, while *Q. shumardi* had less relation to sclerophyllous traits and a strong coordination with LD and hemicellulose content. In the case of *Q. myrsinifolia* (sect. *Cyclobalanopsis*), it had moderate coordination with both main components. In the case of the sect. *Cerris*, the only species with sclerophyllous traits was *Q. suber*. Regarding climate, no significant relationship was found between arid intensity and the main components of PCA (Fig. **5b**; Fig. **S5**).

4.3.5. Differences in construction cost and underlying traits among the evergreen oaks

Leaf construction cost of the seven species ranged from 1.43 ± 0.05 g glucose g^{-1} (*Q. ilex* subsp. *rotundifolia*) to 1.61 ± 0.06 g glucose g^{-1} (*Q. agrifolia* and *Q. coccifera*), while the mean leaf construction cost for the seven studied species was 1.53 ± 0.07 g glucose g^{-1} (**Table S2**). All the studied species showed construction cost values in the range published for sclerophyllous evergreen leaves (Villar and Merino, 2001). The ash fraction does not contribute to the construction cost, while the nitrogen concentration increases the construction cost due to extra costs required for nitrogen assimilation. However, the energy content (H_c) was the main determinant of the leaf construction cost; therefore, the highest H_c values corresponded to the highest construction cost values (**Table S2**). Thus, *Q. suber*, with the highest leaf N concentration, but also with the highest ash content, and low energy content had the lowest leaf construction cost (**Table S2**).

4.4. Discussion

In this study, we analysed 41 leaf mechanical, morphological, physicochemical and anatomical traits of 25 *Quercus* species grown in a common garden in Northeastern Spain to determine the leaf traits underlying sclerophylly. Although most ecophysiological studies have used leaf mass per unit area (LMA) as a proxy value for sclerophylly, sclerophylly actually means "hard-leaved," implying that mechanical properties of leaves are important in understanding this attribute (Alonso-Forn *et al*., 2020). As previously observed (Wright *et al*., 2004; Onoda *et al*., 2011), our results showed that, mechanical properties were strongly correlated with LMA in studied *Quercus* species.

The inability of LMA to account adequately for the wide variation in punch strength in our dataset reveals that there are variations in the mechanical properties that do not contribute to an increase in the accumulated mass per surface area. In this sense, we found a variation range in punch strength (PS) measures of 7.5-fold and in work to fracture (WF) of 18-fold, while LMA only varied 3.5-fold. Moreover, leaves showed higher PS and WF at a given LMA as the scaling coefficients of the standardized major axis slopes were significantly steeper than 1 (1.47 for PS and 2.08 for WF; see Fig. **S3**). Although the increase in LMA in *Quercus* has previously been attributed mainly to an increase in leaf thickness (LT) (Sancho-Knapik *et al*., 2021), we found that when accounting for the effect of LT, the specific work to fracture (SWF) still increased with LMA (Fig. **2**; **S3**). These results suggest that LMA could have more implications besides leaf strength. Although high LMA species can have lower photosynthetic rate per unit leaf area (Wright *et al*., 2004), Sancho-Knapik *et al*. (2021) suggested that accumulation of photosynthetic tissues enable plants to generate higher assimilation rates when facing certain environmental limitations. Moreover, some sclerophyllous evergreen oaks, despite their larger LMA, were able to achieve area-based net CO_2 assimilation values equivalent to congeneric deciduous species due to an increased chloroplast surface area exposed to intercellular air space (Peguero-Pina *et al*., 2017; Onoda *et al*., 2017).

According to our models, the upper epidermis outer wall had a strong and direct contribution to the leaf mechanical strength (measured as specific punch strength, SPS) (Fig. **3a**) which is consistent with previous studies (Onoda *et al*., 2012; Westbrook *et al*., 2011; He *et al*., 2019). Thus, an increase in the thickness of this layer appears to lead to a tougher epidermis without great changes in the accumulated mass per unit leaf surface area. Onoda *et al*. (2012) showed that leaves with relatively thicker cuticles had higher leaf mechanical resistance because the cuticle is made of very stiff material. Although a thicker cuticle may imply a higher investment (Poorter and Villar, 1997), it does not result in substantial changes in leaf thickness, total accumulated mass per surface nor construction cost (Villar and Merino, 2001; **Table S2**). In this sense, although *Q. chrysolepis* a notably higher (>60%) LMA than *Q. agrifolia*, their construction costs are very similar (**Table S2**). In addition, its extra cost can be offset by its greater mechanical resistance to biotic and abiotic stresses which might confer longer leaf lifespans among evergreen species (Krauss *et al*., 1997; Riederer and Schreiber, 2001; Wright *et al*., 2004; Carver and Gurr, 2006; Onoda *et al*., 2012).

The cellulose concentration per unit volume had a strong direct contribution to SPS/LD (Fig. **3b**) which indicates that cellulose plays a critical role in increasing leaf strength and toughness, a result also found in tropical plants (Kitajima and Poorter, 2010). In the correlation analysis (Fig. **S2**), cellulose concentration was tightly correlated with the bundle sheath extension width and cover percentage, and the outer wall thickness, suggesting that these anatomical components, which are rich in cellulose, play key roles in mechanical resistance.

The PCA plots based on foliar trait values indicated a clear separation of two groups along the first component axis corresponding to evergreen and deciduous species (Fig. **4**). Foliar mechanical properties, including punch strength, LMA, cellulose content, mesophylls thickness upper epidermis outer wall thickness, Bundle Sheath Extension width and cover percentage separated these contrasting leaf habits. Deciduous oaks from *Quercus*, *Cerris* and *Lobatae* sections, showed common leaf attributes regardless of their phylogenetic lineage. This group of species is characterized by softer and thinner leaves, narrower bundle sheath extension, less coverage of bundle sheath extension, lower cellulose content, as well as thinner cuticles and outer walls that are not lignified. Thus, *Q. robur* (section *Quercus*) from central Europe and *Q. shumardii* (section *Lobatae*) from eastern North America share these leaf traits (Fig. **5**). Moreover, these leaf features do not seem to depend on the average climate of the native habitat. Temperate species (e.g., *Q. robur*) share characters with species that inhabit Mediterranean climates (e.g., *Q. faginea* and *Q. cerris*; Gil-Pelegrín *et al*., 2017).

Evergreen oaks also share common features irrespective of their phylogenetic lineage. These species share harder and thicker leaves, wider bundle sheath extensions, a higher coverage of wider bundle sheath extensions, higher cellulose content and an epidermis with thick cuticles and cell walls that are often lignified. Regarding this second group, some interesting insights emerge. Species of sect. *Ilex* show similar foliar features, regardless of their climate of origin. They occupy quite contrasting climates that range in arid intensity from 0 (e.g., *Q. phyllireoides*) to 261 (e.g., *Q. calliprinus*) (Fig. **5b**) and there is still a great variation when we consider the phylogeny (Fig. **S5)**. These shared features may be explained by their common phylogenetic origin in the palaeotropical flora in East Asia (Jiang *et al.*, 2019) (Fig. **5a**). Species from this section are currently found in diverse climates, from humid temperate or subtropical climates (e.g., *Q, phillyreoides* and *Q. semecarpifolia*; cf. Jiang *et al.*, 2019), to semi-arid Mediterranean areas (e.g., *Q. coccifera*; cf. Vilagrosa *et al.*, 2003; Gil-Plegrín *et al.*, 2017). According to fossil record, xeromorphic-like traits were already present in the ancestor of the sect. *Ilex*, *Q. yangyiensis* that lived in warm aseasonal tropical conditions (Zhou *et al.*, 2007; He *et al.*, 2014). Under conditions favorable year-round growth, maintaining an evergreen leaf habit would have been favored over a deciduous leaf habit (Kikuzawa 1991, 1995). Evergreen species with longer leaf lifespan necessitated more durable leaves with traits that allow them to resist tearing and wear due to abiotic (Nikklas, 1999) and biotic (Wrigth and Vincent, 1996; Peeters *et al.*, 2007) interactions with the environment (Turner, 1994). The increase in the level of sclerophyll through the accumulation of structural carbohydrates could improve leaf persistence. In this way, the improved leaf protection would help to resist for a longer time (Turner, 1994; Takashima *et al.*, 2004). Moreover, habitats with several ecological limitations (e.g., drought, nutrient scarcity, low temperatures during the vegetative period or mechanical damage) are the ones where sclerophylly is more prevalent, as exemplified by the case of Mediterranean evergreens (Alonso-Forn *et al.*, 2020 and references therein).

Although the leaf features of evergreen sclerophylls are extremely functional in Mediterranean-type climate conditions, Ackerly (2004) proposed that these qualities originated in ancestral non-Mediterranean-type habitats in many lineages. Thus, previously mentioned sclerophyllous features can be inferred to have contributed to the later success of section *Ilex* with the expansion of the relatively recent Mediterranean-type climates (Ackerly, 2004; Deng *et al.*, 2017). Sect. *Ilex* species may thus have evolved foliar traits under ancestral climates that pre-adapted them to Mediterranean-type climates (Verdú *et al.*, 2003). All American oak clades share a common high latitude temperate ancestor (Hipp *et al.*, 2018). Thus, some oaks studied here that occupy habitats in

Mediterranean climates in the Nearctic (e.g., *Q. agrifolia* or *Q. wislizeni,* section *Lobatae*), have been suggested to have evolved from temperate ancestors (Hipp *et al.*, 2018). Nevertheless, species of section *Protobalanus* are evergreen and are restricted to mild climates. It appears likely that both conservatism in foliar traits linked to shared ancestry and evolutionary legacies of adaptation to ancient climates as well as evolutionary convergence in foliar traits from different sections contributes to the observed patterns of sclerophyllous traits among lineages and leaf habits. To conclude, there is an evolutionary progression towards sclerophyllous leaf traits in sect. *Protobalanus* and sect. *Lobatae* that inhabit dry climates, while sect. *Ilex* may have evolved to a lesser degree because its tropical ancestors had foliar traits that were pre-adaptated to some extent.

Funding

This work was supported by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) [grant number RTA2015-00054-C02-01] and funded by Ministerio de Ciencia e Innovación/ Agencia Estatal de Investigación (Spain) [grant PID2019-106701RR-I00 /AEI/ 10.13039/501100011033] and from Gobierno de Aragón H09_20R research group. Work of DA-F is supported by an INIA contract [BES-2017-081208]. Work of MDF is supported by a Juan de la Cierva-Incorporación contract [JC2020-043487-I] provided by Ministerio de Ciencia e Innovación (Spain) and by European Union (NextGenerationEU). Work of R.M-S. is supported by a predoctoral Gobierno de Aragón scholarship. Work of MN is supported by postdoctoral fellowship Juan de la Cierva-Formación [FJC2020-043902-I], financed by Ministerio de Ciencia e Innovación/ Agencia Estatal de Investigación (Spain) and the European Union (NextGenerationEU/PRTR).

Author Contribution

DA-F, DS-K, JJP-P, TGA-A and EG-P planned and designed the research. DA-F, DS-K, MDF, RM-S, JJP-P, JPF and EG-P performed the measurements. DA-F, DS-K, MN, JPF and VRD analysed data. DA-F, DS-K, YO and JC-B drafted the manuscript. All authors edited the manuscript with valuable inputs.

Data availability

Data that support the findings of this study are openly available in TRY Plant Trait Database at http://doi.org/10.17871/TRY.86.

David Alonso-Forn, Domingo Sancho-Knapik, María Dolores Fariñas, Rubén Martín-Sánchez, Juan Pedro Ferrio, José Javier Peguero-Pina, Tomás Gómez Álvarez Arenas, Eustaquio Gil-Pelegrín. 2022. Mean leaf trait values of 25 *Quercus* species. TRY Plant Data Base. http://doi.org/10.17871/TRY.86

Competing interests

None declared.

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4.6. Supporting Information

The following Supporting Information is available for this article:

Table S1 Mean value, maximum (Max.) and minimum (Min.) values, standard deviation (SD) and coefficient of variation (CV) of leaf traits for *Quercus* species.

Table S2 Heat of combustion, ash, nitrogen concentration (N) and construction cost of leaves for the seven studied *Quercus* species.

Fig. S1 Example of force-displacement curves and points of the leaf were punch and die tests were conducted.

Fig. S2 Correlation coefficients matrix of leaf mechanical properties, compositional and anatomical traits.

Fig. S3 The bivariate trait relationships between leaf structural properties and leaf mass per area (LMA).

Fig. S4 Corrected main relationships using phylogenetic generalized least squares analysis.

Fig. S5 Corrected relationship between PC1 and PC2 and Arid intensity (AI) using phylogenetic generalized least squares analysis.

Supporting Information

Article title: **Disentangling leaf structural and material properties in relation to their anatomical and chemical compositional traits in oaks (*Quercus* L.)**

Authors: David Alonso-Forn, Domingo Sancho-Knapik, María Dolores Fariñas, Miquel Nadal, Rubén Martín-Sánchez, Juan Pedro Ferrio, Víctor Resco de Dios, José Javier Peguero-Pina, Yusuke Onoda, Jeannine Cavender-Bares, Tomás Gómez Álvarez Arenas, Eustaquio Gil-Pelegrín

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Abbr.	Variable	Units	Mean	Min.	Max.	SD	CV
PS	Punch strength	kN m^{-1}	1.81	0.59	4.47	1.01	0.56
SPS	Specific Punch Strength	MN m^{-2}	5.79	2.63	11.1	1.98	0.34
WF	Work of fracture	J m^{-1}	0.42	0.07	1.31	0.35	0.81
SWF	Specific Work to Fracture	kJ m^{-2}	1.26	0.33	3.26	0.76	0.60
LMA	Leaf mass per unit area	g m^{-2}	134	64.0	224	48.5	0.36
LT	Leaf thickness	μm	300	169	487	92.4	0.31
LD	Leaf density	mg cm^{-3}	448	273	579	86.0	0.19
HC	Hemicellulose content	mg g^{-1}	192	134	262	28.1	0.15
CC	Cellulose content	mg g^{-1}	160	96.0	246	41.3	0.26
LCC	Lignin and cutin content	mg g^{-1}	74.6	32.0	110	18.4	0.25
HCD	Hemicellulose per unit volume	mg cm^{-3}	86.5	39.1	139	23.5	0.27
CD	Cellulose per unit volume	mg cm^{-3}	72.4	31.0	132	26.0	0.36
LCD	Lignin and cutin per unit volume	mg cm^{-3}	32.9	16.2	53.4	8.93	0.27
Ntotal	Nitrogen content	g 100^{-1} g^{-1}	1.37	0.95	1.78	0.26	0.19
Ctotal	Carbon content	g 100^{-1} g^{-1}	47.7	41.2	50.7	2.30	0.05
N.LD	Nitrogen per unit volume	mg cm^{-3}	6.11	2.86	8.37	1.46	0.24
C.LD	Carbon per unit volume	mg cm^{-3}	213	126	280	41.5	0.19
Ncw_Ntotal	Nitrogen in cell wall:Nitrogen		0.41	0.23	0.74	0.13	0.32
Ccw_Ctotal	Carbon in cell wall:Carbon		0.48	0.31	0.66	0.08	0.17
C_N	Ratio carbon:nitrogen		36.0	26.0	52.3	7.38	0.20

PMT	Palisade mesophyll thickness	µm	79.7	42.5	148	29.3	0.37
PM_nl	Palisade mesophyll layers		1.52	1	3	0.59	0.39
PM_cl	Palisade mesophyll cell length	µm	54.3	32.6	80.2	12.4	0.23
PM_ct	Palisade mesophyll cell width	µm	8.74	6.99	12.1	1.56	0.18
SMT	Spongy mesophyll thickness	µm	96.9	49.3	163	36.2	0.37
SM_p	Spongy mesophyll porosity	%	36.5	21.5	47.5	6.37	0.17
UET	Upper epidermis thickness	µm	18.2	14.1	24.4	2.78	0.15
UE_ow	Upper epidermis outer wall	µm	5.49	2.80	9.66	2.07	0.38
UE_luw	Upper epidermis lumen width	µm	11.6	6.60	18.1	3.34	0.29
UE_lul	Upper epidermis lumen length	µm	18.16	10.6	24.6	4.41	0.24
UE_latw	Upper epidermis lateral wall	µm	3.12	1.30	6.42	1.16	0.37
UE_lu	Upper epidermis lumen cell	μm^2	200	70.4	312	77.2	0.39
LET	Lower epidermis thickness	µm	11.8	8.30	16.7	2.02	0.17
LE_ow	Lower epidermis outer wall	µm	3.56	1.10	6.20	1.50	0.42
LE_luw	Lower epidermis lumen width	µm	7.12	4.53	9.46	1.08	0.15
LE_lul	Lower epidermis lumen length	µm	10.9	7.00	14.1	2.10	0.19
LE_latw	Lower epidermis lateral wall	µm	1.31	0.71	2.14	0.43	0.33

Abbr.	**Variable**	**Units**	**Mean**	**Min.**	**Max.**	**SD**	**CV**
BSE_d	Bundle sheath extension density	mm mm^{-2}	7.40	4.96	9.60	1.38	0.19
BSE_cp	Bundle sheath extension cover percentage	%	34.3	8.87	59.0	15.1	0.44
BSE_w	Bundle sheath extension width	µm	26.1	12.3	35.9	7.02	0.34

Table S2 Heat of combustion, ash, nitrogen concentration (N) and construction cost (CC) of leaves for the seven studied *Quercus* species. Data are mean ± SE. Different letters indicate significant differences among species (Tukey's test, $P < 0.05$).

Species	Heat of combustion (kJ g^{-1})	Ash (g g^{-1})	N (g g^{-1})	CC (g glucose g^{-1})
Q. chrysolepis	22.2 ± 0.3^{b}	0.0736 ± 0.0004^{c}	0.012 ± 0.001^{a}	1.58 ± 0.02^{bc}
Q. agrifolia	21.9 ± 0.3^{ab}	0.0465 ± 0.0004^{b}	0.014 ± 0.002^{a}	1.61 ± 0.02^{bc}
Q. wislizeni	19.8 ± 0.6^{a}	0.0392 ± 0.0005^{b}	0.014 ± 0.003^{a}	1.46 ± 0.04^{a}
Q. coccifera	22.0 ± 0.9^{ab}	0.0487 ± 0.0002^{b}	0.014 ± 0.002^{a}	1.61 ± 0.06^{bc}
Q. ilex subsp. *ilex*	20.8 ± 2.1^{ab}	0.0558 ± 0.0006^{bc}	0.011 ± 0.003^{a}	1.50 ± 0.16^{abc}
Q. ilex subsp. *rotundifolia*	20.0 ± 0.7^{a}	0.0257 ± 0.0004^{a}	0.011 ± 0.001^{a}	1.49 ± 0.06^{ab}
Q. suber	20.3 ± 0.7^{a}	0.0856 ± 0.0004^{c}	0.017 ± 0.002^{a}	1.43 ± 0.05^{a}

Fig. S1 (a) Example of force-displacement curves of *Quercus robur* obtained from punch and die test showing maximum force (F_{max}) and work to fracture (WF) calculated as the area below the curve between the initial contact of the punch with the leaf and Fmax. (b) Examples of the points were punch and die tests were conducted in leaves of *Q. coccifera* (left) and *Q. robur* (right), avoiding major veins and delimiting upper and lower borders of the intercostal panel.

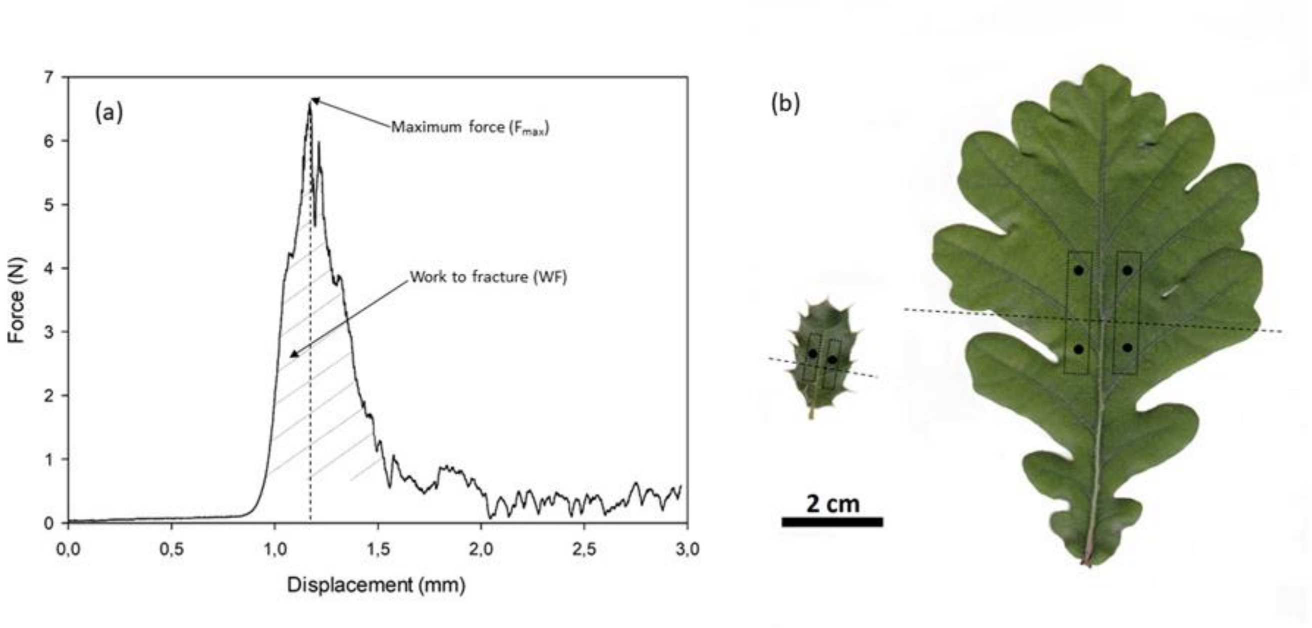


Fig. S2 Correlation coefficients in matrix form between leaf mechanical properties, compositional and anatomical traits. The matrix includes the correlations graduated according to the legend. Abbreviations as in Table 1.

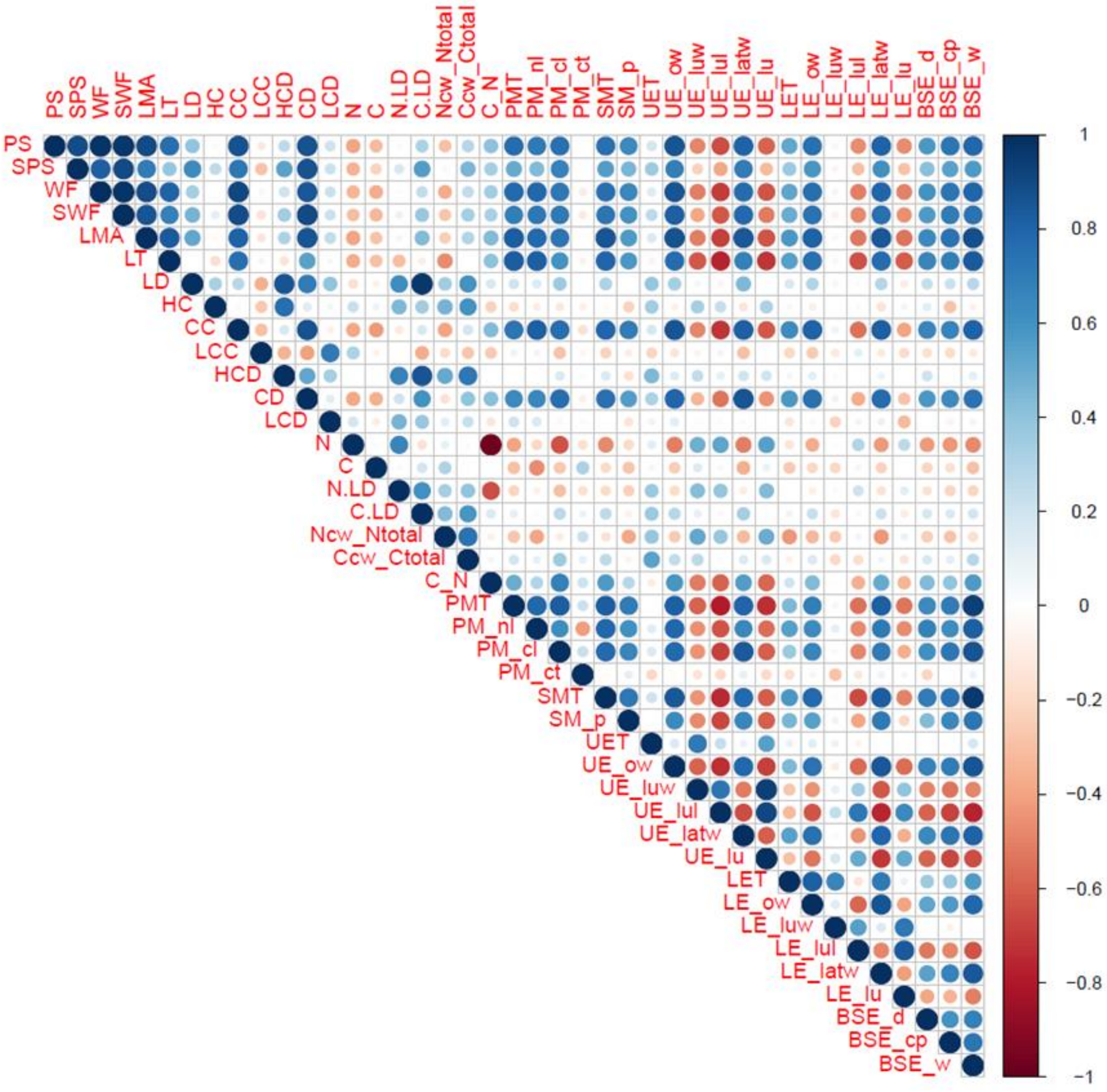


Fig. S3 The bivariate trait relationships between leaf structural properties and leaf mass per area (LMA). Note that graph axes are log10 scaled.

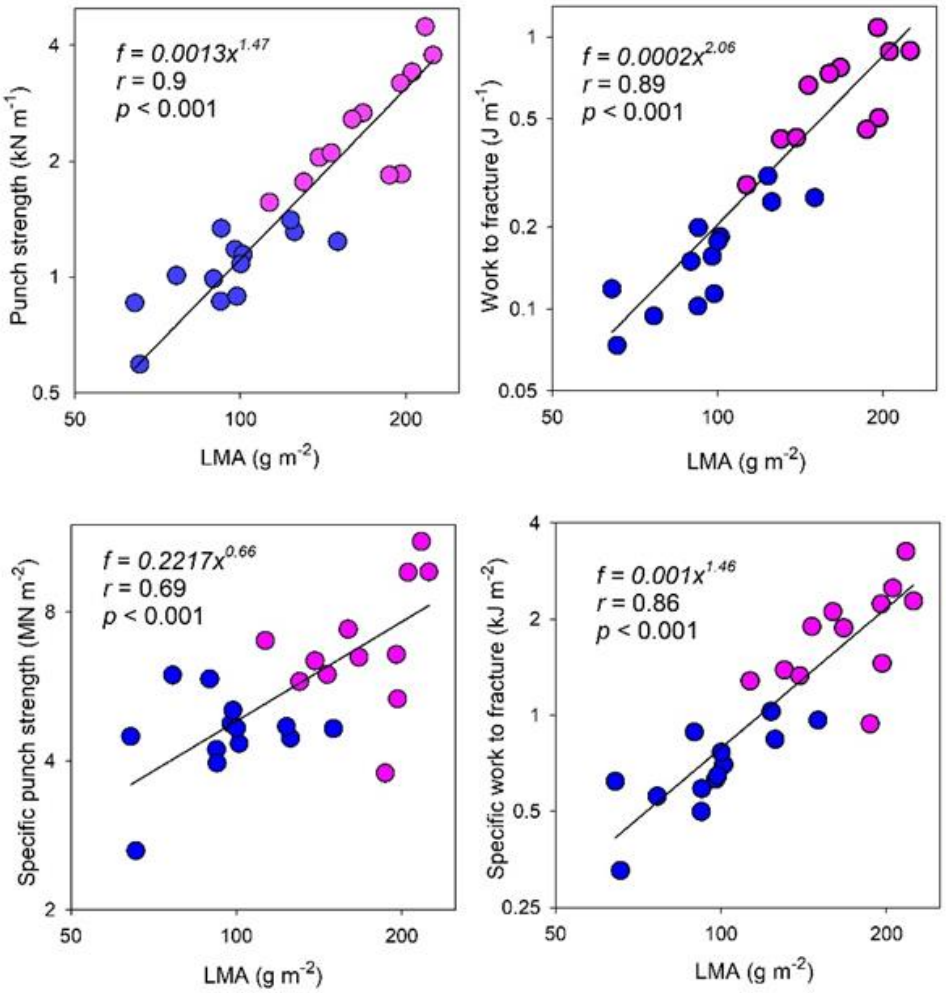


Fig. S4 Corrected main relationships using phylogenetic generalized least squares analysis assuming that trait evolution mimics Brownian motion and using the phylogeny from Hipp *et al*. (2020). Physical parameters (punch strength and specific punch strength) and leaf mass per area, leaf thickness, and leaf density, for deciduous (DEC; blue) and evergreen (EVE; pink) *Quercus* species. Each circle belongs to a *Quercus* species and represents its mean value. The blue continuous line is the correlation considering all species.

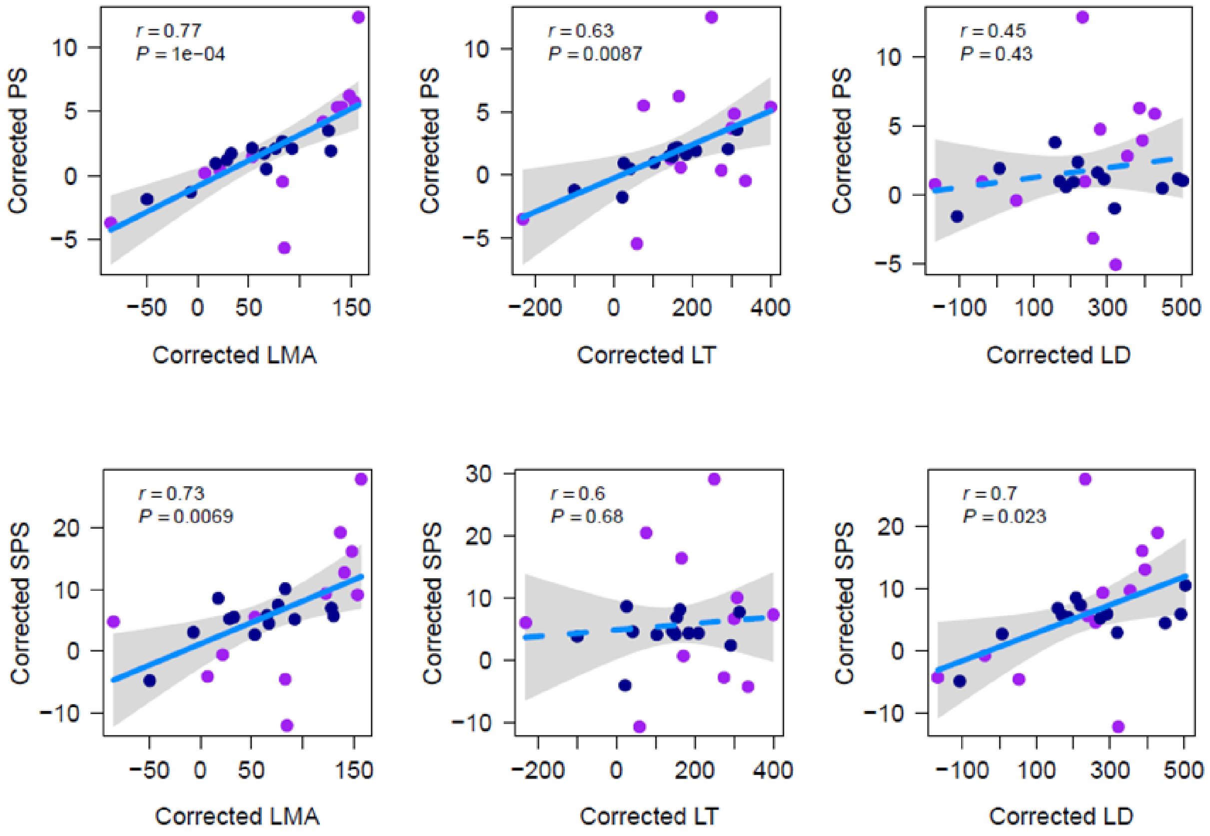
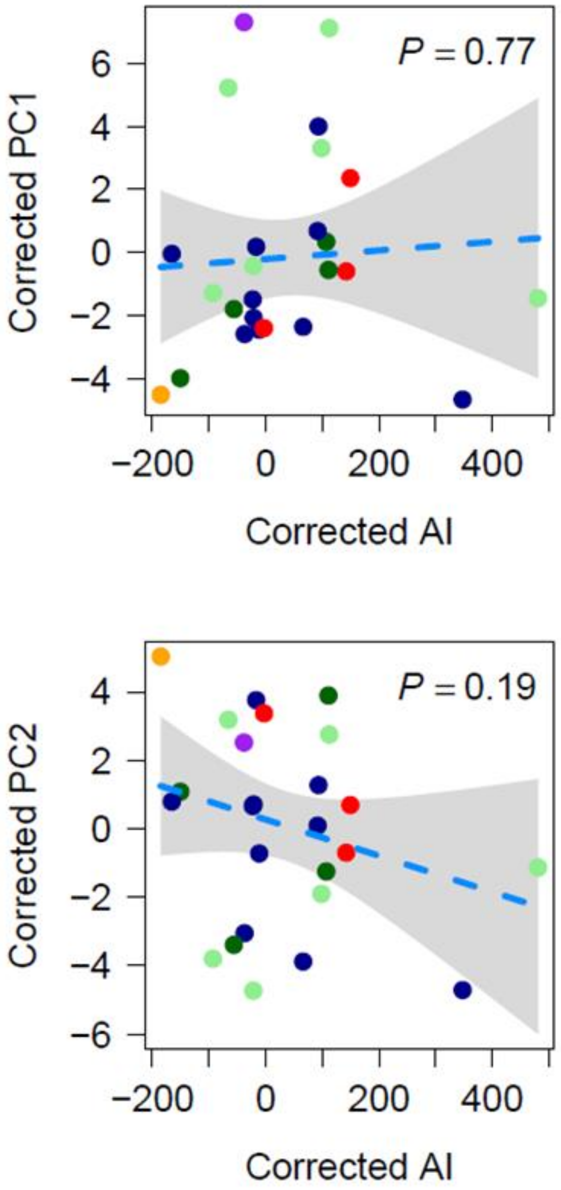


Fig. S5 Corrected relationship between PC1 and PC2 and Arid intensity (AI) using phylogenetic generalized least squares analysis assuming that trait evolution mimics Brownian motion and using the phylogeny from Hipp *et al.* (2020). Each circle belongs to a Quercus species and represents its mean value. The colours of the circles represent the different sections of studied *Quercus* (see **Fig. 5**).



Capítulo V

Cell-level anatomy explains leaf age-dependent declines in mesophyll conductance and photosynthetic capacity in the evergreen Mediterranean oak *Quercus ilex* subsp. *rotundifolia*

Tree Physiology 00, 1–15
https://doi.org/10.1093/treephys/tpac049

Research paper

Cell-level anatomy explains leaf age-dependent declines in mesophyll conductance and photosynthetic capacity in the evergreen Mediterranean oak *Quercus ilex* subsp. *rotundifolia*

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Received January 11, 2022; accepted April 13, 2022; handling Editor Antonio Diaz-Espejo

Leaves of Mediterranean evergreen tree species experience a reduction in net CO_2 assimilation (A_N) and mesophyll conductance to CO_2 (g_m) during aging and senescence, which would be influenced by changes in leaf anatomical traits at cell level. Anatomical modifications can be accompanied by the dismantling of photosynthetic apparatus associated to leaf senescence, manifested through changes at the biochemical level (i.e., lower nitrogen investment in photosynthetic machinery). However, the role of changes in leaf anatomy at cell level and nitrogen content in g_m and A_N decline experienced by old non-senescent leaves of evergreen trees with long leaf lifespan is far from being elucidated. We evaluated age-dependent changes in morphological, anatomical, chemical and photosynthetic traits in *Quercus ilex* subsp. *rotundifolia* Lam., an evergreen oak with high leaf longevity. All photosynthetic traits decreased with increasing leaf age. The relative change in cell wall thickness (T_{cw}) was less than in chloroplast surface area exposed to intercellular air space (S_c/S), and S_c/S was a key anatomical trait explaining variations in g_m and A_N among different age classes. The reduction of S_c/S was related to ultrastructural changes in chloroplasts associated to leaf aging, with a concomitant reduction in cytoplasmic nitrogen. Changes in leaf anatomy and biochemistry were responsible for the age-dependent modifications in g_m and A_N. These findings revealed a gradual physiological deterioration related to the dismantling of the photosynthetic apparatus in older leaves of *Q. ilex* subsp. *rotundifolia*.

Keywords: holm oak, leaf aging, leaf anatomy, mesophyll conductance, nitrogen, photosynthesis.

Introduction

The ecological implications of differences in leaf lifespan have been analyzed by cost–benefit models that optimize leaf lifetime carbon gain in different environments (Chabot and Hicks 1982, Kikuzawa 1991, Ackerly 1999). These models postulate that leaves should be replaced when leaf maintenance costs during unfavorable periods surpass the carbon gain in favorable periods or, alternatively, the potential future gain is less than the cost of losing the leaves in terms of carbon and nutrient investment (Chabot and Hicks 1982). It is widely acknowledged that developing leaves show a rise in photosynthesis to maximum levels that is followed by an age-dependent decrease in photosynthetic potential until leaf senescence (Freeland 1952, Chabot and Hicks 1982, Niinemets et al. 2004, 2006, 2009, 2012, Harayama et al. 2016).

Evergreen tree species with long leaf lifespan retain several cohorts of foliage that can contribute significantly to canopy photosynthesis (Niinemets et al. 2005, Warren 2006, Peguero-Pina et al. 2007, Yasumura and Ishida 2011). Indeed, Escudero and Mediavilla (2003) analyzed the decline in photosynthetic performance of several evergreen species from Mediterranean-type climates with long lifespan, concluding that the retention of old leaves resulted in a higher whole-canopy net CO_2 assimilation, despite their lower assimilation rate.

Studies have also found evidence for increased photosynthetic limitation in older leaves of different plant species due to a reduced mesophyll conductance to CO_2 (g_m) (Loreto et al. 1994, Flexas et al. 2007*a*, Zhang et al. 2008, Niinemets et al. 2009), including Mediterranean evergreen tree species during aging and senescence (Niinemets et al. 2005, 2006). In fact, g_m plays a predominant role in the photosynthetic process of Mediterranean evergreen tree species, being often the most limiting factor for net CO_2 assimilation (Flexas et al. 2014, Galmés et al. 2014, Niinemets and Keenan 2014, Peguero-Pina et al. 2016*a*, Peguero-Pina et al. 2017*a*, Peguero-Pina et al. 2018, Alonso-Forn et al. 2021).

Values of g_m for a given plant species can be influenced by different leaf anatomical traits, mainly the cell wall thickness, the mesophyll and chloroplast surface area exposed to intercellular air space per unit leaf area (S_m/S and S_c/S, respectively) and the chloroplast size (Terashima et al. 2011, Tomás et al. 2013, Peguero-Pina et al. 2016*b*, 2017*b*, Sáez et al. 2017, Sáez et al. 2018, Carriquí et al. 2019). Given the strong control of leaf anatomy on g_m, Niinemets et al. (2005) suggested that the drawdown of photosynthesis and g_m in older leaves of Mediterranean evergreen tree species could be associated with increases in the thickness of mesophyll cell walls with increasing leaf age. Moreover, Niinemets et al. (2009) proposed that the dismantling of the photosynthetic apparatus associated to leaf senescence would reduce S_c/S, which could explain the reduction of both g_m and net CO_2 assimilation. Recently, Clarke et al. (2021) concluded that reduced g_m in older leaves of the annual forb *Nicotiana tabacum* was associated with cell wall thickening and reduction in S_c/S. However, to the best of our knowledge, there is a lack of empirical studies of age-dependent changes in leaf anatomical traits (i.e., cell wall thickness, S_c/S, chloroplast number and size) as the mechanistic explanation for the decline in photosynthetic potential of older leaves in species with long leaf lifespan.

Moreover, the dismantling of photosynthetic apparatus associated to leaf senescence can be manifested through a decrease in leaf nitrogen content and/or lower fractions of nitrogen in photosynthetic enzymes of functional chloroplasts (i.e., Rubisco and rate-limiting components of photosynthetic electron transport). Reallocation of N from older to younger leaves has been interpreted as a mechanism to improve whole-canopy photosynthesis (Thomas and Stoddart 1980, Chabot and Hicks 1982, Field 1983, Field and Mooney 1983, Smart 1994, Kitajima et al. 1997). Indeed, Warren (2006) and Kuusk et al. (2018) found that the concentrations of nitrogen and the maximum rate of carboxylation (V_{cmax}) decreased with needle age in different *Pinus* species. On the other hand, Hikosaka (2005) suggested that the decrease in photosynthetic potential in evergreen species with a longer leaf lifespan is not necessarily coupled with leaf nitrogen content. In this regard, Niinemets et al. (2005) only found minor changes in nitrogen content per unit area in older leaves of Mediterranean evergreens despite the fact that the capacities for photosynthetic electron transport (J_{max}) and V_{cmax} decreased fivefold on an area basis.

Therefore, the role of leaf anatomy and nitrogen content in determining g_m and photosynthetic capacity in old non-senescent leaves of evergreen trees with long leaf lifespan is far from being elucidated. We hypothesized that (i) changes in leaf anatomical traits (e.g., increased cell wall thickness and/or decreased S_c/S) in older leaves of evergreen species with long leaf lifespan are primarily responsible for decreases in foliage photosynthetic potentials in older leaves and (ii) anatomical modifications are accompanied by age-dependent changes at biochemical level (e.g., the decrease in nitrogen investment in photosynthetic machinery). To test these hypotheses, we evaluated the age-dependent changes in morphological, anatomical, chemical and photosynthetic traits in *Q. ilex* subsp. *rotundifolia* Lam., a Mediterranean evergreen oak with high leaf longevity (ca 28 months, according to Mediavilla and Escudero 2003) and a high fraction of leaves >1 year old that play an important role in canopy photosynthesis (Sala i Serra 1992, Corcuera et al. 2005).

Materials and methods

Plant material and experimental conditions

This study was carried out with adult ca 5 m tall trees of *Q. ilex* subsp. *rotundifolia* ('Cazorla' provenance, seed origin: 38°06′ N, 02°33′ W, 1236 m above sea level, Spain) growing outdoors at CITA de Aragón (41°39′ N, 0°52′ W, Zaragoza, Spain) under Mediterranean conditions (mean annual temperature 15.4 °C, total annual precipitation 298 mm). All trees were exposed to the same environmental conditions and drip-irrigated every 2 days. Physiological measurements were carried out in September 2018 in current-year (0-year-old), 1-year-old and 2-year-old leaves from three branches of three 15-year-old trees. The same measurements were repeated during September 2020 in current-year (0-year-old), 1-year-old and 2-year-old leaves from the same three branches of the same trees. Thus, the current-year leaves (0-year-old) measured in September 2018 correspond to the 2-year-old leaves measured in September 2020.

Light environment was characterized with three HOBO Pendant temp/light sensors (ONSET, Bourne, MA, USA) placed along a branch to determine the average light intensity above each of the three leaf cohorts studied (0-year-old, 1-year-old and 2-year-old). Measurements were recorded every 1 min during three sunny days of September 2018 (Figure S1 available as Supplementary data at *Tree Physiology* Online). The integrated daily quantum flux density (Q_{int}, mol m^{-2} day^{-1}) was calculated for each leaf cohort from the mean values of incident solar radiation (Cescatti and Zorer 2003) (Figure S2 available as Supplementary data at *Tree Physiology* Online).

Leaf gas-exchange and chlorophyll fluorescence measurements

Simultaneous gas-exchange and chlorophyll fluorescence measurements were carried out between 09:00 and 10:00 h (solar time) with a portable photosystem system CIRAS-2 (PP-Systems, Amesbury, MA, USA) fitted with an automatic universal leaf cuvette (PLC6-U, PP-Systems) and an FMS II portable pulse amplitude modulated fluorometer (Hansatech Instruments Ltd, Norfolk, UK). All measurements were conducted under the following standard environmental conditions: CO_2 concentration surrounding the leaf (C_a) of 400 μmol mol^{-1}, leaf temperature of 25 °C, vapor pressure deficit of 1.25 kPa and saturating photosynthetic photon flux density (PPFD) of 1500 μmol m^{-2} s^{-1}. Additional measurements were also performed under lower PPFD of 350 and 750 μmol m^{-2} s^{-1} in September 2018. After steady state gas-exchange rate was reached, the net assimilation rate (A_N), the stomatal conductance (g_s) and the effective quantum yield of PSII (Φ_{PSII}) were estimated. Φ_{PSII} was calculated as $(F'_M{-}F_S)/F'_M$, where F_S is the steady-state fluorescence yield and F'_M is the maximum fluorescence yield during an 1 s saturating light pulse of ca 8000 μmol m^{-2} s^{-1} (Genty et al. 1989). Photosynthetic electron transport rate (J_{flu}) was then calculated according to Krall and Edwards (1992), following the methodology described in Peguero-Pina et al. (2016*a*). Leakage of CO_2 in and out of the cuvette was determined for the same range of CO_2 concentrations as described in Flexas et al. (2007*b*) and used to correct the values of A_N and corresponding substomatal CO_2 concentration (C_i).

Estimation of mesophyll conductance, g_m and maximum velocity of carboxylation (V_{cmax}) by gas exchange and chlorophyll fluorescence

Mesophyll conductance (g_m) and maximum velocity of carboxylation (V_{cmax}) were estimated according to the variable *J* method of Harley et al. (1992) (Eq. (1)) and the one-point method of De Kauwe et al. (2016) (Eq. (2)), respectively, as follows:

$$g_m = \frac{A_N}{C_i - \frac{\Gamma^*(J_F+8(A_N+R_L))}{J_F-4(A_N+R_L)}} \tag{1}$$

$$V_{\text{cmax}} = \frac{A_N}{\left(\frac{C_i-\Gamma^*}{C_i+K_m}\right) - 0.015} \tag{2}$$

where A_N and C_i values used correspond to the gas-exchange measurements at saturating light. The chloroplastic CO_2 compensation point in the absence of mitochondrial respiration (Γ^*) and the respiration rate in the light (R_L) were estimated as in Flexas et al. (2007*a*), whereas K_m (the effective Michaelis–Menten constant) was estimated as in De Kauwe et al. (2016).

Morphological and anatomical measurements and leaf nitrogen concentration

Sections of 1 mm × 1 mm were cut between the main veins from the same leaves used for gas-exchange and processed for anatomical measurements following the methodology described in Peguero-Pina et al. (2016*c*). Semi-thin (0.8 μm) and ultrathin (90 nm) cross-sections were cut with an ultramicrotome (Reichert and Jung model Ultracut E). Semi-thin cross-sections were stained with 1% toluidine blue and viewed under a light microscope (Optika B-600TiFL, Optika Microscopes, Ponteranica, Italy). Ultrathin cross-sections were contrasted with uranyl acetate and lead citrate and viewed under a transmission electron microscope (H600, Hitachi, Tokyo, Japan). Light and electron microscopy images were analyzed with ImageJ software (http://rsb.info.nih.gov/nih-image/) to determine leaf anatomical characteristics. Light micrographs were used to measure leaf thickness, mesophyll thickness between the two epidermal layers, number of palisade layers, fraction of the mesophyll tissue occupied by the intercellular air spaces (f_{ias}), and mesophyll (S_m/S) and chloroplast (S_c/S) surface area facing intercellular air spaces per leaf area (Evans et al. 1994, Syvertsen et al. 1995, Tomás et al. 2013). Electron micrographs were used to measure the cell wall thickness (T_{cw}), cytoplasm thickness (T_{cyt}), chloroplast length (L_{chl}) and chloroplast thickness (T_{chl}) (Tomás et al. 2013). Moreover, total chloroplast area (A_{chl}) and total area occupied by starch grains and plastoglobuli (A_{st} and A_{plg}, respectively) were also measured to estimate the percentage of functional area per chloroplast as $(A_{\text{chl}}{-}A_{\text{stg}}{-}A_{\text{plg}})$ × 100. Each anatomical trait was measured in three different sections and four to six different fields of view.

Leaf dry mass per unit area (LMA) was measured in September 2018 and September 2020 in nine leaves per cohort from the same branches previously used for gas exchange and chlorophyll fluorescence measurements (three leaves of each age from each of the three trees used for measurements). Leaf area was measured after scanning the leaves with the ImageJ software. Leaf dry mass was determined after leaves were oven dried at 70 °C for 3 days. The LMA was calculated as the ratio of foliage dry mass to foliage area.

Total leaf nitrogen (N_{total}) was quantified in dried leaves using an Organic Elemental Analyzer (Flash EA 112, Thermo Fisher Scientific Inc., Waltham, MA, USA). For the leaves measured in September 2020, the cell wall fraction was obtained after performing the neutral fiber detergent (NDF) following the method of Goering and Van Soest (1970). Nitrogen content of the cell wall fraction ($N_{\text{cell wall}}$) was further estimated using the elemental analyzer as aforementioned. The fraction of nitrogen allocated in the cytoplasm ($N_{cytoplasm}$) was calculated as follows: $N_{cytoplasm}$ = (N_{total} × 100–$N_{cell\ wall}$ × NDF)/(100–NDF).

Mesophyll conductance modeled on the basis of anatomical traits

Mesophyll diffusion conductance estimated using the measured leaf anatomical traits ($g_{m,a}$) was calculated as a composite conductance of within-leaf gas and liquid diffusion pathways, according to the one-dimensional gas diffusion model of Niinemets and Reichstein (2003) as applied by Tosens et al. (2012):

$$g_{m,a} = \frac{1}{\frac{1}{g_{ias}} + \frac{R \bullet T_k}{H \bullet g_{liq}}} \tag{3}$$

where g_{ias} is the gas phase conductance from substomatal cavities to outer surface of cell walls, g_{liq} is the conductance in the liquid and lipid phases from the outer surface of cell walls to chloroplasts, R is the gas constant (Pa m³ K⁻¹ mol⁻¹), T_k is the absolute temperature (K) and H is the Henry's law constant for CO$_2$ (Pa m³ mol⁻¹). $g_{m,a}$ is defined as a gas-phase conductance, and thus $H/(RT_k)$, the dimensionless form of the Henry's law constant converts g_{liq} to the corresponding gas-phase equivalent conductance (Niinemets and Reichstein 2003).

The gas-phase conductance (and the reciprocal term, r_{ias}) was calculated as described in Niinemets and Reichstein (2003):

$$g_{ias} = \frac{1}{r_{ias}} = \frac{D_A \bullet f_{ias}}{\Delta L_{ias} \bullet \tau} \tag{4}$$

where ΔL_{ias} (m) is the average gas-phase thickness, τ is the diffusion path tortuosity (1.57 m m^{-1}, Syvertsen et al. 1995), D_A is the diffusivity of the CO$_2$ in the air (1.51·10^{-5} m^2 s^{-1} at 25 °C) and f_{ias} is the fraction of intercellular air spaces. ΔL_{ias} was taken as the half of the mesophyll thickness. Total liquid phase conductance (g_{liq}) from the outer surface of cell walls to the carboxylation sites in the chloroplasts is the sum of serial resistances of the cell wall (r_{cw}), the plasmalemma (r_{pl}) and the liquid phase inside the cell ($r_{cel,tot}$) (Tomás et al. 2013):

$$g_{liq} = \frac{S_m}{\left(r_{cw} + r_{pl} + r_{cel,tot}\right) \bullet S} \tag{5}$$

Cell wall conductance was calculated as described in Peguero-Pina et al. (2012). We used a value of 0.028 for the porosity of cell wall as previously estimated by Tomás et al. (2013) for *Q. ilex*. We used an estimate of 0.0035 m s^{-1} for the conductance of plasma membrane (Tosens et al. 2012). The conductance inside the cell was calculated considering two different pathways of CO$_2$ (one for cell wall parts lined with chloroplasts and the other for interchloroplastial areas, Tholen et al. 2012) as described by Tomás et al. (2013).

Quantitative limitations analyses of A_N

The relative controls on A_N were separated into their functional components according to the quantitative limitation analysis of Grassi and Magnani (2005) as applied in Tomás et al. (2013). This methodology allows comparing relative changes in limitations to net CO$_2$ assimilation into limitations due to limited g_s to CO$_2$ ($g_{s,CO2}$; stomatal limitations, l_s), g_m (mesophyll limitations, l_m) and leaf biochemistry (biochemical limitations, l_b). Each of the three components, l_s, l_m and l_b, can vary between zero and one ($l_s + l_m + l_b = 1$). They were calculated as:

$$l_s = \frac{g_{tot}/g_{s,CO2} \bullet \delta A_N/\delta C_c}{g_{tot} + \delta A_N/\delta C_c} \tag{6}$$

$$l_m = \frac{g_{tot}/g_m \bullet \delta A_N/\delta C_c}{g_{tot} + \delta A_N/\delta C_c} \tag{7}$$

$$l_b = \frac{g_{tot}}{g_{tot} + \delta A_N/\delta C_c} \tag{8}$$

where g_{tot} is the total conductance to CO$_2$ from leaf surface to carboxylation sites in the chloroplasts ($1/g_{tot} = 1/g_{s,CO2} + 1/g_m$). The values of g_m (Eq. (1)) were used to calculate the chloroplastic CO$_2$ concentration (C_c) as $C_c = C_i – A_N/g_m$. $\delta A_N/\delta C_c$ was calculated as the slope of the relationship between C_c and A_N, considering a C_c range of 50–100 μmol mol^{-1}.

Chlorophyll measurements

Discs from the leaves measured in September 2020 were wrapped in aluminum foil, frozen in liquid nitrogen and stored at −20 °C. Pigments were extracted with acetone (100%) in presence of Na-ascorbate. Extracts were thawed on ice, filtered through a 0.45-μm filter and chlorophylls were determined spectrophotometrically (V-1100, J.P. Selecta, Abrera, Spain) according to Lichtenthaler (1987).

Statistical analysis

Data are expressed as means ± standard error of the mean. One-way analyses of variance were performed to identify the leaf age effect on each of the measured traits. Multiple comparisons were carried out among 0-, 1- and 2-year-old leaves using the post hoc Tukey's honestly significant difference test. Student's *t*-tests were used to compare current-year leaves (0-year-old) measured in September 2018 with 2-year-old leaves measured in September 2020 to evaluate the changes of the measured traits due to leaf aging within the same cohort of leaves. Principal components analysis (PCA) was used to summarize the multivariate relationships among the measured traits of 0-, 1- and 2-year-old leaves. All statistical analyses were performed in the R software environment (version 4.0.0, R Development Core Team 2018).

Results

For measurements performed both in 2018 and in 2020, all photosynthetic characteristics decreased with increasing leaf

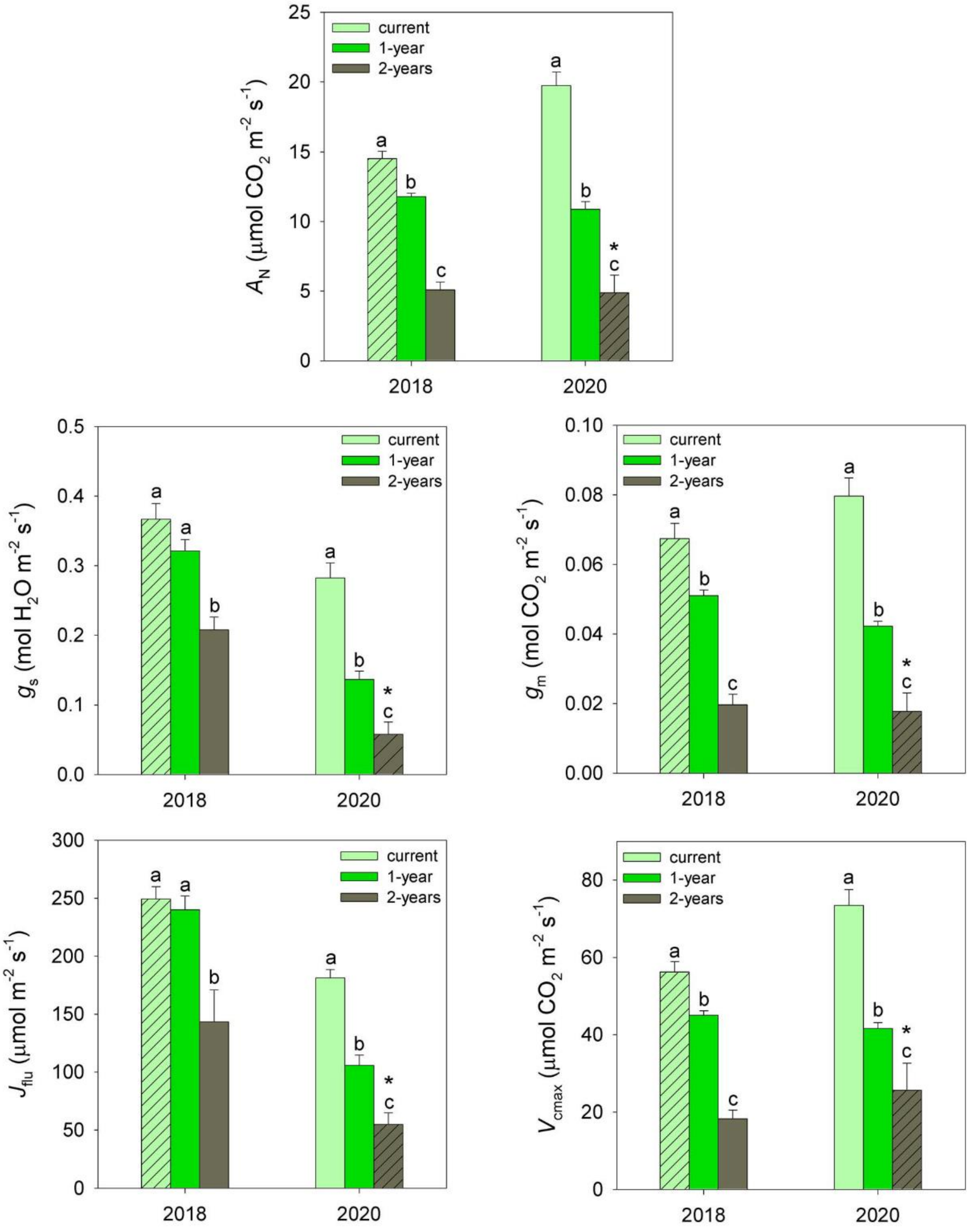

Figure 1. Photosynthetic characteristics for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020: A_N, net assimilation rate; g_s, stomatal conductance; g_m, mesophyll conductance; J_{flu}, photosynthetic electron transport rate; V_{cmax}, maximum velocity of carboxylation. Data are means ± SE. Different letters indicate significant differences among 0-, 1- and 2-year-old leaves (Tukey's test, $P < 0.05$). Asterisks indicate significant differences between current-year leaves measured in 2018 and 2-year-old leaves measured in September 2020 (striped bars) (Student's *t*-test, $P < 0.05$).

age within the individual branches (Figure 1). In addition, for the same cohorts of leaves measured in two different years (0-year-old leaves in 2018 and 2-year-old leaves in 2020), leaf photosynthetic traits were also greater in 0-year-old leaves measured in 2018 than in 2-year-old leaves measured in 2020 (Figure 1). Analogously, leaf nitrogen concentration (N) decreased with increasing leaf age, although exclusively in terms of nitrogen allocated in the cytoplasm (Figure 2). The LMA varied less, and only the current-year leaves measured in 2018 had a lower LMA than 1- and 2-year-old leaves (Figure 2).

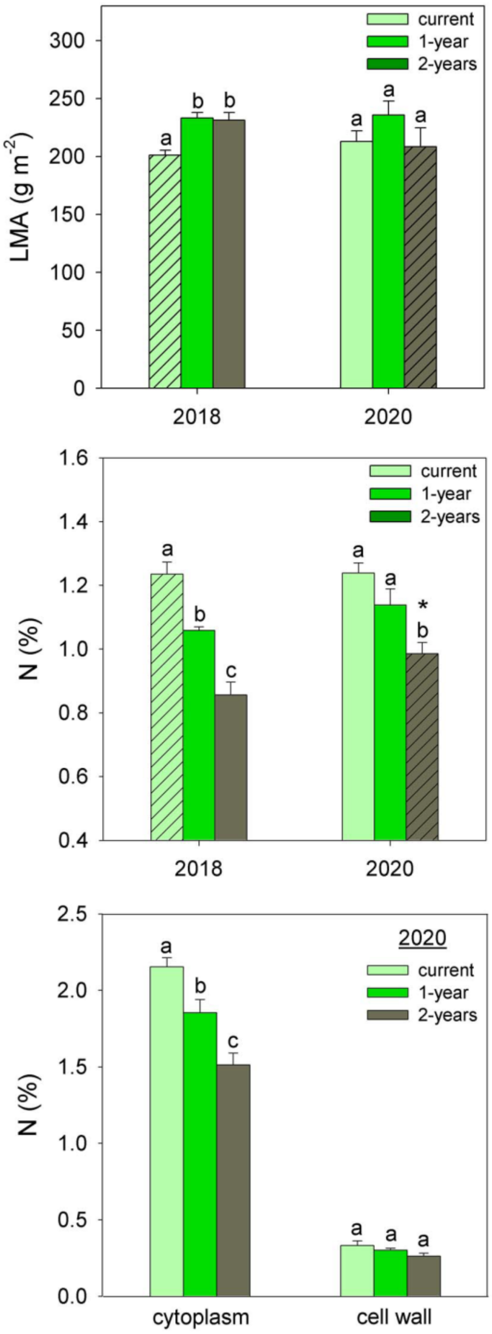

Figure 2. Leaf mass per area (LMA) (upper panel) and total leaf nitrogen concentration (N) on mass basis (medium panel) for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. Lower panel shows cytoplasmic and cell wall N concentrations on mass basis for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2020. Data are means ± SE. Different letters indicate significant differences among 0-, 1- and 2-year-old leaves (Tukey's test, $P < 0.05$). Asterisk indicates significant differences between current-year leaves measured in 2018 and 2-year-old leaves measured in September 2020 (striped bars) (Student's *t*-test, $P < 0.05$).

Leaf age-dependent differences in net assimilation rate (A_N) were primarily driven by mesophyll conductance (g_m) and maximum velocity of carboxylation (V_{cmax}). Thus, the correlations between A_N and g_m ($r^2 = 0.94$, $P < 0.001$) and between A_N and V_{cmax} ($r^2 = 0.91$, $P < 0.001$) were stronger than those found between A_N and g_s ($r^2 = 0.32$, $P < 0.001$) and between A_N and J_{flu} ($r^2 = 0.26$, $P < 0.001$) (Figure 3). Analysis of the partitioning of photosynthetic limitations further confirmed that A_N was mainly limited by g_m and the relative importance of mesophyll limitation (l_m) increased with leaf age ($P < 0.05$, Figure S3 available as Supplementary data at *Tree Physiology* Online). A_N ($r^2 = 0.40$, $P < 0.001$) and V_{cmax} ($r^2 = 0.36$, $P < 0.001$) increased with increasing N concentration on area basis (Figure 4). No significant correlations were found between LMA and A_N and between LMA and N ($P > 0.01$, Figure 4).

Two-year-old leaves had a lower chloroplast surface area facing intercellular air spaces (S_c/S) than current and 1-year-old leaves (Figure 5). Moreover, S_c/S for 2-year-old leaves measured in 2020 was much lower than that for 0-year-old leaves measured in 2018 (Student's *t*-test, $P < 0.05$) (Figure 5). This primarily reflected presence of fewer chloroplasts in older leaves (Figure 6). Furthermore, a sharp decrease in the percentage of functional area per chloroplast was observed in 2-year-old leaves, both in 2018 and 2020, mainly due to a strong increase in the size of starch grains and plastoglobuli (Figures 6 and 7). The percentage of functional area per chloroplast was also lower for 2-year-old leaves measured in 2020 than in current-year leaves measured in 2018 (Figure 7). Cell wall thickness (T_{cw}) was greater in 2-year-old leaves than in 0- and 1-year-old leaves both in 2018 and 2020 (Figure 5). There were no age-related changes in chloroplast thickness (T_{chl}) (Figure 5). Mesophyll surface area facing intercellular air spaces per leaf area (S_m/S) also did not differ among leaf cohorts, except for a higher value in 0-year-old than in 1- and 2-year-old leaves measured in 2018 (Figure 5). T_{cw}, T_{chl} and S_m/S did not display significant differences between 0-year-old leaves measured in 2018 and 2-year-old leaves measured in 2020 (Figure 5).

Across leaf ages, a strong positive relationship was observed between S_c/S and A_N ($r^2 = 0.61$, $P < 0.001$) and a negative relationship between T_{cw} and A_N ($r^2 = 0.39$, $P < 0.05$) (Figure 8). No correlations were detected between T_{chl} and S_m/S and A_N ($P > 0.05$, Figure 8). A positive linear relationship was observed between measured values of g_m (Eq. (1)) and g_m values modeled using leaf anatomical traits ($g_{m,a}$, Eq. (3)) ($r^2 = 0.44$, $P < 0.001$, Figure S4 available as Supplementary data at *Tree Physiology* Online). Together with the correlations of A_N with S_c/S and T_{cw} (Figure 8), this underscores the importance of leaf anatomy in determining the differences in g_m and A_N among the different leaf age classes in *Q. ilex* subsp. *rotundifolia*.

In the PCA, the first and second principal components accounted for 53% and 15% of the total variation (Figure S5

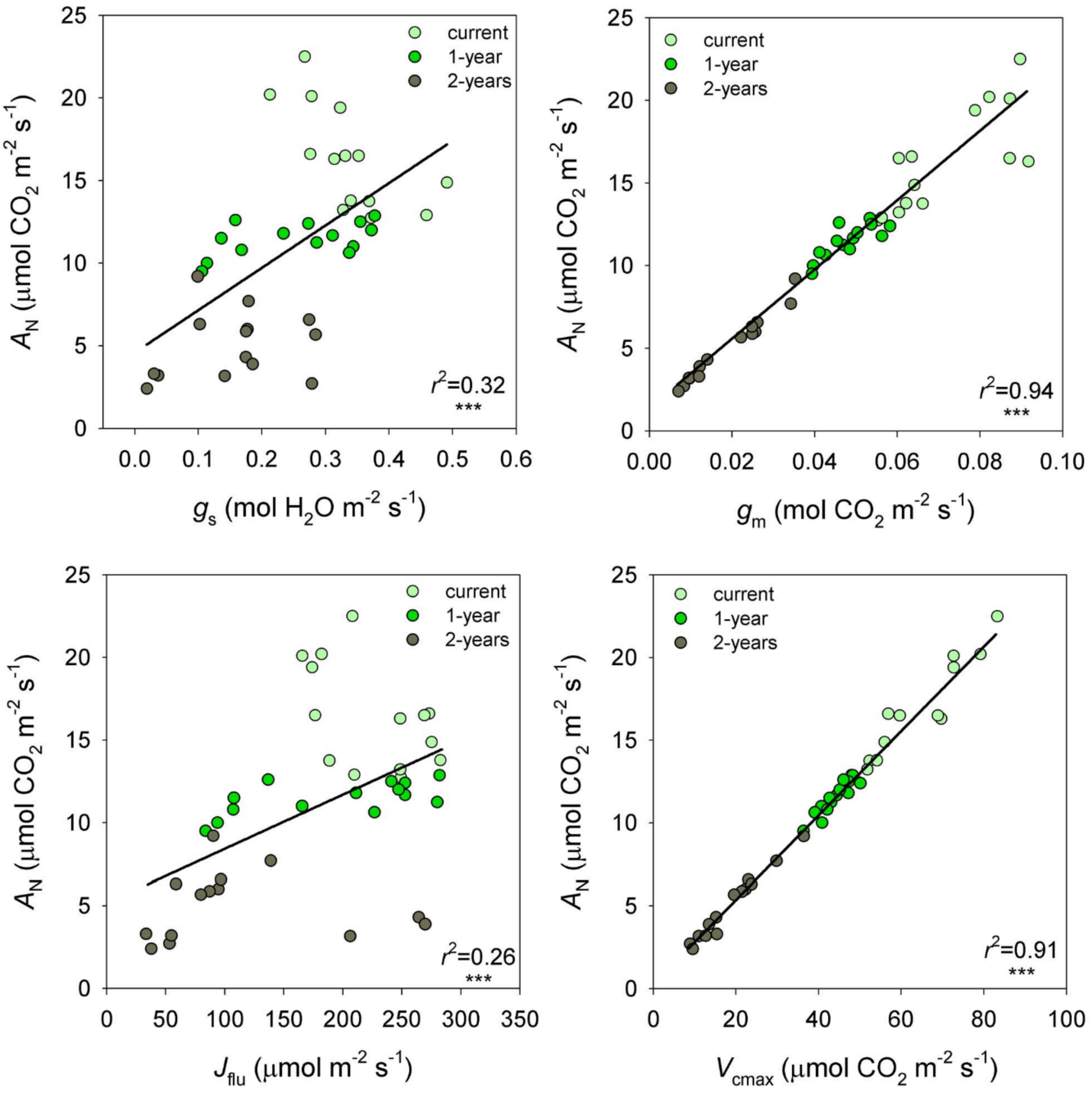

Figure 3. Relationships between net assimilation rate (A_N) and (i) stomatal conductance (g_s) (upper left panel), (ii) mesophyll conductance (g_m) (upper right panel), (iii) photosynthetic electron transport rate (J_{flu}) (lower left panel) and (iv) maximum velocity of carboxylation (V_{cmax}) (lower right panel) for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. ***$P < 0.001$.

available as Supplementary data at *Tree Physiology* Online). The first component integrated the variation in A_n, positively associated with g_m, S_c/S, V_{cmax} (and, to a lesser extent, N, g_s and J_{flu}), but negatively associated with T_{cw}. Conversely, the second component was not related with A_n, but showed positive weights for S_m/S and T_{chl}, and negative weights for g_s and J_{flu}. The scores of the studied leaf cohorts in the PCA biplot indicated that the functional and anatomical traits analyzed clearly differentiated 0-, 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia*. In particular, the three leaf age classes differed clearly along the first component (A_n and associated traits), whereas the second component explained the variability within each leaf cohort.

Discussion

Our study demonstrated a major decline in the photosynthetic activity with leaf age in *Q. ilex* subsp. *rotundifolia*, an evergreen Mediterranean oak with long leaf lifespan (Figure 1). Thus, this work agrees with the previous findings for this species by Escudero and Mediavilla (2003) and Niinemets et al. (2005, 2006). Furthermore, we have established a clear link between

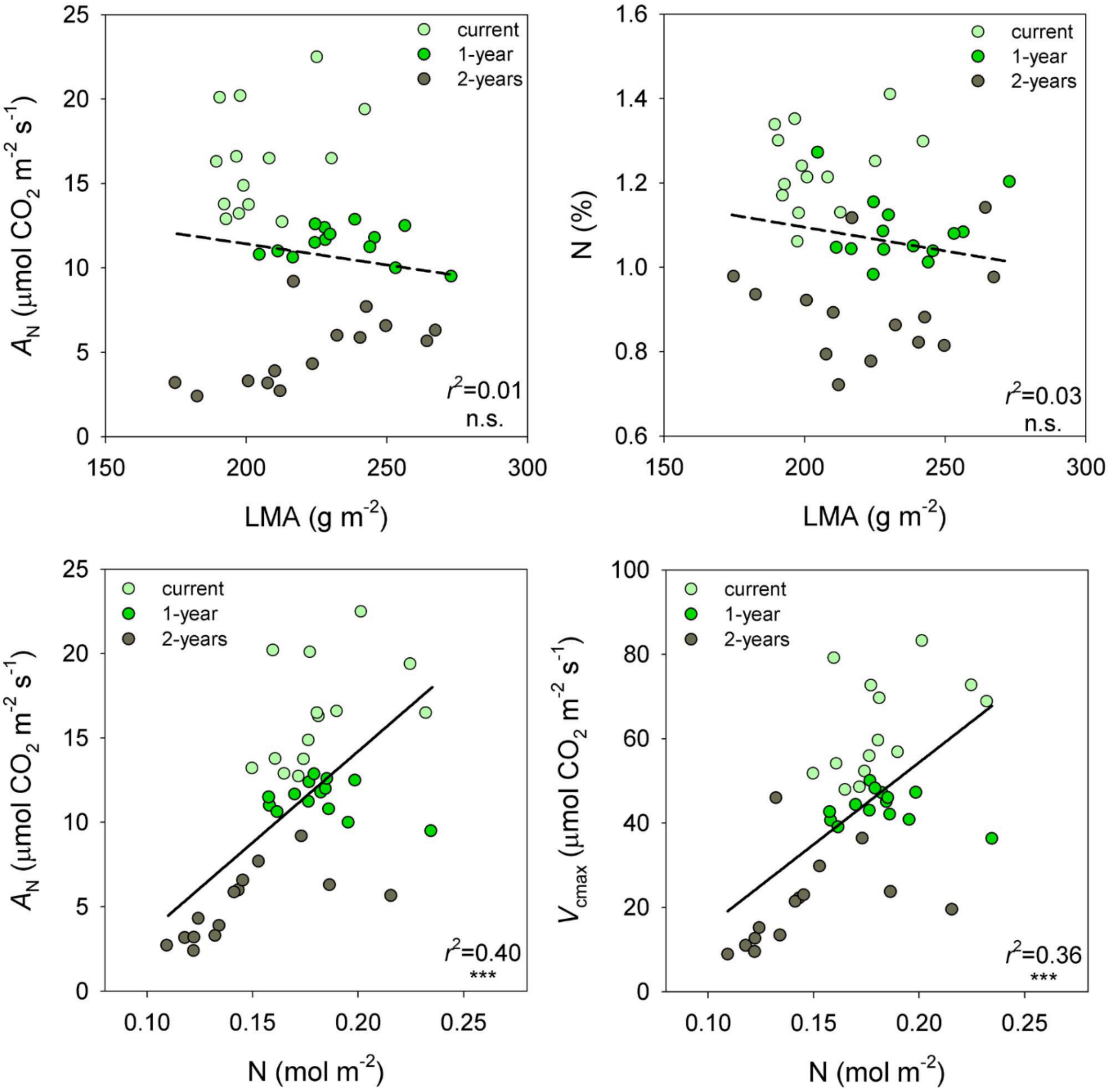

Figure 4. Relationships between LMA and (i) net assimilation rate (A_N) (upper left panel) and (ii) total leaf nitrogen concentration (N) on mass basis (upper right panel) for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. Relationships between total leaf nitrogen concentration (N) on area basis and (iii) net assimilation rate (A_N) (lower left panel), and (iv) maximum velocity of carboxylation (V_{cmax}) (lower right panel) for 0-, 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. N.s. means non-significant relationship ($P > 0.05$). ***$P < 0.001$.

the decrease in A_N and the changes in g_m experienced by the different leaf cohorts (Figure 3) as indicated by Niinemets et al. (2005) for some Mediterranean evergreen tree species. This link has been also evidenced for *Q. ilex* subsp. *rotundifolia* when describing the within-species variation in g_m and A_N (Peguero-Pina et al. 2017*b*) and the response of these traits to water stress (Peguero-Pina et al. 2018, Alonso-Forn et al. 2021). In fact, the results here obtained support the idea that A_N was mainly limited by g_m and the relative importance of mesophyll limitation (l_m) increased with increasing leaf age ($P < 0.05$, Figure S3 available as Supplementary data at *Tree Physiology* Online).

The good relationship between modeled and measured g_m (Figure S4 available as Supplementary data at *Tree Physiology* Online) largely underpins a predominant role of anatomical traits in determining photosynthetic differences among different leaf age classes in *Q. ilex* subsp. *rotundifolia*. However, it should be noted that g_m measured with the Harley et al. (1992)

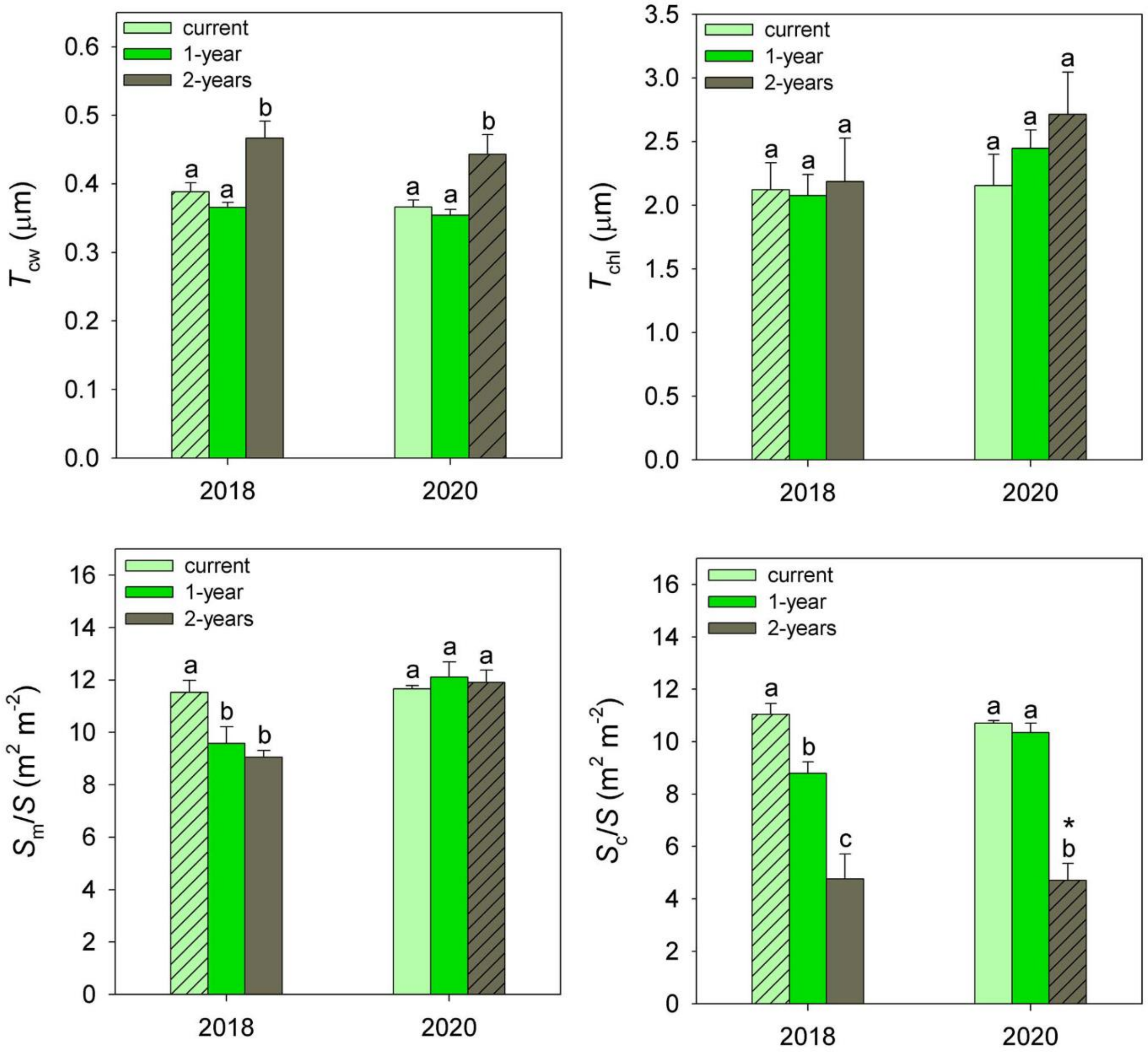

Figure 5. Cell wall thickness (T_{cw}), chloroplast thickness (T_{chl}) and mesophyll (S_m/S) and chloroplast (S_c/S) surface area facing intercellular air spaces per leaf area for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. Data are means ± SE. Different letters indicate significant differences among 0-, 1- and 2-year-old leaves (Tukey's test, $P < 0.05$). Asterisk indicates significant differences between current-year leaves measured in 2018 and 2-year-old leaves measured in September 2020 (striped bars) (Student's *t*-test, $P < 0.05$).

method was higher than g_m modeled with anatomical traits, which is contrary to expectations as modeled g_m usually gives the maximum theoretical value. This fact suggests the existence of other factors that might also influence the age-dependent changes in g_m displayed by *Q. ilex* subsp. *rotundifolia*. In this regard, Roig-Oliver et al. (2021) demonstrated that short-term changes in cell wall composition (i.e., pectins enhancement) were associated with g_m decline under water deficit in *Helianthus annuus* L. Therefore, it would be possible that long-term changes in cell wall composition might affect cell wall conductance and photosynthesis in *Q. ilex* subsp. *rotundifolia*, although this is a matter that deserves further investigation. In any case, regarding leaf anatomy, the reduction of both g_m and A_N in older leaves of this species was clearly induced by increases in cell wall thickness (T_{cw}) and reductions in chloroplast surface area exposed to intercellular air space per unit leaf area (S_c/S) (Figures 8 and S5 available as Supplementary data at *Tree Physiology* Online). To the extent of our knowledge, this is the first study that provides empirical evidence demonstrating changes in ultrastructural leaf anatomical traits as the explanation for the drawdown of photosynthesis and g_m with leaf age in species with high leaf longevity. Concerning cell wall thickness, Sugiura et al. (2020) stated that the decrease in g_m in old senescing leaves of *Glycine max* and *Phaseolus vulgaris* could

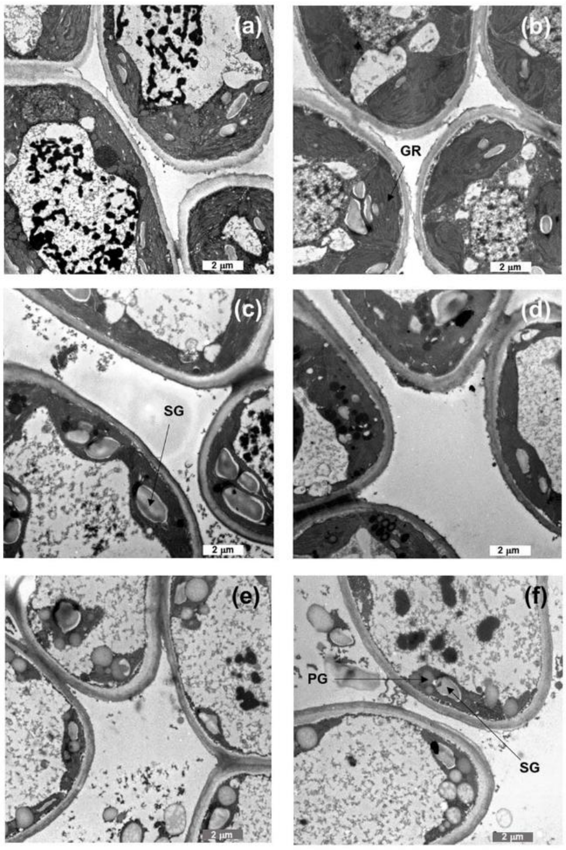

Figure 6. Transverse sections of 0- (current) (a, b), 1- (c, d) and 2-year-old (e, f) leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 (a, c, e) and 2020 (b, d, f). GR, grana; SG, starch grain; PG, plastoglobulus. Bars, 2 μm.

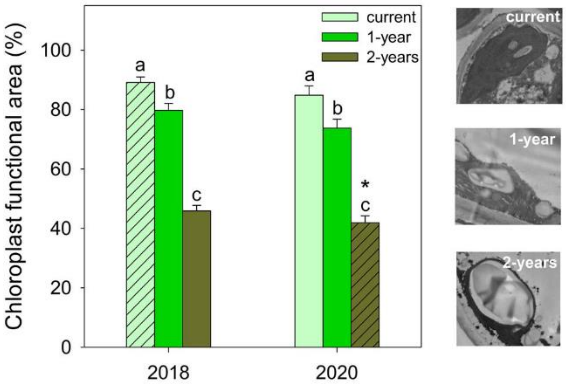

Figure 7. Functional area per chloroplast (%) for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. Data are means ± SE. Different letters indicate significant differences among 0-, 1- and 2-year-old leaves (Tukey's test, $P < 0.05$). Asterisk indicates significant differences between current-year leaves measured in 2018 and 2-year-old leaves measured in September 2020 (striped bars) (Student's *t*-test, $P < 0.05$). Micrographs located at the right of the panel show a representative chloroplast for each of the studied age classes.

be partly attributable to an increase in cell wall mass. Carriquí et al. (2021) also reported that leaf aging was associated with increases in T_{cw} in *Arabidopsis thaliana*, albeit without effects on g_m and photosynthesis probably due to a compensatory association between increased T_{cw} and decreased chloroplast thickness. Niinemets et al. (2005) suggested that the age-dependent increase in LMA in Mediterranean species reflected the higher investments in cell wall during leaf aging, with a concomitant effect on the CO_2 drawdown from internal air spaces to chloroplasts due to limited g_m. However, in our study, we only found minor changes in LMA among different leaf cohorts (Figure 2), and found no correlation between LMA and A_N (Figure 4). In addition, changes in T_{cw} during leaf aging, although correlated with A_N, were relatively small (Figures 8 and 9). By contrast, the stronger relationship found between A_N and S_c/S (Figure 8) demonstrated that S_c/S is the key anatomical trait dominating the variations in g_m and A_N among different leaf age classes in this species. Although the cell walls typically exert the strongest control on g_m (Peguero-Pina et al. 2017*a*, Veromann et al. 2017), other components such as S_c/S as observed in our study and in Veromann-Jürgenson et al. (2020), chloroplast thickness (Peguero-Pina et al. 2012) or the liquid-phase pathway length between cytosol and chloroplasts (Lei et al. 2021) can dominate changes in g_m. In fact, irrespective of the leaf age class, all T_{cw} values found in our study were relatively high, implying a major control on g_m (Terashima et al. 2011). However, the relative age-dependent change in T_{cw} was less than S_c/S, implying that age-dependent modifications in g_m were dominated by S_c/S in our study. Analogously, needle age-dependent reductions in g_m in three Mediterranean *Pinus* species were associated with reductions in S_c/S (Kuusk et al. 2018).

The reduction of S_c/S in old leaves may be related to the progressive dismantling of the photosynthetic apparatus associated to leaf aging (Niinemets et al. 2009, 2012). Chloroplast dismantling, i.e., the transition to the so-called 'gerontoplasts', is an essential process in leaf senescence that is characterized by ultrastructural changes such as the increase in size and number of plastoglobuli (Niinemets et al. 2012, Mulisch and Krupinska 2013, Domínguez and Cejudo 2021). In our study, chloroplasts in older leaves were characterized by a sharp decrease in their functional area (Figure 7), which could be associated to the size increase experienced by starch grains and plastoglobuli in 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* (Figure 6). Leaf aging was also manifested in a decrease in total leaf nitrogen concentration, mainly in terms of nitrogen allocated in the cytoplasm of 2-year-old leaves (Figure 2). Consequently, it might be expected that the dismantling of the photosynthetic apparatus could have a strong impact on the amount of Rubisco, explaining the sharp decrease in V_{cmax}

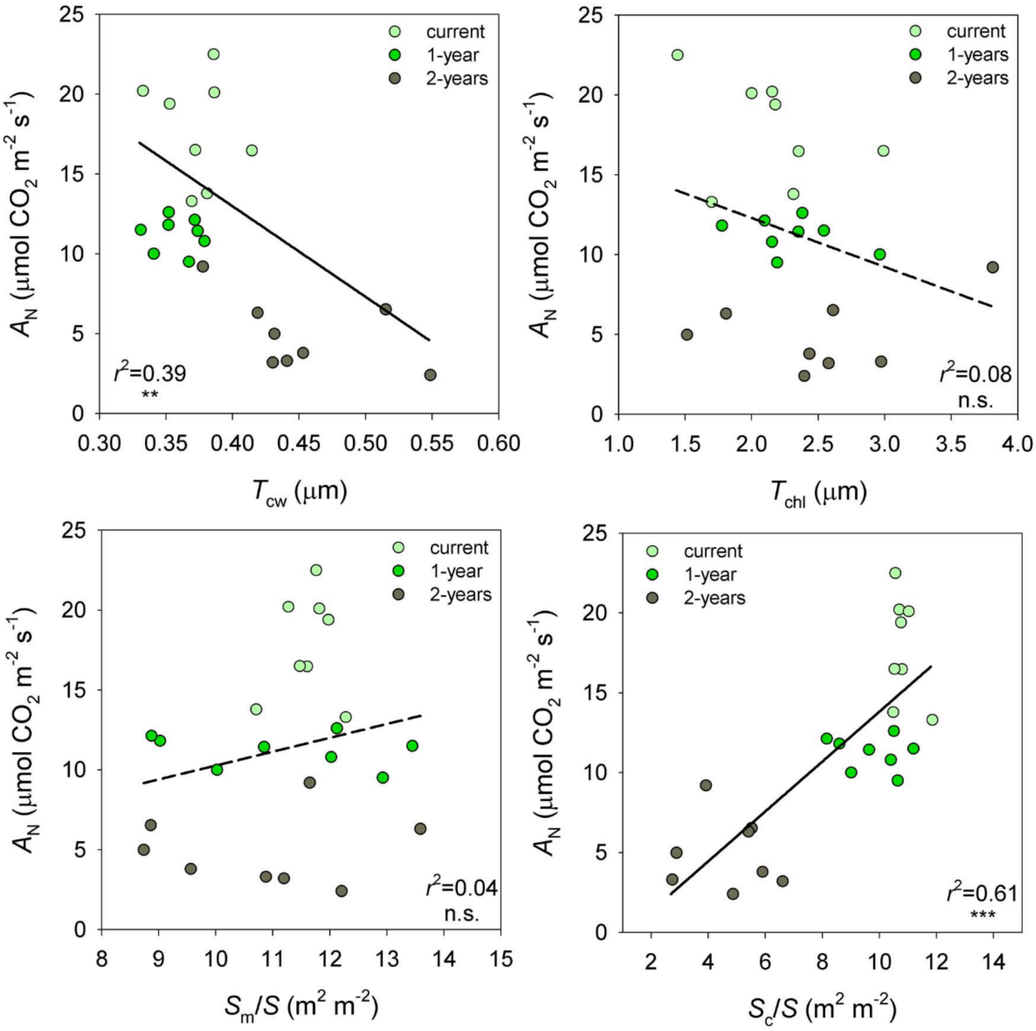

Figure 8. Relationships between net assimilation rate (A_N) and (i) cell wall thickness (T_{cw}) (upper left panel), (ii) chloroplast thickness (T_{chl}) (upper right panel), (iii) mesophyll surface area facing intercellular air spaces per leaf area (S_m/S) (lower left panel) and (iv) chloroplast surface area facing intercellular air spaces per leaf area (S_c/S) (lower right panel) for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. N.s. denotes a non-significant relationship ($P > 0.05$). $^{**}P < 0.05$. $^{***}P < 0.001$.

experienced by 2-year-old leaves (Figure 1), with a substantial impact on the photosynthetic capacity of this species (Figure 3 and 4).

At this point, the next question is, which is triggering the age-dependent decline of photosynthetic potential in *Q. ilex* subsp. *rotundifolia*? Different authors have argued that this could be the consequence of self-shading of older leaves within the crown as they age (Field and Mooney 1983, Kitajima et al. 1997, Ackerly 1999, Ishida et al. 1999, Niinemets et al. 2004, 2006, 2009). Thus, Terashima et al. (2005) stated that self-shading of older leaves might be involved in chlorophyll degradation and the regulation of leaf senescence. On the other hand, reacclimation of evergreen leaves to reduced light conditions is typically associated with increased chlorophyll content to harvest more light (Brooks et al. 1994, 1996). In addition, in these studies, chlorophyll a to b content also decreased upon reacclimation of older leaves after shading (Brooks et al. 1996), indicating a greater share of chlorophyll associated with the light harvesting complex II (Lichtenthaler et al. 2007). We observed a decrease in light availability in 1- and 2-year-old leaves with respect to current-year leaves of *Q. ilex* subsp. *rotundifolia* (Figures S1 and S2 available as Supplementary data at *Tree Physiology* Online), but without major consequences for the total amount of chlorophylls (Chl a + Chl b, Table S2 available as Supplementary data at *Tree Physiology* Online). However, contrary to other studies, the ratio Chl a/Chl b was higher in 2-year-old than in 1-year-old leaves (Table S2 available as Supplementary data at *Tree Physiology* Online), indicating limited acclimation of pigment-protein complexes to altered light level but an acclimation to a higher light energy excess in *Q. ilex* subsp. *rotundifolia*.

Self-shading could also promote a decrease of nitrogen in older foliage due to the retranslocation from shaded to sunlit leaves (Hikosaka 2005). The concurrent decrease of A_N and nitrogen should imply the absence of changes in photosynthetic nitrogen-use efficiency (PNUE) with leaf age (Mooney et al. 1981, Field and Mooney 1983). However, in our study, we observed a strong decrease in PNUE with leaf age in *Q. ilex* subsp. *rotundifolia*, as demonstrated in other evergreen oak species (Escudero and Mediavilla 2003, Niinemets et al. 2004, Yasumura and Ishida 2011). This reflected a much greater decrease in net CO_2 assimilation than in leaf nitrogen content (Table S1 available as Supplementary data at *Tree Physiology* Online). Thus, this species has a substantial fraction of older leaves with an inherently low photosynthetic potential and nitrogen-use efficiency, irrespective of the amount of incident light (Table S1 available as Supplementary data at *Tree Physiology* Online). Despite this, 2-year-old leaves maintained a positive carbon balance, so the retention of these leaves would yield a higher whole-canopy net CO_2 assimilation despite their low PNUE and intrinsic water-use efficiency (iWUE = A_N/g_s) (Table S1 available as Supplementary data at *Tree Physiology* Online).

Besides self-shading, a possible increase in the allocation of nitrogen to cell walls with leaf age could also decrease photosynthetic activity and PNUE (Niinemets et al. 2009). A higher investment on cell wall biomass is expected to lead to an increase of LMA (Onoda et al. 2004). However, as stated above, we only found minor changes in LMA among different leaf cohorts (Figure 2), which was in line with the results obtained by Yasumura and Ishida (2011) for the evergreen oak *Quercus myrsinaefolia*. Moreover, we did not observe an increase in the nitrogen content per dry mass in the cell wall with leaf age (Figure 2), and T_{cw} did not show differences between the same cohort of leaves measured in 2018 (current-year) and in 2020 (2-year-old) (Figure 5).

Therefore, it is not well established the triggering factor for the age-dependent physiological deterioration evidenced by leaves of *Q. ilex* subsp. *rotundifolia*. In this regard, the role of leaf hydraulic conductance decline as the causing factor of photosynthetic decay in senescent leaves should be also considered. The role of leaf hydraulics in leaf senescence was suggested early by Neumann and Stein (1984), who hypothesized that the sequential pattern of leaf senescence observed during plant development in *P. vulgaris* L. may be related to progressive changes in the hydraulic architecture of the plant. Brodribb and Holbrook (2003) found a declining leaf hydraulic conductance that was probably linked to the loss of photosynthetic capacity during leaf senescence in two deciduous tree species (*Calycophyllum candidissimum* and *Rhedera trinervis*). In line with this, Locke and Ort (2014) showed that hydraulic decline in *G. max* during senescence was accompanied by gradual decreases in leaf water status and photosynthesis, although they could not conclude if this decline triggered photosynthetic decline and senescence. This mechanism was described by Giraldo et al. (2013), who proposed that the timing of leaf senescence in *Solanum lycopersicum* L. was determined by an age-dependent decline in leaf hydraulic conductance limiting gas exchange. However, these authors did not find evidence of this phenomenon in deciduous tree species such as *Acer saccharum* Marsh. and *Quercus rubra* L. In any case, to the best of our knowledge, no studies have dealt with this issue in evergreen trees with long leaf lifespan. More research is needed to clarify the possible involvement of leaf hydraulics decline in the age-dependent changes of photosynthetic capacity observed in *Q. ilex* subsp. *rotundifolia*.

Conclusions

In conclusion, our study demonstrates major age-dependent changes in leaf photosynthetic capacity that were driven by alterations in mesophyll conductance due to anatomical modification. However, the age-dependent decrease in the foliage photosynthetic potentials was weakly associated with self-shading and/or an increase in cell wall biomass. By contrast, confirming our hypotheses, we observed that changes in leaf anatomical (i.e., a strong decrease in S_c/S) and biochemical (i.e., a decrease in leaf nitrogen content and nitrogen investments in photosynthetic machinery) traits were responsible for the age-dependent modifications. These changes reflect a gradual physiological deterioration related to the dismantling of the photosynthetic apparatus. Further research is needed to elucidate the ultimate causing factor that triggers the physiological deterioration of leaves of evergreen trees with long leaf lifespan.

Authors' contributions

D.A.-F., J.J.P.–P., J.P.F., D.S.-K. and E.G.-P. planned and designed the research. D.A.-F., J.J.P.–P., J.P.F., R.M.-S. and D.S.-K. performed the experiments. D.A.-F., J.J.P.–P., J.P.F., J.I.G.-P., Ü.N., D.S.-K. and E.G-P. analyzed the data. D.A.-F., J.J.P.–P and E.G.-P. drafted the manuscript. All authors edited the manuscript with valuable inputs.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Funding

This research was funded by Grant PID2019-106701RR-I00 funded by MCIN/AEI/10.13039/501100011033 and by Gobierno de Aragón H09_20R research group. The work of D.A.-F. is supported by an FPI-INIA contract BES-2017-081208 and that of R.M.-S. is supported by a pre-doctoral Gobierno de Aragón scholarship.

Conflict of interest

None declared.

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Supporting Information

Figure S1. Photosynthetic photon flux density (PPFD) during three sunny days of September 2018 for each of the three leaf cohorts studied (0- (current), 1- and 2-year-old leaves) of *Q. ilex* subsp. *rotundifolia*.

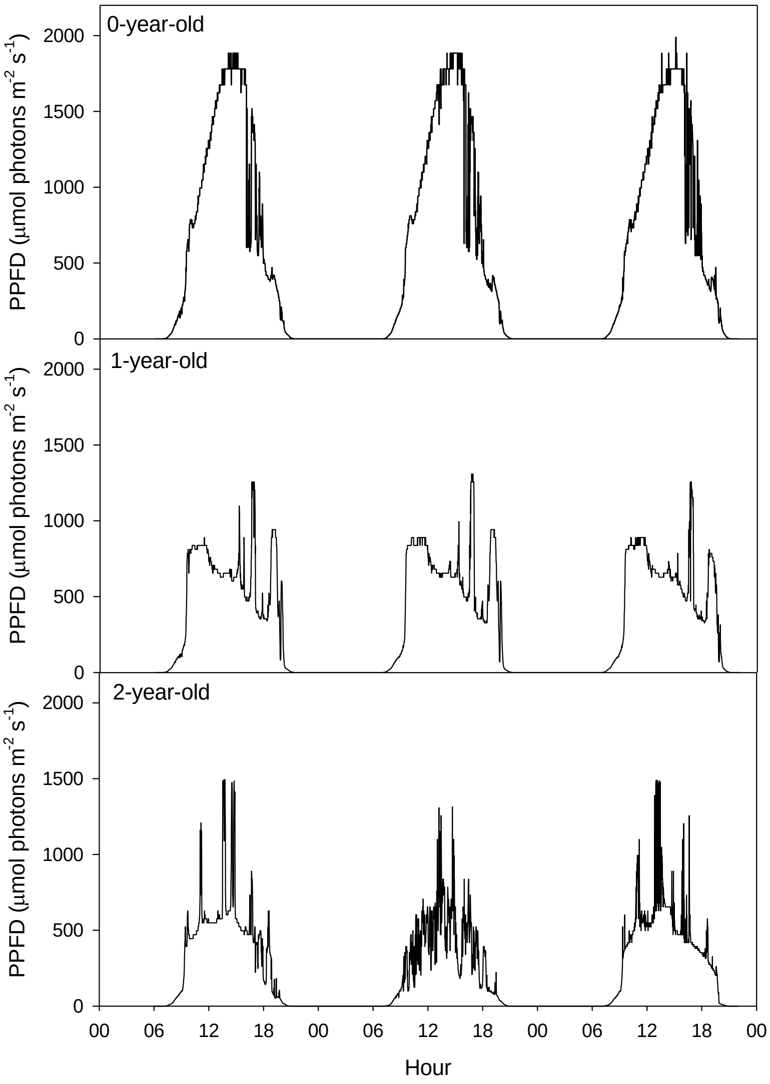

Figure S2. Integrated daily quantum flux density (Q_{int}) during three sunny days of September 2018 for each of the three leaf cohorts studied (0- (current), 1- and 2-year-old) of *Q. ilex* subsp. *rotundifolia*. Different letters indicate significant differences among 0-, 1- and 2-year-old leaves (Tukey's test, $P < 0.05$).

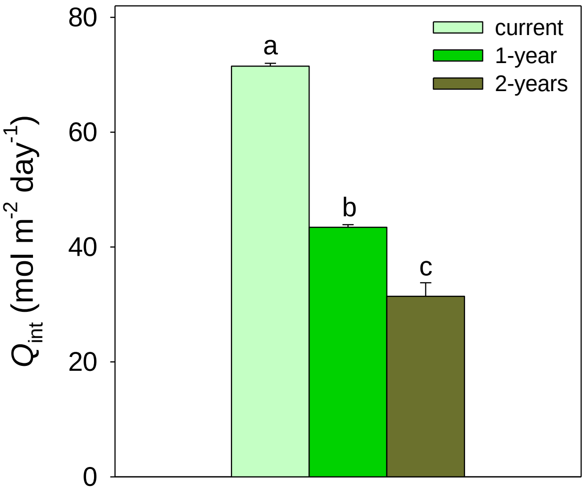

Figure S3. Relative stomatal (l_s), mesophyll (l_m) and biochemical (l_b) photosynthesis limitations for 0-(current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. The increase of l_m with increasing leaf age was statistically significant at $P < 0.05$ both in 2018 and 2020.

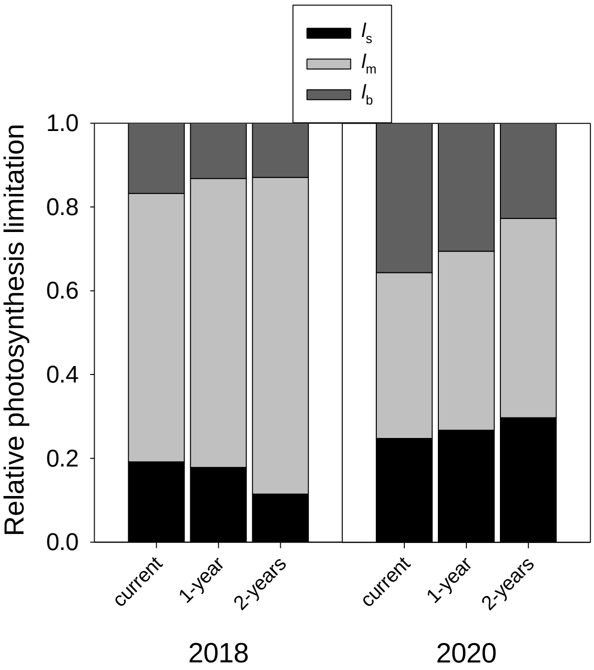

Figure S4. Relationship between mesophyll conductance (g_m) modelled with anatomical traits (Eq. 3) and g_m measured with the Harley et al. (1992) method (Eq. 1) for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. *** - *P* < 0.001.

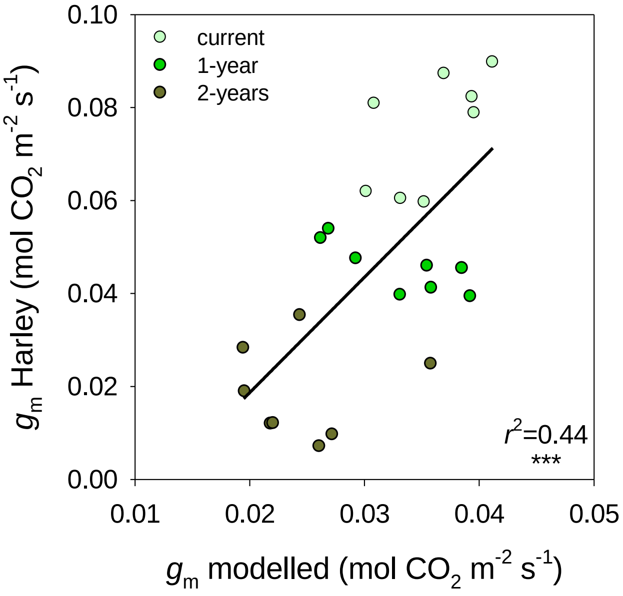

Figure S5. Relationship between the first two principal components (PC1 vs. PC2) of the principal component analysis (PCA) computed on the measured traits obtained for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 and 2020. Variables: A_N, net assimilation rate; g_s, stomatal conductance; g_m, mesophyll conductance; J_{flu}, photosynthetic electron transport rate; V_{cmax}, maximum velocity of carboxylation; LMA, leaf dry mass per unit area; N, total leaf nitrogen concentration; T_{cw}, cell wall thickness; T_{chl}, chloroplast thickness; S_m/S and S_c/S, mesophyll and chloroplast surface area facing intercellular air spaces per leaf area.

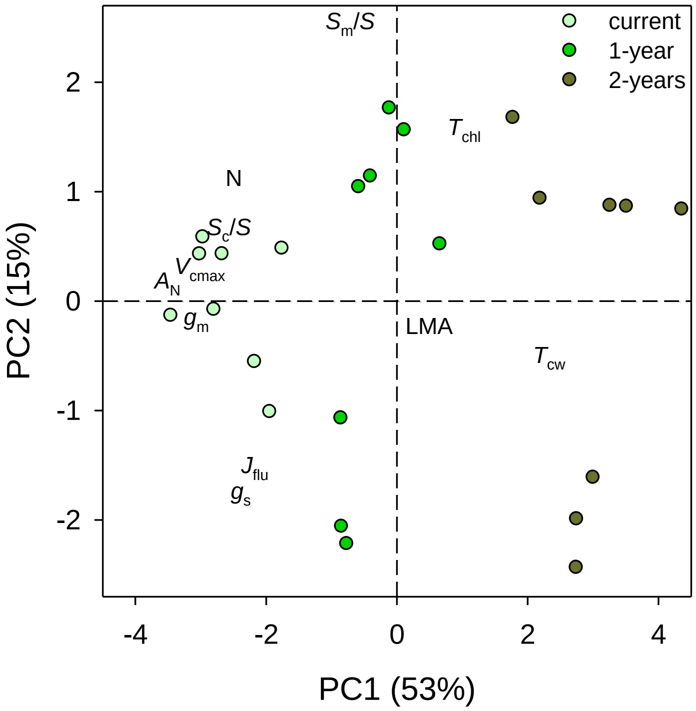

Table S1. Photosynthetic characteristics for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2018 at three photosynthetic photon flux density (PPFD) conditions (1500, 750 and 350 μmol m^{-2} s^{-1}): A_N, net assimilation rate; g_s, stomatal conductance; g_m, mesophyll conductance; V_{cmax}, maximum velocity of carboxylation; Φ_{PSII}, effective quantum yield of PSII; iWUE, intrinsic water use efficiency (A_N/g_s); PNUE, photosynthetic nitrogen use efficiency (A_N/N). Different letters indicate significant differences among 0-, 1- and 2-year-old leaves (Tukey's test, $P < 0.05$).

		Leaf Age	PPFD (μmol photons m^{-2} s^{-1})		
			1500	750	350
		0	14.5 ± 0.5^{a}	11.7 ± 1.6^{a}	7.4 ± 0.6^{a}
A_N	(μmol CO_2 m^{-2} s^{-1})	1	11.8 ± 0.2^{b}	10.7 ± 0.9^{a}	7.1 ± 0.6^{a}
		2	5.1 ± 0.5^{c}	6.3 ± 0.7^{b}	4.9 ± 0.6^{b}
		0	0.367 ± 0.023^{a}	0.265 ± 0.049^{a}	0.276 ± 0.046^{a}
g_s	(mol H_2O m^{-2} s^{-1})	1	0.321 ± 0.016^{a}	0.245 ± 0.032^{a}	0.256 ± 0.035^{a}
		2	0.208 ± 0.018^{b}	0.182 ± 0.029^{a}	0.192 ± 0.031^{a}
		0	0.067 ± 0.004^{a}	0.065 ± 0.011^{a}	0.035 ± 0.004^{a}
g_m	(mol CO_2 m^{-2} s^{-1})	1	0.051 ± 0.002^{b}	0.053 ± 0.005^{a}	0.032 ± 0.004^{a}
		2	0.020 ± 0.003^{c}	0.026 ± 0.004^{b}	0.020 ± 0.003^{b}
		0	0.37 ± 0.02^{a}	0.46 ± 0.03^{a}	0.55 ± 0.02^{a}
Φ_{PSII}		1	0.35 ± 0.02^{a}	0.46 ± 0.02^{a}	0.58 ± 0.01^{a}
		2	0.21 ± 0.04^{b}	0.35 ± 0.02^{b}	0.51 ± 0.02^{b}
		0	41.1 ± 3.4^{a}	47.6 ± 3.2^{a}	31.2 ± 3.6^{a}
iWUE	(μmol CO_2 mol^{-1} H_2O)	1	37.5 ± 2.2^{a}	45.8 ± 2.3^{ab}	30.1 ± 2.7^{a}
		2	25.8 ± 3.2^{b}	38.2 ± 3.7^{b}	28.9 ± 3.3^{a}
		0	82.1 ± 2.2^{a}	65.0 ± 8.3^{a}	41.9 ± 3.3^{a}
PNUE	(μmol CO_2 mol N^{-1} s^{-1})	1	67.0 ± 1.1^{b}	60.9 ± 5.4^{a}	40.4 ± 3.4^{a}
		2	35.7 ± 3.1^{c}	46.3 ± 5.9^{a}	35.8 ± 4.6^{a}

Table S2. Total chlorophyll concentration (Chl a + Chl b) and the ratio between Chl a and Chl b concentrations for 0- (current), 1- and 2-year-old leaves of *Q. ilex* subsp. *rotundifolia* measured in 2020. Different letters indicate significant differences among 0-, 1- and 2-year-old leaves (Tukey's test, $P < 0.05$).

Leaf Age	Chl a + Chl b (μmol m^{-2})	Chl a / Chl b (mol mol^{-1})
0	458.6 ± 4.0^{ab}	3.0 ± 0.2^{ab}
1	541.6 ± 2.9^{a}	2.7 ± 0.1^{a}
2	364.2 ± 2.3^{b}	3.1 ± 0.1^{b}

Capítulo VI

Contrasting functional strategies following severe drought in two Mediterranean oaks with different leaf habit: *Quercus faginea* and *Quercus ilex* subsp. *rotundifolia*

Tree Physiology 41, 371–387
doi:10.1093/treephys/tpaa135

Research paper

Contrasting functional strategies following severe drought in two Mediterranean oaks with different leaf habit: *Quercus faginea* and *Quercus ilex* subsp. *rotundifolia*

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Received May 27, 2020; accepted October 8, 2020; handling Editor Roberto Tognetti

Nowadays, evergreen sclerophyllous and winter-deciduous malacophyllous oaks with different paleogeographical origins coexist under Mediterranean-type climates, such as the mixed forests of the evergreen *Quercus ilex* subsp. *rotundifolia* Lam. and the winter-deciduous *Quercus faginea* Lam. Both Mediterranean oaks constitute two examples of contrasting leaf habit, so it could be expected that they would have different functional strategies to cope with summer drought. In this study, we analysed photosynthetic, photochemical and hydraulic traits of different organs for *Q. faginea* and *Q. ilex* subsp. *rotundifolia* under well-watered conditions and subjected to very severe drought. The coordinated response between photosynthetic and hydraulic traits explained the higher photosynthetic capacity of *Q. faginea* under well-watered conditions, which compensated its shorter leaf life span at the expense of higher water consumption. The progressive imposition of water stress evidenced that both types of Mediterranean oaks displayed different functional strategies to cope with water limitations. Specifically, the decrease in mesophyll conductance associated with edaphic drought seems to be the main factor explaining the differences found in the dynamics of net CO_2 assimilation throughout the drought period. The sharp decline in photosynthetic traits of *Q. faginea* was coupled with a strong decrease in shoot hydraulic conductance in response to drought. This fact probably avoided extensive xylem embolism in the stems (i.e., 'vulnerability segmentation'), which enabled new leaf development after drought period in *Q. faginea*. By contrast, leaves of *Q. ilex* subsp. *rotundifolia* showed effective photoprotective mechanisms and high resistance to drought-induced cavitation, which would be related with the longer leaf life span of the evergreen Mediterranean oaks. The co-occurrence of both types of Mediterranean oaks could be related to edaphic conditions that ensure the maintenance of soil water potential above critical values for *Q. faginea*, which can be severely affected by soil degradation and climate change.

Keywords: hydraulic traits, Mediterranean-type climates, mesophyll conductance, photoprotection, photosynthesis, vulnerability segmentation, water stress.

Introduction

Two types of oaks (*Quercus* L.; Fagaceae) currently coexist in locations under genuine Mediterranean-type climates, i.e., the well-known evergreen sclerophyllous type and the sparsely accepted winter-deciduous malacophyllous type (see Gil-Pelegrín et al. 2017 and references therein). Several authors have reported such co-occurrence (Tognetti et al. 1998, Nardini et al. 1999, Montserrat-Martí et al. 2009) and considered as a peculiarity of the Mediterranean biome (Baldocchi et al. 2010). The evergreen sclerophyllous oaks have been considered keystone Mediterranean tree species that would have their origin in pre-Mediterranean lineages of the Palaeotropical geoflora (Valiente-Banuet et al. 2006, Denk et al. 2017, Jiang et al. 2019) and display morphological traits traditionally associated to Mediterranean-type climates with dry and hot summers (Traiser et al. 2005, Walter 1985). Nevertheless, most of the winter-deciduous Mediterranean oak species, in spite of having an Arcto-Tertiary origin (Grímsson et al. 2015), seem to be able to cope with the extreme summer aridity period in a similar way to their evergreen relatives (Radoglou 1996, Scarascia-Mugnozza et al. 2000), and some of them inhabit under extremely dry Mediterranean sub-types (Dufour-Dror and Ertas 2004).

The mixed forests of the evergreen sclerophyllous *Quercus ilex* subsp. *rotundifolia* Lam. and winter-deciduous *Quercus faginea* Lam. located in the inland areas of the Iberian Peninsula would be a good example of this situation (Forner et al. 2018*a*, Montserrat-Martí et al. 2009). These areas are characterized by the occurrence of two potential stress periods throughout the year, the cold winter and the dry and hot summer (Mediavilla and Escudero 2004, Montserrat-Martí et al. 2009). Leaf shedding during the autumn allows *Q. faginea* to evade the negative effect of low temperatures experienced by the leaves of *Q. ilex* subsp. *rotundifolia* (Corcuera et al. 2005*a*, 2005*b*). However, the incidence of a dry period during summer severely affects the performance and growth of the two co-occurring species (Corcuera et al. 2004*a*, 2004*b*). In this context, it is crucial for Mediterranean tree species to develop different mechanisms and strategies to survive under situations of water deficit (Baldocchi et al. 2010, Forner et al. 2018*b*).

Stomatal closure under drought conditions regulates water consumption at the expense of a reduction in photosynthesis, which has been extensively reported for both types of Mediterranean oaks (Acherar and Rambal 1992, Mediavilla and Escudero 2003, 2004, Peguero-Pina et al. 2008*a*, 2009, 2018). Besides stomatal limitations, the decrease of the diffusion of CO_2 from the sub-stomatal cavities to the sites of carboxylation (i.e., a reduced mesophyll conductance, g_m) is also one of the key factors limiting photosynthesis under water stress (Flexas et al. 2012, Niinemets and Keenan 2014). However, to the extent of our knowledge, only a few studies have dealt with this topic in Mediterranean oaks (Peguero-Pina et al. 2017*a* and references therein). An increase of limitations to photosynthesis in response to drought implies that light incident on the leaf surface may exceed the amount that can be used for photosynthesis. Under this situation, different mechanisms allow the protection of the photosynthetic apparatus, such as the non-photochemical quenching (NPQ) of chlorophyll fluorescence and the de-epoxidation of the xanthophyll cycle, which have been described for some evergreen and deciduous oaks in response to moderate or severe drought conditions (see García-Plazaola et al. 2017 and references therein).

Another efficient strategy to survive under water deficit is the development of xylem with a high resistance to water stress-induced embolism, as the failure of the water transport system is a primary cause of plant mortality during drought (Martin-StPaul et al. 2017, Rodriguez-Dominguez et al. 2018). This seems to be the case in both types of Mediterranean oaks, which show very negative values of water potential inducing 50% and 88% loss of hydraulic conductivity in the stems (PLC_{50} and PLC_{88}, respectively), as reviewed by Gil-Pelegrín et al. (2017). Thus, the combination of an early stomatal closure together with a high resistance of the stem to embolism (i.e., a wide hydraulic safety margin, Martin-StPaul et al. 2017) in Mediterranean oaks would constitute a key factor for increasing the survival under Mediterranean severe drought conditions (Vilagrosa et al. 2010).

Besides stem vulnerability, plant tolerance to drought would be constrained by the capacity of other plant organs to withstand xylem embolism (Skelton et al. 2018). This phenomenon is known as 'vulnerability segmentation' (Hochberg et al. 2017), and could create hydraulic fuses within the plant in order to confine the drought-induced cavitation to the most expendable parts of the plant (e.g., leaves) and further protect the more costly tissues (e.g., stems or roots) (Creek et al. 2018, Rodriguez-Dominguez et al. 2018). However, contrastingly to stem xylem embolism, only a few studies have dealt with the dynamic of leaf or shoot hydraulic conductance under water stress conditions in Mediterranean oaks (Nardini et al. 2012, Nardini and Tyree 1999, Peguero-Pina et al. 2015, Scoffoni et al. 2012, Skelton et al. 2018). Furthermore, leaf hydraulic conductance is also considered a key factor for net CO_2 assimilation (Scoffoni et al. 2012), possibly through its covariation with stomatal and mesophyll conductance (Flexas et al. 2013, Wang et al. 2018).

In particular, regarding *Q. ilex* subsp. *rotundifolia*, a considerable number of studies have dealt with its physiological performance under drought stress from different viewpoints. Thus, a drop in water potential increased the diffusive and non-diffusive limitations to net CO_2 assimilation in this species (Limousin et al. 2010, Gallé et al. 2011, Zhou et al. 2014, Peguero-Pina et al. 2018), which responded through photoprotective mechanisms for dissipating the excess of light energy

(Peguero-Pina et al. 2009, Sancho-Knapik et al. 2018). Moreover, the reported curves of stem xylem cavitation seem to indicate a high resistance to drought-induced cavitation (e.g., PLC_{50} ca −6.5 MPa according to Peguero-Pina et al. 2014). Nevertheless, the tolerance of other tissues (i.e., leaves or shoots) of *Q. ilex* subsp. *rotundifolia* to drought-induced cavitation has not been studied yet. A high resistance would be also expected, as recently found by Rodriguez-Dominguez et al. (2018) in *Olea europaea*, another very resilient Mediterranean evergreen tree that is able to resist high level of water stress. In contrast, concerning *Q. faginea*, several studies have reported an early stomatal closure in response to water scarcity, even more marked than that found for *Q. ilex* subsp. *rotundifolia* (Acherar and Rambal 1992, Forner et al. 2018a, Mediavilla and Escudero 2003, 2004). However, as far as we know, no studies have dealt with the dynamic of mesophyll conductance, the role of non-stomatal limitations to photosynthesis and the existence of efficient photoprotective mechanisms when this species is subjected to severe water shortage. Regarding hydraulic traits, although Esteso-Martínez et al. (2006) reported a relatively low sensitivity of the stem xylem of seedlings of *Q. faginea* to drought-induced cavitation (PLC_{50} = −3.9 MPa), there is a lack of studies about the sensitivity or leaves or shoots to drought-induced cavitation in *Q. faginea*.

Therefore, it could be expected that both species show different functional strategies to cope with water limitations that go beyond that they constitute two examples of contrasting leaf habit (Escudero et al. 2017). Very few comparative ecophysiological studies have analysed simultaneously the response to intense water deficit of photosynthetic, photochemical and hydraulic traits of different organs involving co-occurring Mediterranean oaks with contrasting leaf habits (Ramírez-Valiente et al. 2020). In this sense, the specific objectives of this study are: (i) to analyse the existence of a differential response to very severe drought in photosynthetic, photochemical and hydraulic traits of *Q. faginea* and *Q. ilex* subsp. *rotundifolia*, and (ii) to elucidate the possible existence of a coordination among these traits for both species to withstand water shortage.

Materials and methods

Plant material and experimental conditions

This study was carried out with plants of *Quercus faginea* Lam. and *Quercus ilex* subsp. *rotundifolia* from the same provenance ('Alcarria-Serranía de Cuenca' provenance, 40°19′N, 2°15′W, 950 m above sea level, Spain). Acorns from both species were sown and cultivated in 2009 under the same conditions with a mixture of 80% compost (Neuhaus Humin Substrat N6; Klasman-Deilmann GmbH, Geeste, Germany) and 20% perlite in 0.5 l containers inside a greenhouse. After the first growth cycle, seedlings were transplanted to 25 l containers filled with the same mixture described above and cultivated outdoors in a common garden at CITA de Aragón (41°39′N, 0°52′W, Zaragoza, Spain) under Mediterranean conditions (mean annual temperature 15.4 °C, total annual precipitation 298 mm). A slow-release fertilizer (15:9:12 nitrogen:phosphorous:potassium (N:P:K), Osmocote Plus, Sierra Chemical, Milpitas, CA, USA) was added twice a year to the top 10-cm layer of substrate (3 g l^{-1} growth substrate). All plants were grown under the same environmental conditions, drip-irrigated to field capacity every 2 days and pruned when necessary until just before the beginning of the vegetative period of 2018.

Two weeks before the onset of the experiment, five potted plants per species (10 years old) were placed under a polyethylene roof (200 μm thickness) with open sides that allowed passing 90% of photosynthetic photon flux density (PPFD) (~1800 μmol photons m^{-2} s^{-1} at midday, over the course of the experiment). We used this cover to avoid re-watering by unwanted precipitation events throughout the drought period. Irrigation was stopped on 11 July 2018 and all measurements started on 12 July 2018 under well-watered conditions in the five plants per species (thereafter 'stressed plants'). During the following days, all measurements were simultaneously performed in the same five stressed plants per species every 2–4 days with increasing levels of drought: 12, 16, 19, 23, 25, 27, 30 July and 1 August 2018 for *Q. faginea*; 12, 16, 20, 24, 28 July and 1 August 2018 for *Q. ilex* subsp. *rotundifolia*. Drought stress was imposed until 2 August 2018, when the percentage of green leaves was almost negligible for both species (11% for *Q. faginea* and 12% for *Q. ilex* subsp. *rotundifolia*, Figure 1). Subsequently, stressed plants were irrigated again to field capacity after the last measurement under drought conditions.

Plants of *Q. faginea* burst buds again at the end of the summer; we took advantage of this event to examine the hydraulic capacity of the new stems (hereafter 'second growth') and those that experienced the drought period (hereafter 'first growth') in the stressed plants. For comparative purposes, we also measured the hydraulic capacity of the first and second growth in another set of five plants of *Q. faginea* that were grown under the same environmental conditions than stressed plants although drip-irrigated to field capacity every 2 days throughout the summer (hereafter 'control plants').

Water potential measurements

Leaf water potential was measured at predawn and midday (Ψ_{PD} and Ψ_{MD}, respectively, MPa) throughout the drought period in one shoot of each one of the five studied stressed plants per species (with leaves still attached to the shoots) with a Scholander pressure chamber, according to the methodology described by Turner (1988).

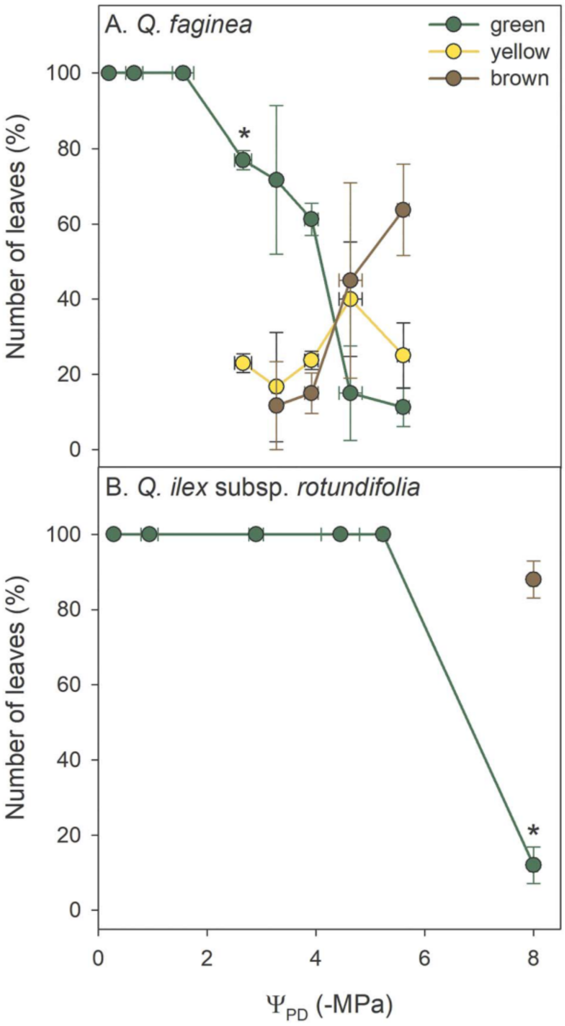

Figure 1. Time course of the percentage of green, yellow and brown leaves with predawn water potential (Ψ_{PD}) for *Q. faginea* (A) and *Q. ilex* subsp. *rotundifolia* (B) during the drought period. Data are mean ± standard error (SE) of each date when stressed plants were measured (see Materials and methods for details). Asterisks indicate the point of the drought period when the decrease of the percentage of green leaves was statistically significant (pairwise *t*-test, $P < 0.05$) compared with well-watered conditions (first point of the graph).

Percentage of leaf damage using visual scoring and spectral reflectance

Percentage of green, yellow and brown leaves was estimated by means of visual scoring (VS) throughout the drought period for each one of the five studied stressed plants per species. To test that colour changes were associated to changes in leaf chlorophyll concentration, we made spectral reflectance measurements between 9 and 10 h (solar time) in one green, yellow and brown fully developed current-year attached leaf for each one of the five studied stressed plants per species throughout the drought period. To do this, we used a visible/near-infrared spectroradiometer USB-2000 (Ocean Optics, Dunedin, FL, USA) connected into one end of a bifurcated fibre optic cable, which were also connected into the other end to a tungsten halogen light source LS-1-LL (Ocean Optics). Leaf reflectance was calculated by dividing leaf spectral radiance by the radiance of a white standard (Spectralon, Labsphere, North Sutton, NH, USA). Integration time was 200 ms. The normalized difference vegetation index (NDVI) was used for the estimation of changes in leaf chlorophyll concentration and calculated as $(R_{750} - R_{705})/(R_{750} + R_{705})$, where R_{750} and R_{705} represent, respectively, the reflectance at 750 and 705 nm (Richardson and Berlyn 2002).

Leaf gas exchange and chlorophyll fluorescence measurements

Chlorophyll (Chl) fluorescence parameters were measured firstly at predawn on fully developed current-year attached leaves and cortical bark chlorenchyma of each one of the five studied stressed plants of *Q. faginea* and *Q. ilex* subsp. *rotundifolia*. Measurements were performed with a FMS II modulated fluorometer (Hansatech Instruments, Norfolk, UK) on one green, yellow and brown leaf per studied stressed plant, and the weighted mean was calculated for each plant according to the percentage of each leaf type (Figure 1). Initial Chl fluorescence in the dark-adapted state (F_O) was measured only with the modulated light (0.6 kHz, PPFD <0.4 µmol m^{-2} s^{-1}), which avoids electron transport through photosystem II (PSII). Maximal Chl fluorescence in the dark-adapted state (F_M) was measured by closing reaction centres with a 0.8-s pulse of 6000 µmol m^{-2} s^{-1} of white light (20 kHz). F_O was measured in presence of far-red light that fully oxidizes the PSII acceptor side (Belkhodja et al. 1998). The dark-adapted, maximum quantum efficiency of PSII was calculated as F_V/F_M (Abadía et al. 1999), being $F_V = F_M - F_O$, both for leaves and cortical bark chlorenchyma (Peguero-Pina et al. 2008b).

Afterwards, simultaneous gas-exchange and chlorophyll fluorescence measurements were carried out in the same leaves between 9 and 10 h (solar time) with the portable photosystem CIRAS-2 (PP-Systems, Amesbury, MA, USA) fitted with an automatic universal leaf cuvette (PLC6-U, PP-Systems) and the FMS II portable fluorometer mentioned above. All measurements were conducted under controlled conditions for CO_2 concentration surrounding the leaf (C_a = 400 µmol mol^{-1}), leaf temperature (25 °C), vapour pressure deficit (1.25 kPa) and saturating PPFD (1500 µmol m^{-2} s^{-1}). After steady-state in terms of gas exchange was reached (typically ~10 min after leaf clamping), net photosynthesis (A_N, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) and Chl fluorescence at steady-state photosynthesis (F_S) were recorded. The maximum Chl fluorescence in the light-adapted state (F'_M) was subsequently determined with a 0.8-s pulse of 6000 µmol m^{-2} s^{-1} of white light. Finally, the minimum Chl fluorescence in the light-adapted state (F'_0) was determined after switching off the actinic light and in presence of far-red light (7 μmol m^{-2} s^{-1}).

The actual (Φ_{PSII}) and intrinsic (Φ_{exc}) PSII efficiency were calculated according to Genty et al. (1989) as $(F'_M - F_S)/F'_M$ and $(F'_M - F'_0)/F'_M$, respectively. NPQ was calculated as $(F_M/F'_M) - 1$ (Bilger and Björkman 1990). The fraction of light absorbed that is dissipated in the PSII antenna $(1 - \Phi_{exc})$ was also estimated (Morales et al. 1998). Photosynthetic electron transport rate (J_F) was determined according to Krall and Edwards (1992). The measured fluxes were corrected by quantifying the leakage of CO$_2$ in and out of the cuvette with a photosynthetically inactive leaf obtained by heating (Flexas et al. 2007*a*).

Mesophyll conductance (g_m) and maximum velocity of carboxylation (V_{cmax}) were estimated according to the variable *J* method of Harley et al. (1992) (Eq. 1) and the one-point method of De Kauwe et al. (2016) (Eq. 2), respectively, as follows:

$$g_m = \frac{A_N}{C_i - \frac{\Gamma^*(J_F + 8(A_N + R_L))}{J_F - 4(A_N + R_L)}} \tag{1}$$

$$V_{c\,\mathbf{max}} = \frac{A_N}{\left(\frac{C_i - \Gamma^*}{C_i + K_m}\right) - 0.015}, \tag{2}$$

where A_N and the substomatal CO$_2$ concentration (C_i) were taken from the gas-exchange measurements at saturating light. The chloroplastic CO$_2$ compensation point in the absence of mitochondrial respiration (Γ^*) and the respiration rate in the light (R_L) were estimated as described in Flexas et al. (2007b), whereas K_m (the Michaelis–Menten constant) was estimated as described in De Kauwe et al. (2016).

The relative controls on A_N were separated into their functional components according to the proposal by Jones (1985) as implemented and comprehensively described by Grassi and Magnani (2005). This methodology allows comparing relative changes in limitations to net CO$_2$ assimilation into different components related to g_s (stomatal limitations, l_s), g_m (mesophyll limitations, l_m) and leaf biochemistry (biochemical limitations, l_b). l_s, l_m and l_b, with value between zero and one ($l_s + l_m + l_b = 1$), were calculated as:

$$l_s = \frac{g_{tot}/g_s \bullet \delta A_N/\delta C_c}{g_{tot} + \delta A_N/\delta C_c}, \tag{3}$$

$$l_m = \frac{g_{tot}/g_m \bullet \delta A_N/\delta C_c}{g_{tot} + \delta A_N/\delta C_c}, \tag{4}$$

$$l_b = \frac{g_{tot}}{g_{tot} + \delta A_N/\delta C_c}, \tag{5}$$

where g_s is the stomatal conductance to CO$_2$, g_m is the mesophyll conductance according to Harley et al. (1992) (Eq. 1), and g_{tot} is the total conductance to CO$_2$ from leaf surface to carboxylation sites in the chloroplasts ($1/g_{tot} = 1/g_s + 1/g_m$). The values of g_m obtained were used to calculate the chloroplastic CO$_2$ concentration (C_c) using the equation $C_c = C_i - A_N/g_m$. $\delta A_N/\delta C_c$ was calculated for *Q. faginea* and *Q. ilex* subsp. *rotundifolia* as the slope of the relationship between C_c and A_N obtained for each species, considering a C_c range of 50–100 μmol mol^{-1} (Peguero-Pina et al. 2017b).

Native xylem embolism

Native xylem embolism was determined throughout the drought period in current-year twigs of *Q. faginea* and *Q. ilex* subsp. *rotundifolia* collected from branches of each one of the five studied stressed plants per species where Ψ_{MD} had previously been measured. We used the same experimental procedure that is described in Peguero-Pina et al. (2018). Twigs were cut under water in the experimental field and immediately transported to the laboratory while maintaining them under water and covered with a plastic bag. Once there, stem segments of the twigs were recut under water (50 mm long), and both ends were shaved with a razor blade. Segments were placed in a tubing immersed in distilled water to avoid desiccation and to maintain a near constant temperature, and connected to a digital mass flowmeter Liqui-Flow (Bronkhorst High-Tech, Ruurlo, The Netherlands). We perfused distilled, degassed and filtered (0.22 μm) water containing 0.005% (volume/volume) Micropur (Katadyn Products, Wallisellen, Switzerland) to prevent microbial growth. The hydraulic conductivity (K_{stem}, kg m s^{-1} MPa^{-1}) was measured at a pressure of 0.005 MPa and after removing air embolisms at a pressure of 0.15 MPa for 60–90 s. Native embolism was calculated as the ratio between K_{stem} values obtained before and after removing embolism.

Shoot hydraulic conductance (K_{shoot})

Shoot hydraulic conductance (K_{shoot}, mmol m^{-2} s^{-1} MPa^{-1}) was measured for *Q. faginea* and *Q. ilex* subsp. *rotundifolia* throughout the drought period following the methodology detailed in Brodribb et al. (2005). One sun-exposed branch of each one of the five studied stressed plants per species was sampled at 07:00–08:00 h (solar time), minimizing the possibility for midday reduction in leaf hydraulic conductance (K_{leaf}) (Brodribb and Holbrook 2004). Branches were enclosed in sealed plastic bags to impede water loss, and stored in complete darkness during at least 1 h to achieve complete stomatal closure. This procedure ensures that all leaves from the same branch reached the same water potential, which is assumed to be that of the leaves before rehydration (Ψ_0). Afterwards, one shoot per branch was cut under water to avoid air entry and allowed to take up water for 30 to 60 s (t). The water potential after rehydration was subsequently measured (Ψ_f). The leaf hydraulic conductance was calculated as:

$$K_{shoot} = \frac{C \bullet \ln\left(\frac{\psi_0}{\Psi_f}\right)}{t}, \tag{6}$$

where C (mol MPa^{-1} m^{-2}) is the shoot capacitance for each species, calculated as the initial slope of the pressure-volume relationships, normalized by the leaf area (Brodribb et al. 2005). pressure-volume relationships for *Q. faginea* and *Q. ilex* subsp. *rotundifolia* were determined in six leaves per species following the free-transpiration method (Vilagrosa et al. 2003).

Nitrogen, phosphorus and potassium concentration in green, yellow and brown leaves

The macro-nutrients N, P and K were selected to document nutrient resorption prior to abscission in response to leaf damage for *Q. faginea* and *Q. ilex* subsp. *rotundifolia*. Nitrogen P and K concentration were analysed for each one of the studied stressed plants in (i) green leaves at the beginning of the experiment, and (ii) green, yellow and brown leaves at the end of the drought period. N was quantified using an Organic Elemental Analyzer (Flash EA 112, Thermo Fisher Scientific Inc., Waltham, MA, USA). Phosphorus and K were quantified using an inductively coupled plasma optical emission spectrometer (Varian 725 ES, Varian Inc., Palo Alto, CA, USA).

Stem hydraulic conductivity in Q. faginea after the drought period

As explained above, we examined the hydraulic capacity of the new stems ('second growth') and the possible hydraulic recovery of the stems that experienced the drought period ('first growth') in the five stressed plants of *Q. faginea*. Moreover, for comparative purposes, we also measured the hydraulic capacity of the first and second growth in another set of five control plants of *Q. faginea* that were well irrigated throughout the summer. To do this, K_{stem} was determined 2 months after the end of experiment (1–2 October 2018) in one stem segment of each one of the five control and the five stressed plants of *Q. faginea* before and after removing air embolisms, and native embolism was then calculated as described above and expressed as percentage loss of conductivity (PLC, %). Leaf-specific conductivity (LSC, kg m^{-1} s^{-1} MPa^{-1}) was calculated as the ratio between K_{stem} before removing air embolism and total leaf surface area supplied. Leaf area was measured by digitalizing the leaves and using the ImageJ image analysis software (http://rsb.info.nih.gov/nih-image/).

Statistical analysis

Data are expressed as means ± standard error of the mean. Student's *t*-tests were used to determine the point during the drought period when the change for each measured parameter was statistically significant compared with well-watered conditions. PLC$_{50}$ values were calculated for stems and shoots by adjusting pairs of data (Ψ_{MD} vs. percentage of maximum K_{stem} and Ψ_{MD} vs percentage of maximum K_{shoot}, respectively) by using a sigmoidal function according to Pammenter and Van der Willigen (1998). One-way analyses of variance were performed to identify the effect of the type of leaf (green, yellow and brown) on NDVI and nutrient composition for stressed plants of both species. Multiple comparisons were carried out among types of leaf for NDVI and nutrient composition using post hoc Tukey's Honest Significant Difference test. Student's *t*-tests were used to compare the values of K_{stem}, PLC, LSC and total leaf area between control and stressed plants both for the first and the second growth of *Q. faginea* 2 months after the drought period. All statistical analyses were performed in the R software environment (version 4.0.0, R Development Core Team, 2018).

Results

We found a differential response to changes in water potential between *Q. faginea* and *Q. ilex* subsp. *rotundifolia* in terms of leaf damage (Figure 1). Thus, *Q. faginea* showed a gradual decrease in the percentage of green leaves for a Ψ_{PD} range between ca −2 MPa and −4 MPa, as the percentage of yellow and brown leaves gradually increased (Figure 1A). The percentage of green leaves experienced a drastic reduction (15%) when Ψ_{PD} was below −4.5 MPa in comparison with the values under well-watered conditions (100%) and the percentage of brown leaves showed its maximum value (64%) at the end of the drought period ($\Psi_{\mathrm{PD}} = -5.6$ MPa) (Figure 1A). By contrast, *Q. ilex* subsp. *rotundifolia* maintained all the leaves green at this stage ($\Psi_{\mathrm{PD}} = -5.2$ MPa), and only experienced a sharp increase in the percentage of brown leaves (88%) at the end of the drought period ($\Psi_{\mathrm{PD}} = -8$ MPa) (Figure 1B). It should be noted that *Q. ilex* subsp. *rotundifolia* never showed yellow leaves throughout the drought period (Figure 1B).

Leaf damage in *Q. faginea* was subsequently followed by a decrease in F_V/F_M in yellow and, especially, brown leaves with respect to green leaves (Figure 2A). Thus, at the end of the drought period, when the percentage of green leaves was almost negligible, F_V/F_M in yellow and brown leaves were ~0.5 and 0.1, respectively (Figure 2A). Nonetheless, F_V/F_M in stems of *Q. faginea* remained high throughout the drought period (Figure 2B). By contrast, *Q. ilex* subsp. *rotundifolia* did not show this pattern, as F_V/F_M showed a decrease in green leaves and, albeit delayed, also in stems, reaching very low values at the end of the drought period (~0.2 and 0.3, respectively) (Figure 2C and D).

The values of the NDVI of green leaves were much higher than those measured for yellow or brown leaves for drought stressed plants of both species ($P < 0.05$, Figure S1 available as Supplementary Data at *Tree Physiology* Online), suggesting that leaf discoloration was associated to a decrease in chlorophyll concentration. The NDVI did not show statistically significant differences ($P > 0.05$) throughout the drought period within each leaf type (i.e., green, yellow or brown) for drought stressed plants of either *Q. faginea* or *Q. ilex* subsp. *rotundifolia* (data not shown). The drop in NDVI associated to changes in

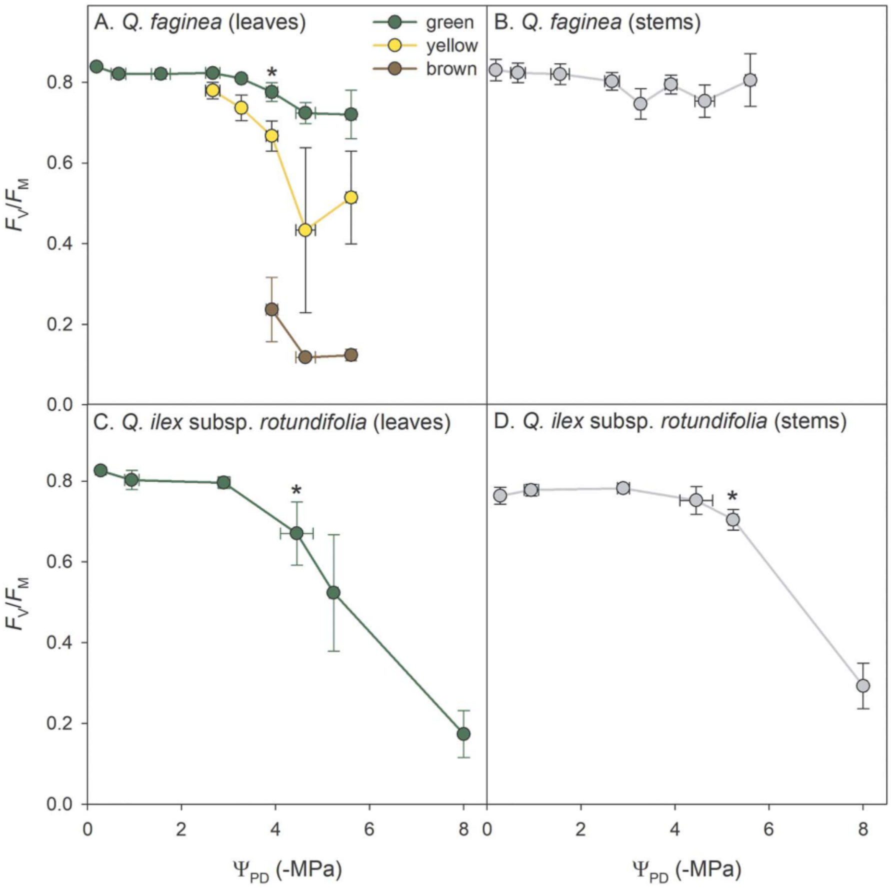

Figure 2. Time course of the maximum potential PSII efficiency (F_V/F_M) with predawn water potential (Ψ_{PD}) for *Q. faginea* and *Q. ilex* subsp. *rotundifolia* leaves (A and C, respectively) and stems (B and D, respectively) during the drought period. Data are mean ± SE of each date when stressed plants were measured (see Materials and methods for details). Asterisks indicate the point of the drought period when the decrease was statistically significant (pairwise *t*-test, $P < 0.05$) compared with well-watered conditions (first point of the graph).

leaf colour was accompanied in *Q. faginea* by a decrease in N, P and K concentrations, especially for brown leaves (Figure S2 available as Supplementary Data at *Tree Physiology* Online). In contrast, *Q. ilex* subsp. *rotundifolia* did not experience changes in N/P/K content between green and brown leaves (Figure S2 available as Supplementary Data at *Tree Physiology* Online). No differences in N/P/K concentrations were found between green leaves under well-watered conditions at the beginning of the drought period (Gr_i) and the leaves that remained green at the end of the drought period (Gr_f) for stressed plants of *Q. faginea* and *Q. ilex* subsp. *rotundifolia* (Figure S2 available as Supplementary Data at *Tree Physiology* Online).

The values of net photosynthesis and stomatal conductance (A_N and g_s) for *Q. faginea* were 1.4- and 1.6-fold higher, respectively, than those recorded for *Q. ilex* subsp. *rotundifolia* at the beginning of the experiment (i.e., when Ψ_{PD} was about −0.2 MPa) (Figure 3A and B). The intrinsic water-use efficiency (iWUE = A_N/g_s) did not show statistically significant differences ($P > 0.05$) between for both species at this stage (Figure 3C). Both A_N and g_s decreased in *Q. faginea* and *Q. ilex* subsp. *rotundifolia* when Ψ_{PD} became more negative, reaching negative values for A_N and almost negligible values for g_s at the end of the drought period (Figure 3). The main difference in the response to changes in water potential between the two species was found when water stress was moderate (i.e., when Ψ_{PD} ranged between ca −1 and −2 MPa). At this stage, *Q. faginea* experienced a sharp decrease in A_N (Figure 3B), even higher than that found for g_s (Figure 3A), with a concomitant reduction in iWUE when Ψ_{PD} reached −1.5 MPa (Figure 3C). By contrast, *Q. ilex* subsp *rotundifolia* showed a more gradual decrease in A_N and g_s, displaying higher values that *Q. faginea* at this stage (Figure 3A and B). Moreover, the drops in A_N and g_s in *Q. ilex* subsp. *rotundifolia* when Ψ_{PD} became more negative were coordinated, such that iWUE remained fairly constant for this species throughout the drought period (Figure 3C). This pattern was also observed for mesophyll conductance (g_m) and maximum velocity of carboxylation (V_{cmax}), with higher values for *Q. faginea* at the beginning of the experiment but a sharp decrease than *Q. ilex* subsp. *rotundifolia* throughout the drought period (Figure 4). The analysis of the partitioning of photosynthetic limitations revealed that A_N was mainly limited by mesophyll conductance (l_m) for *Q. ilex* subsp. *rotundifolia*, with a gradual increase throughout the drought period (Figure 5B). Stomatal (l_s) and biochemical (l_b) limitations for this species slightly decreased and remained fairly constant, respectively, during the drought period (Figure 5A and C). Regarding *Q. faginea*, A_N was mainly limited by l_m and l_b at the beginning of the experiment (Figure 5). However, when Ψ_{PD} became more negative, l_b strongly decreased at the expense of a sharp increase in l_m, whereas l_s only experienced a slight reduction (Figure 5).

The rest of parameters derived from Chl fluorescence measurements also showed outstanding differences between the two species. The actual PSII efficiency (Φ_{PSII}) was much higher in *Q. ilex* subsp. *rotundifolia* than in *Q. faginea* at the beginning of the drought period (i.e., when Ψ_{PD} was about −0.2 MPa) (Figure 6A). However, contrary to *Q. faginea*, Φ_{PSII} in *Q. ilex* subsp. *rotundifolia* experienced a strong decrease when Ψ_{PD} diminished, showing lower values than *Q. faginea* even when stress was moderate (i.e., when Ψ_{PD} was ca −1 MPa) (Figure 6A). The fraction of light absorbed that is dissipated in the PSII antenna ($1 - \Phi_{exc}$) and NPQ were much lower for *Q. ilex* subsp. *rotundifolia* at the beginning of the drought period, while both parameters experienced a sharp increase in this species when water stress was moderate (Figure 6B and C). By contrast, the increase in Φ_{exc} and NPQ throughout the drought period was more gradual in *Q. faginea* (Figure 6B and C).

The increasing levels of maximum daily drought stress (estimated through the measurement of Ψ_{MD}) induced a progressive loss of hydraulic conductivity in *Q. faginea* and *Q. ilex* subsp. *rotundifolia*, both in shoots (K_{shoot}) and stems (K_{stem}) (Figure 7). Regarding K_{stem}, *Q. ilex* subsp. *rotundifolia* reached values of native xylem embolism $\sim$50% at water potential values similar than *Q. faginea* (ca −5.9 and −5.5 MPa, respectively, Figure 7B). By contrast, the water potential inducing the 50% loss of hydraulic conductivity in the shoots occurred at water potential values much more negative in *Q. ilex* subsp. *rotundifolia* than in *Q. faginea* (ca −6.8 and − 4.9 MPa, respectively, Figure 7A).

Two months after the end of the experiment, the values of the PLC in the stems that were subjected to the drought period (first growth) of stressed plants of *Q. faginea* were much higher than that for the first growth of control plants (Figure 8B), which implied a strong reduction in K_{stem} (Figure 8A). PLC in the second growth was almost negligible for both control and stressed plants (Figure 8B). In spite of this, K_{stem} in the second growth of stressed plants was much lower than that for control plants (Figure 8A), which can be explained by the strong decrease in the conductive xylem area (data not shown).

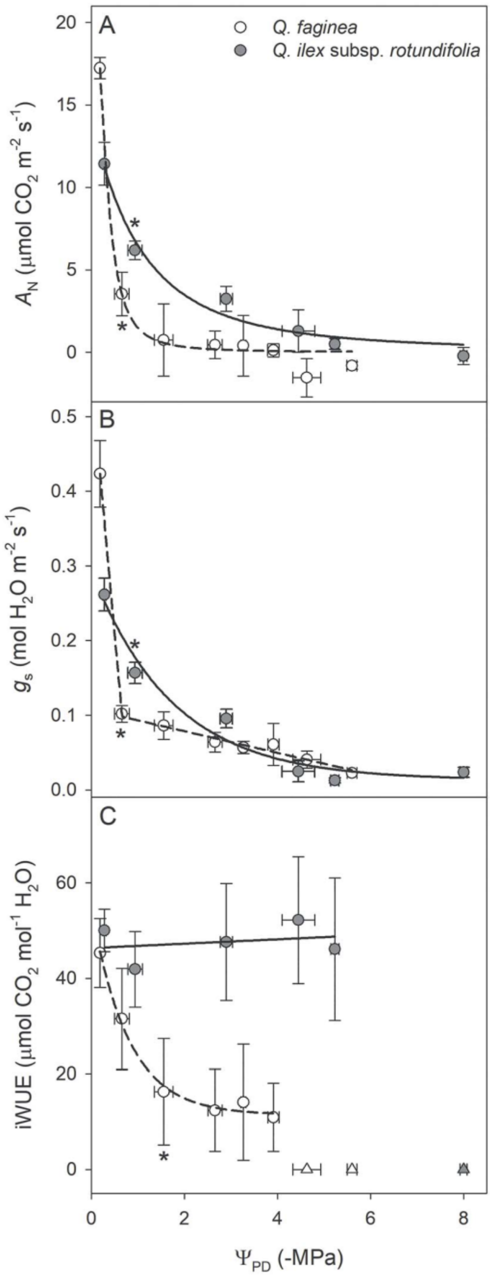

Figure 3. Time course of (A) net photosynthesis (A_N), (B) stomatal conductance (g_s) and (C) iWUE with predawn water potential (Ψ_{PD}) for *Q. faginea* (white symbols) and *Q. ilex* subsp. *rotundifolia* (grey symbols) leaves during the drought period. Data are mean ± SE of each date when stressed plants were measured (see Materials and methods for details). Measurements were performed on green, yellow and brown leaves when present, and the weighted mean was calculated for each plant according to the percentage of each leaf type (Figure 1). Data were adjusted for a better visualization and interpretation of the results. Asterisks indicate the point of the drought period when the decrease was statistically significant (pairwise *t*-test, $P < 0.05$) compared with well-watered conditions (first point of the graph). Triangles represent unreliable iWUE values due to the combination of negative A_N and extremely low g_s at the end of the drought period.

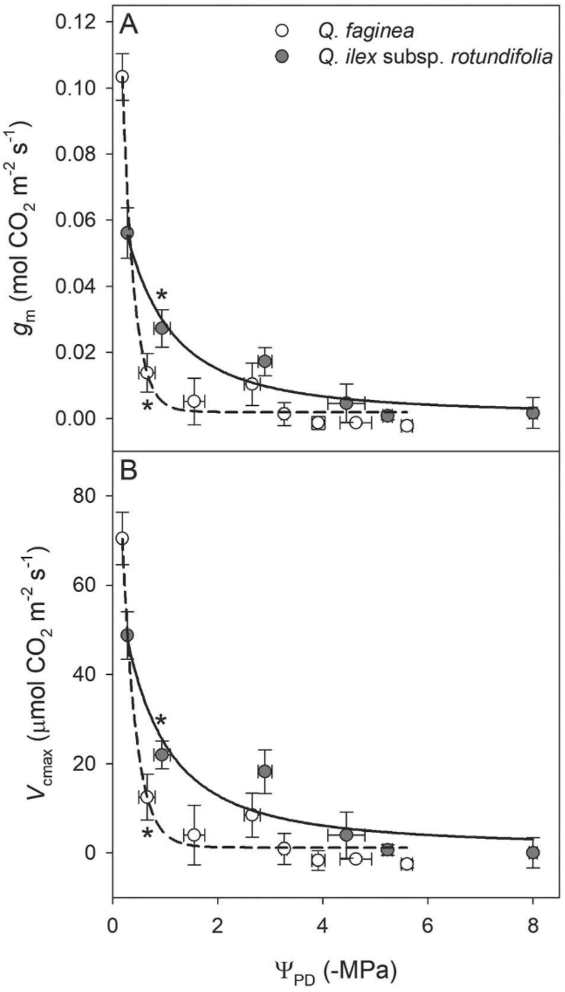

Figure 4. Time course of (A) mesophyll conductance (g_m) and (B) maximum velocity of carboxylation (V_{cmax}) with predawn water potential (Ψ_{PD}) for *Q. faginea* (white symbols) and *Q. ilex* subsp. *rotundifolia* (grey symbols) leaves during the drought period. Data are mean ± SE of each date when stressed plants were measured (see Materials and methods for details). Measurements were performed on green, yellow and brown leaves when present, and the weighted mean was calculated for each plant according to the percentage of each leaf type (Figure 1). Data were adjusted for a better visualization and interpretation of the results. Asterisks indicate the point of the drought period when the decrease was statistically significant (pairwise *t*-test, $P < 0.05$) compared with well-watered conditions (first point of the graph).

LSC did not show differences ($P > 0.05$) between control and stressed plants for the first growth (Figure 8C) due to the strong reduction in supported leaf area experienced by stressed plants (Figure 8D), which compensated the differences in K_{stem} (Figure 8A). The differences in LSC between control and stressed plant for the second growth (Figure 8C) reflected the differences in K_{stem} (Figure 8A), as supported leaf area did not show differences ($P > 0.05$) between both types of plants for the second growth (Figure 8D).

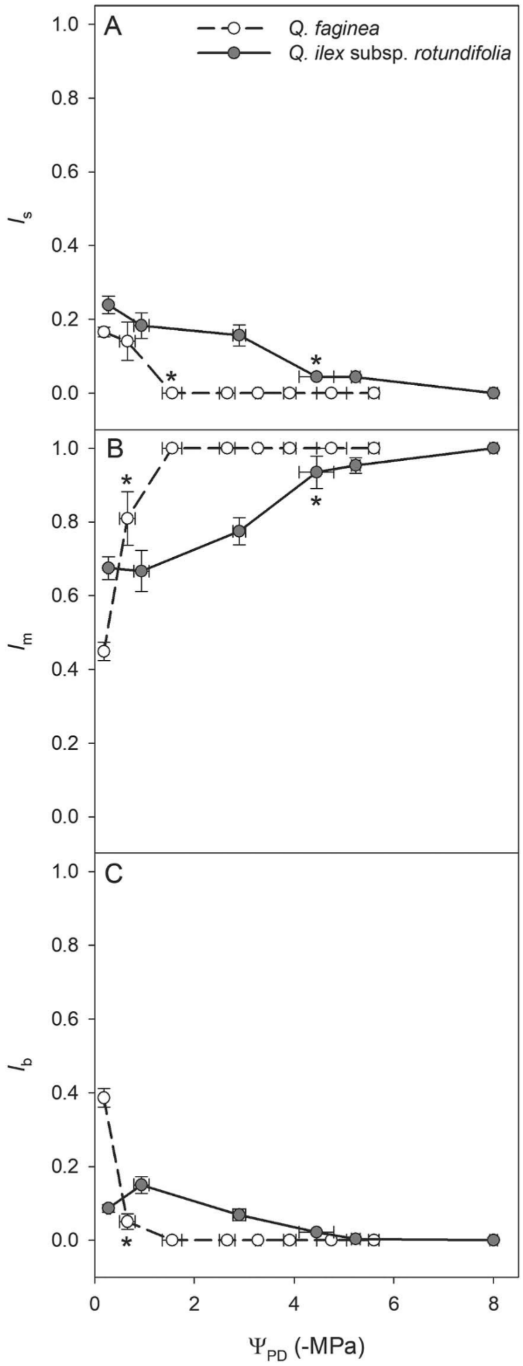

Figure 5. Time course of relative (A) stomatal (l_s), (B) mesophyll (l_m) and (C) biochemical (l_b) photosynthesis limitations with predawn water potential (Ψ_{PD}) for *Q. faginea* (white symbols) and *Q. ilex* subsp. *rotundifolia* (grey symbols) leaves during the drought period. Data are mean ± SE of each date when stressed plants were measured (see Materials and methods for details). Measurements were performed on green, yellow and brown leaves when present, and the weighted mean was calculated for each plant according to the percentage of each leaf type (Figure 1). Asterisks indicate the point of the drought period when the change was statistically significant (pairwise *t*-test, $P < 0.05$) compared with well-watered conditions (first point of the graph).

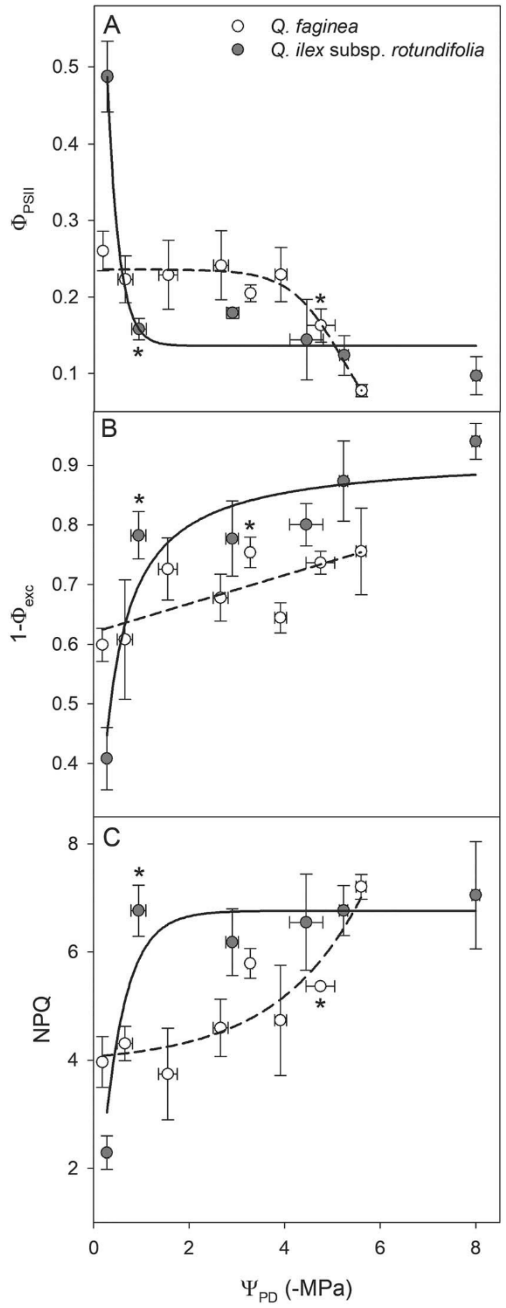

Figure 6. Time course of (A) actual PSII efficiency (Φ_{PSII}), (B) the fraction of light absorbed that is dissipated in the PSII antenna ($1 - \Phi_{exc}$) and (C) NPQ with predawn water potential (Ψ_{PD}) for *Q. faginea* (white symbols) and *Q. ilex* subsp. *rotundifolia* (grey symbols) leaves during the drought period. Data are mean ± SE of each date when stressed plants were measured (see Materials and methods for details). Measurements were performed on green, yellow and brown leaves when present, and the weighted mean was calculated for each plant according to the percentage of each leaf type (Figure 1). Data were adjusted for a better visualization and interpretation of the results. Asterisks indicate the point of the drought period when the change was statistically significant (pairwise *t*-test, $P < 0.05$) compared with well-watered conditions (first point of the graph).

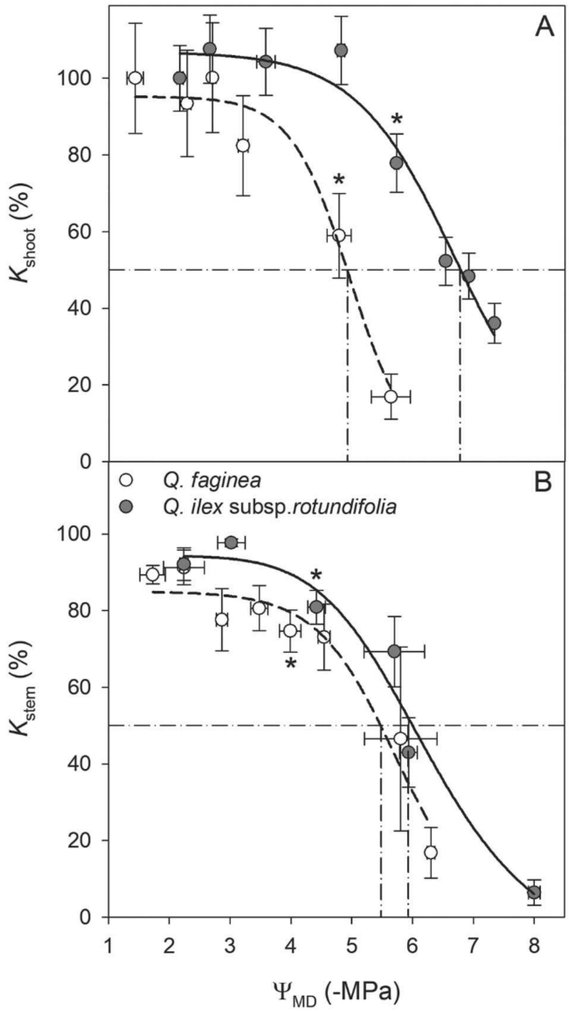

Figure 7. Relationship between midday water potential (Ψ_{MD}) and the percentage of (A) shoot hydraulic conductance (K_{shoot}) and (B) stem hydraulic conductivity (K_{stem}) for *Q. faginea* (white symbols) and *Q. ilex* subsp. *rotundifolia* (grey symbols) leaves during the drought period. Data are mean ± SE of each date when stressed plants were measured (see Materials and methods for details). Asterisks indicate the point of the drought period when the decrease was statistically significant (pairwise *t*-test, $P < 0.05$) compared with well-watered conditions (first point of the graph). Vertical dashed lines indicate the water potential inducing 50% loss of hydraulic conductivity (PLC_{50}) in shoots and stems.

Discussion

Segregation between Q. faginea and Q. ilex subsp. rotundifolia in the absence of water stress

This study has evidenced that *Q. faginea* displayed higher values of A_N, g_s and g_m than *Q. ilex* subsp. rotundifolia under well-watered conditions at the beginning of the experiment (first point of the graphs in Figures 3 and 4), with similar values than those previously reported for *Q. faginea* (Peguero-Pina et al. 2016) and for *Q. ilex* subsp. rotundifolia (Peguero-Pina

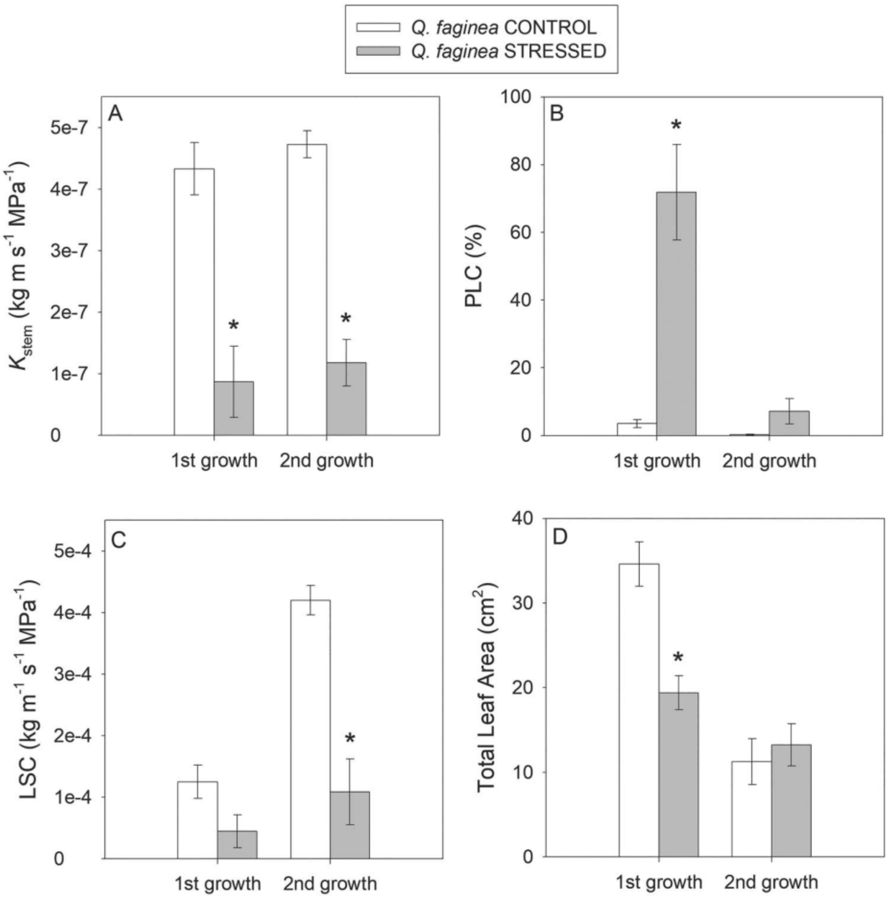

Figure 8. (A) Stem hydraulic conductivity (K_{stem}), (B) PLC, (C) LSC and (D) supported leaf area for the first and the second growth in control (white bars) and stressed (grey bars) plants of *Q. faginea* 2 months after the end of the drought period. Data are mean ± SE. Asterisks indicate statistically significant differences between control and stressed plants ($P < 0.05$).

et al. 2017b, 2018). Regarding this, some comparative studies already stated the lower g_s and A_N in *Q. ilex* subsp. *rotundifolia* when compared with *Q. faginea* in the absence of water stress (Forner et al. 2018a, Juárez-López et al. 2008, Mediavilla and Escudero 2003, 2004). Our results also show that, under well-watered conditions at the beginning of the experiment, K_{shoot} was much higher for *Q. faginea* than for *Q. ilex* subsp. *rotundifolia* (45.4 ± 3.9 and 27.2 ± 1.4 mmol m^{-2} s^{-1} MPa^{-1}, respectively) (data not shown). Taken together, these facts reflect the existence of a coordinated response between gas exchange and leaf hydraulic when these species are compared under well-watered conditions, as the evergreen *Q. ilex* subsp. *rotundifolia* showed a similar percentage of decrease for these parameters with respect to the winter-deciduous *Q. faginea* (i.e., 34%, 39%, 45% and 40% for A_N, g_s, g_m and K_{shoot}, respectively). Previous studies have established that the link between photosynthesis and leaf hydraulic arises from g_s (Brodribb et al. 2007, Scoffoni et al. 2016), as water loss and CO_2 uptake share a common pathway through stomata (Boyer 2015). Moreover, several studies have also pointed out the existence of a coordination of g_m and leaf hydraulic (Flexas et al. 2013, Lu et al. 2019, Xiong et al. 2017, 2018), as recently reviewed by Xiong and Nadal (2020). These authors suggested that water movement in mesophyll tissues also share a common pathway with CO_2 transport through the gas phase that would connect g_m and the outside-xylem component of leaf hydraulic conductance (K_{ox}). Overall, this coordination would explain the increased ability for carbon assimilation found in *Q. faginea* when compared with *Q. ilex* subsp. *rotundifolia* in the absence of water stress, albeit at the expense of a higher water consumption under the atmospheric evaporative demand experienced by this species during summer (Peguero-Pina et al. 2016).

The progressive imposition of water stress induced a differential physiological response between Q. faginea and Q. ilex subsp. rotundifolia

We confirmed that winter-deciduous Mediterranean oaks such as *Q. faginea* showed a higher stomatal sensitivity to soil water deficit than evergreen Mediterranean oaks such as *Q. ilex* subsp. *rotundifolia* (Figure 3B), as previously reported by Acherar and Rambal (1992) and Mediavilla and Escudero (2003). This phenomenon minimized water losses in *Q. faginea* at the expense of an extreme reduction in A_N, and may explain the slightly higher rates of A_N in *Q. ilex* subsp. *rotundifolia* at low water potentials (Figure 3) (Forner et al. 2018a). However, unlike previous studies, this is the first comparative research between two species representing the two types of Mediterranean oaks dealing with the role of non-stomatal factors constraining photosynthetic activity under drought conditions.

According to our results, the differential response of g_m to edaphic drought seems to be the main factor explaining the differences found between *Q. faginea* and *Q. ilex* subsp. *rotundifolia* in the dynamics of net CO_2 assimilation when Ψ_{PD} became more negative (Figure 4). Thus, we have demonstrated for the first time the important role of g_m in determining the photosynthetic response of *Q. faginea* to water stress, with increasing relative importance under progressive drought (Figure 5). Specifically, when water stress was moderate, the decrease in g_m in *Q. faginea* (~95%) were more pronounced than those estimated for *Q. ilex* subsp. *rotundifolia* (~51%) (Figure 4), which could explain the additional decrease in A_N (~96%) with respect to g_s (~80%) experienced by *Q. faginea* (Figure 3). Similar observations were obtained in other winter-deciduous oaks, such as *Quercus pubescens* (Zhou et al. 2014), *Quercus robur* (Grassi and Magnani 2005) and *Quercus petraea* (Cano et al. 2013). The underlying mechanisms to the response of g_m to drought are yet to be elucidated, but they may be associated to changes in aquaporin conductance and/or carbonic anhydrase expression (Cano et al. 2013). In parallel to g_m, we also observed a sharp decrease in V_{cmax} for *Q. faginea* under progressive water deficit, which strongly limited the fixation of CO_2 into sugars (Figure 5). By contrast, *Q. ilex* subsp. *rotundifolia*, regardless of the predominant role of mesophyll limitations throughout the drought period (Figure 5), showed a higher degree of co-regulation in the response of A_N, g_s, g_m and V_{cmax} to progressive drought, which allowed this species to maintain fairly constant values of iWUE throughout the drought period (Figures 3 and 4).

Besides photosynthetic traits, the ability of plants to supply water to transpiring leaves can be strongly limited under drought conditions, as it happens to both species here studied (Figure 7). In spite of this common trend, *Q. faginea* experienced an early decrease of K_{shoot} in response to drought, with a PLC_{50} value less negative than that estimated for *Q. ilex* subsp. *rotundifolia* (ca −4.9 and −6.8 MPa, respectively, Figure 7A). This differential response in leaf hydraulic resembled the one observed in photosynthetic traits. Recently, Xiong and Nadal (2020) stated that only a few studies have examined the coordination of photosynthetic and leaf hydraulic traits upon water stress, especially considering g_m. Thus, Wang et al. (2018) proposed that the drought-induced decrease in leaf hydraulic conductance could act as a potential trigger for declines in g_s and g_m in *Oryza sativa*. In contrast, Theroux-Rancourt et al. (2014) reported a delayed response of g_m with respect to g_s and leaf hydraulic conductance in poplar clones exposed to soil drying. Therefore, the interdependence between photosynthetic and hydraulic traits could be species-dependant (Xiong and Nadal 2020). In our case, we observed an early response in g_s and g_m with respect to K_{shoot} for both species, but especially pronounced in *Q. ilex* subsp. *rotundifolia*. This situation agrees with the hypothesis of stomata as a safety valve to prevent hydraulic failure under moderate drought conditions (Flexas et al. 2018, Hochberg et al. 2017).

In contrast to K_{shoot}, both species showed a similar evolution of K_{stem} with increasing levels of drought stress (Figure 7). In other words, PLC_{50} in shoots was lower than in stems of *Q. faginea* (ca −4.9 and −5.5 MPa, respectively), which is consistent with the 'vulnerability segmentation' hypothesis (Hochberg et al. 2017). Skelton et al. (2018) also found that leaves were more vulnerable than stems in *Q. douglasii*, a winter-deciduous Mediterranean oak that experiences leaf shedding under severe drought associated with leaf embolism and avoidance of extensive xylem embolism (i.e., 'vulnerability segmentation'). In our study, a similar behaviour was described for *Q. faginea*, as the percentage of green leaves experienced a drastic reduction when Ψ_{PD} was ca. −4.5 MPa (Figure 1A), as reported by Hinckley et al. (1983) for the winter-deciduous Mediterranean oak *Q. pubescens*. This Ψ_{PD} value corresponded to a Ψ_{MD} value ca −5.7 MPa during the drought period (data not shown), which would imply a reduction of K_{shoot} <20% of its initial value (Figure 7A) while K_{stem} was reduced only to ~50% of the initial value (Figure 7B). Peguero-Pina et al. (2015) suggested that 'vulnerability segmentation' in the winter-deciduous Mediterranean *Quercus subpyrenaica* explained the premature leaf withering during intense summer drought as a way to reduce water losses by transpiration, ensuring bud survival and subsequent new leaf development. This last phenomenon was observed in *Q. faginea* several weeks after the end of the drought period, probably because the embolism threshold for irreversible drought damage (88% loss of stem conductivity, Urli et al. 2013) was not reached (Figure 7B), as also indicated the lack of F_V/F_M decrease in the stems of this species (Figure 2B). The new bud bursting in previously stressed plants of *Q. faginea* was not associated to xylem refilling in the stems that experienced water stress (i.e., first growth), but to the formation of new xylem without embolism (i.e., second growth) after drought period (Figure 8B). In line with this, Creek et al. (2018) also found that three arid zone Australian species with 'vulnerability segmentation' did not refill xylem embolisms, and hydraulic recovery was dependent on new plant growth after drought. However, the hydraulic capacity of the second growth of *Q. faginea* was much lower than that for control plants

(Figure 2A) for an equivalent supported leaf area (Figure 2D), which implied a strong reduction of LSC in the new xylem of previously stressed plants (Figure 2C). Another negative consequence of premature withering in *Q. faginea* could be a decrease in nutrient-use efficiency (Montserrat-Martí et al. 2009), as resorption of N, P and K was only partial in yellow and brown leaves (Figure S2 available as Supplementary Data at *Tree Physiology* Online).

By contrast, PLC_{50} in shoots of *Q. ilex* subsp. *rotundifolia* was even higher than in stems (ca −6.8 and −5.9 MPa, respectively). Indeed, Skelton et al. (2018) did not find evidences of 'vulnerability segmentation' for the evergreen Mediterranean oaks of California, and even *Quercus sadleriana* showed the opposite trend, in line with the results here obtained for *Q. ilex* subsp. *rotundifolia*. In this way, *Q. ilex* subsp. *rotundifolia* maintained all the leaves green throughout almost all the drought period (Figure 1B), which pointed out the high resistance of their leaves to drought-induced cavitation. Peguero-Pina et al. (2015) suggested that this strategy could be related with the longer leaf life span of the evergreen species and the ability of *Q. ilex* subsp. *rotundifolia* for maintaining its photosynthetic capacity for more than one vegetative period (Corcuera et al. 2005*a*). Therefore, this species ensures the keeping of foliage by avoiding irreversible damage even under intense drought conditions (i.e., when Ψ_{PD} was ca −5 MPa).

To do this, besides leaf hydraulic resistance, Mediterranean evergreen oaks have developed a great capacity for protecting the photosynthetic apparatus through the thermal dissipation of the excess of energy in response to water scarcity (García–Plazaola et al. 2017, Peguero-Pina et al. 2009, Sancho-Knapik et al. 2018). Thus, we have shown that *Q. ilex* subsp. *rotundifolia* showed an early strong decrease in Φ_{PSII} in response to drought (Figure 6A), downregulating the photosynthetic electron transport when CO_2 assimilation is severely limited. Furthermore, this species also showed a sharp increase in the fraction of light absorbed that is dissipated in the PSII antenna ($1 - \Phi_{exc}$) and in the NPQ in response to drought (Figure 6B and C, respectively). However, although these mechanisms were also present in *Q. faginea*, our results indicated a limited capacity to cope with water stress in terms of photoprotection when compared with *Q. ilex* subsp. *rotundifolia* (Figure 6).

Ecophysiological implications

Both *Q. faginea* and *Q. ilex* subsp. *rotundifolia* are well-known representatives of the two co-occurring types of Mediterranean oaks, with different leaf type, paleogeographical origin and contrasting strategies to cope with water limitations. Winter-deciduous Mediterranean oaks have been considered components of the so-called 'sub-Mediterranean' vegetation (Sánchez de Dios et al. 2009), inhabiting transitional areas where the Mediterranean summer aridity is partially mitigated by a higher annual precipitation and a lower mean temperature (Martín-Gómez et al. 2017, Río and Penas 2006). Their co-existence with the evergreen congeneric species in many areas of the western Mediterranean basin is a complex matter that has been examined in several ecophysiological studies (e.g., Montserrat-Martí et al. 2009, Nardini et al. 1999, Peguero-Pina et al. 2015), but further research is needed to fully understand it.

On one hand, the leaf life span of the winter-deciduous Mediterranean oaks limits the photosynthetic activity to a shorter period, which would explain the coordination between hydraulic and photosynthetic traits in order to maximize the ability for carbon assimilation in *Q. faginea* under favourable conditions. On the other hand, the great water consumption required under the high atmospheric evaporative demand experienced by this species during summer (Peguero-Pina et al. 2016) could induce an additional drop in soil water availability that could negatively affect leaf hydraulics and gas exchange (Mediavilla and Escudero 2003, 2004), and, ultimately, can induce a premature leaf withering. This fact may definitively shorten the vegetative period and explain the large dependence of *Q. faginea* on edaphic conditions that ensure the maintenance soil water potential values above critical values for this species (Esteso-Martínez et al. 2006). Thus, when soil water was depleted, leaves of *Q. ilex* subsp. *rotundifolia* exhibited a more conservative water-use strategy and a higher resistance to drought-induced cavitation than *Q. faginea* (Figures 3C and 6B, respectively). This conservative leaf strategy of the evergreen Mediterranean oaks allows them to assimilate carbon throughout a longer time period during summer and other periods of the year such as the early spring or late autumn, as reported by Corcuera et al. (2005a) for *Q. ilex* subsp. *rotundifolia*. Therefore, the co-occurrence of both types of Mediterranean oaks in these transitional areas of the western Mediterranean basin could be associated with local edaphic conditions that result in a lower water storage capacity as a consequence of the soil degradation associated to the human management Corcuera et al. (2005*a*, 2005*b*). This situation would be exacerbated in the context of climate change, which predicts an increase in temperature and aridity in several regions, especially in the Mediterranean basin (Klausmeyer and Shaw 2009, Ruiz-Labourdette et al. 2012). For this reason, there are some prediction models that indicate a notable reduction in the potential distribution range of winter-deciduous Mediterranean oaks (e.g., *Q. faginea*) and their substitution by evergreen Mediterranean oaks such as *Q. ilex* (Sánchez de Dios et al. 2009).

Conclusions

We have evidenced in the present study that *Q. faginea* and *Q. ilex* subsp. *rotundifolia* show a differential response in terms of photosynthetic, photochemical and hydraulic traits, both in the absence of water stress and under intense drought. The contrasting leaf life span may condition the different functional strategies shown by different organs (i.e., leaves, shoots and stems) of these species, with important consequences for the co-occurrence of both types of Mediterranean oaks, which could be dependent on local conditions related to soil water availability.

Thus, the shorter leaf life span of the winter deciduous *Q. faginea* was compensated through an increased ability for carbon assimilation by means of enhanced g_m, V_{cmax} and K_{shoot}, although at the expense of a higher water consumption under favourable conditions. However, this advantage in terms of carbon gain was rapidly supressed, even when soil water deficit was moderate, due to the sharp decline in g_m, the most limiting factor for photosynthetic activity in *Q. faginea*. This fact was coupled with an early increase in leaf embolism and the lack of effective photoprotective mechanisms, which could trigger the drastic reduction of green leaves in response to drought. As a counterpart, stems of *Q. faginea* were protected from extensive xylem embolism, which allowed new leaf development after drought period. This fact could be crucial for the survival and growth of this species, as well as partially offsetting the loss of competitiveness with co-occurring evergreen Mediterranean oaks with longer leaf life span. This was the case of *Q. ilex* subsp. *rotundifolia*, which showed a conservative leaf strategy, characterized by a high resistance to drought-induced cavitation and effective photoprotective mechanisms, which kept their leaves functional even under intense drought conditions for more than one vegetative period. This conservative strategy would confer a competitive advantage with respect to winter-deciduous Mediterranean oaks when soil water availability is restricted.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

Funding

This research was funded by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) grant number RTA2015-00054-C02-01, by Ministerio de Ciencia e Innovación grant number PID2019-106701RR-I00/AEI/10.13039/501100011033 and from Gobierno de Aragón H09_20R research group. The work of D.A.-F. is supported by a FPI-INIA contract BES-2017-081208.

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Supplementary Data

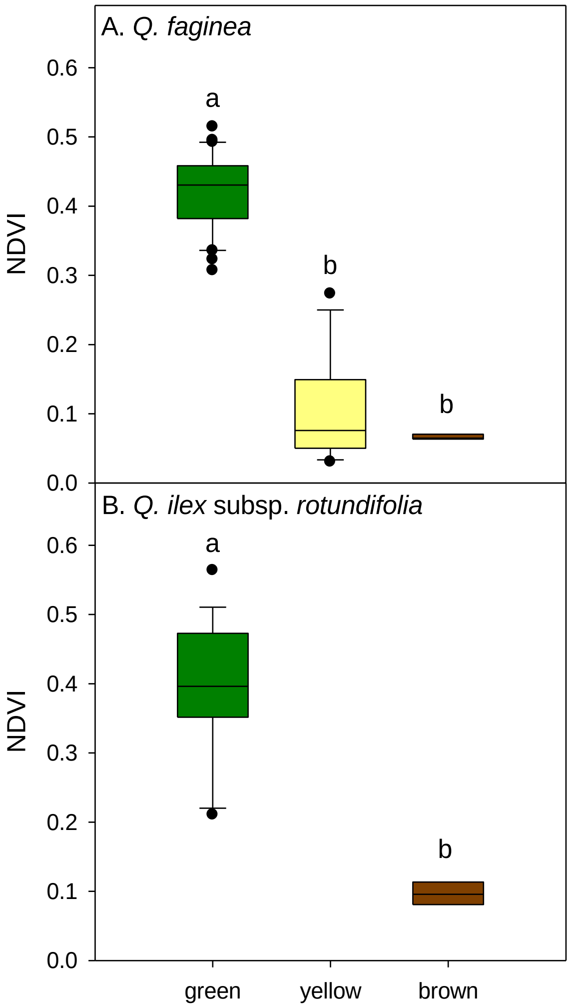

Fig. S1. Box-plot representation of the normalized difference vegetation index (NDVI) for green, yellow and brown leaves of (A) *Q. faginea* and (B) *Q. ilex* subsp. *rotundifolia*. Different letters indicate significant differences among leaf types ($P < 0.05$).

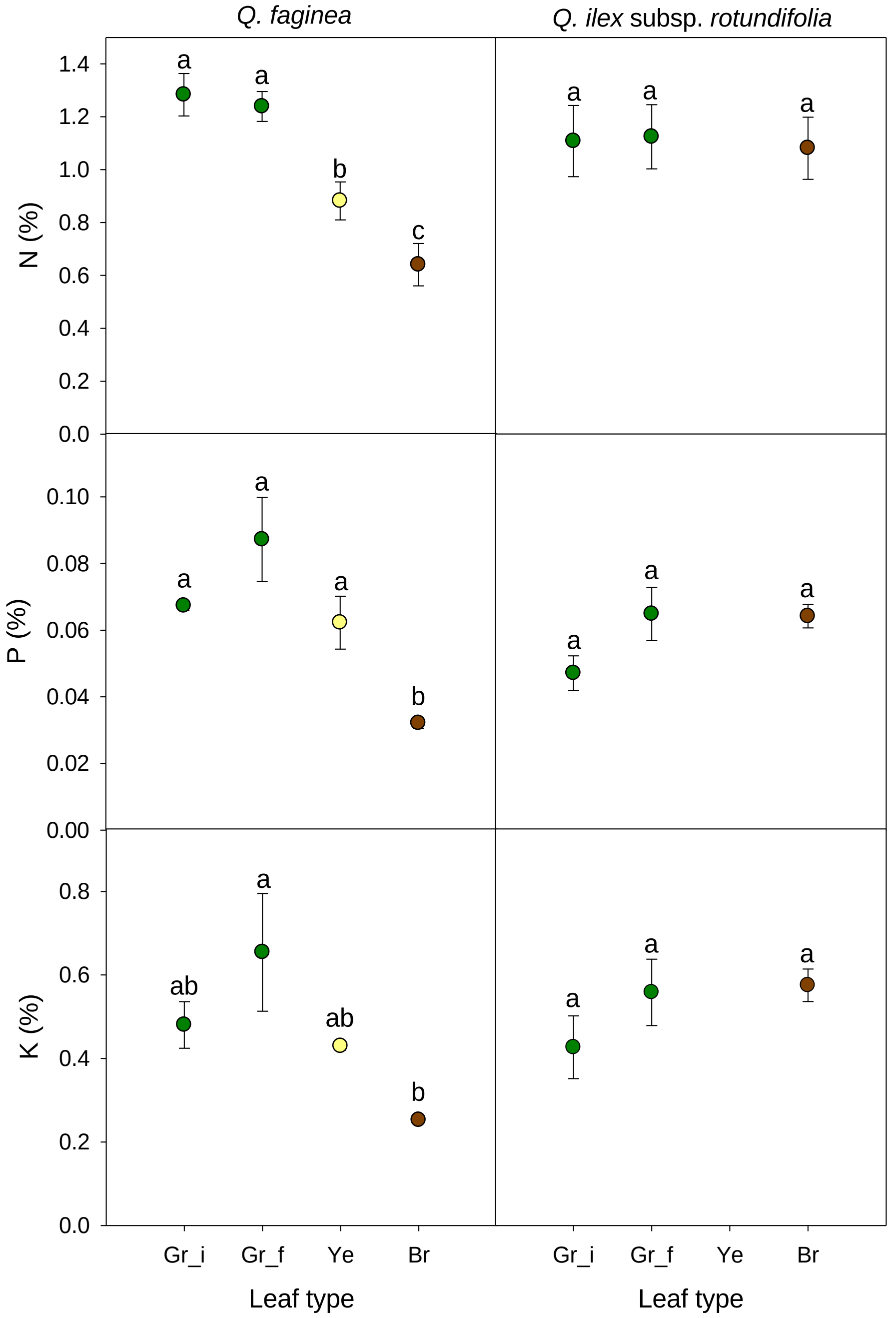

Fig. S2. N, P and K concentration (%) for green leaves at the beginning of the experiment, green, yellow and brown leaves at the end of the drought period (Gr_i, Gr_f, Ye, Br, respectively) of *Q. faginea* and *Q. ilex* subsp. *rotundifolia*. Data are mean ± SE. Different letters indicate significant differences among leaf types ($P < 0.05$).

Capítulo VII

Discusión general y conclusiones

7.1. Discusión general

Como se vio en el **primer capítulo** de esta tesis, la diversidad dentro del denominado genéricamente clima mediterráneo, así como la gran variabilidad fisionómica y funcional de los robles de estos ecosistemas, no permite definir un único y genuino prototipo de roble mediterráneo. En la actualidad se distinguen dos tipos de robles bien diferenciados que coexisten en las regiones mediterráneas: el roble perennifolio esclerófilo con un origen en la Flora Paleotropical y el caducifolio malacófilo con un origen arctoterciario (Barbero *et al.*, 1992; Damesin *et al.*, 1998; Mediavilla y Escudero, 2004). La coexistencia de *Quercus* perennifolios y caducifolios permite estudiar el papel de los dos patrones fenológicos desde un punto de vista funcional (Kikuzawa, 1991), especialmente bajo las restricciones ambientales impuestas por el clima mediterráneo (Montserrat-Martí *et al.*, 2009).

Las condiciones poco favorables a las que están sometidos los robles mediterráneos acortan su periodo vegetativo y disminuyen su actividad fotosintética durante el invierno y el verano (García-Plazaola *et al.*, 1999; Corcuera *et al.*, 2005). Estas restricciones producen una fragmentación del periodo vegetativo (Mitrakos, 1980) y una limitación del funcionamiento en estas especies. Por lo general, los robles caducifolios mediterráneos, como es el caso de *Q. faginea*, compensan la corta duración del periodo vegetativo con altas tasas de asimilación de carbono cuando las condiciones son favorables (van Ommen Kloeke *et al.*, 2012; Peguero-Pina *et al.*, 2015). Por su parte, los perennifolios mediterráneos, como *Q. ilex*, contrarrestan esas restricciones con una mayor longevidad foliar que les permite asimilar carbono tan pronto como las condiciones son propicias (Corcuera *et al.*, 2005).

Para abordar el estudio de las adaptaciones a las restricciones impuestas por el clima mediterráneo, en esta tesis se han usado *Q. faginea* y *Q. ilex* subsp. *rotundifolia* como paradigmas de los dos tipos de robles coexistentes. El primero es un claro ejemplo de roble caducifolio mediterráneo con origen arctoterciario (sección *Quercus*), mientras que el segundo representa el modelo de perennifolio esclerófilo de origen paleotropical (sección *Ilex*). Ambos provienen de ambientes no mediterráneos, por lo que han pasado por procesos de adaptación a este clima (Suc, 1984; Minnich, 1985; Blumler, 1991; Peguero-Pina *et al.*, 2016).

Estos procesos convergen en algunas características comunes, como es la reducción del área foliar, que puede alcanzar valores 10 veces más bajos en los robles mediterráneos o áridos respecto de aquellos que viven en climas templados (Gil-Pelegrín *et al*., 2017; Peguero-Pina *et al*., 2020). Esta característica permite soportar la escasez de agua en los robles mediterráneos (Baldocchi y Xu, 2007) al reducir la superficie transpirante, a la vez que modera el sobrecalentamiento foliar (Voguel, 1970, 2009; Schuepp, 1993) y mejora la relación entre la conductividad hidráulica del tallo en relación con la superficie foliar sustentada, o conductividad foliar específica (Peguero-Pina *et al*., 2015). Otra característica común de los robles mencionados es la mayor resistencia hidráulica del tallo respecto de otros robles que viven en ambientes templados (Peguero-Pina *et al*., 2015). Así, *Q. faginea* registra valores de conductividad hidráulica en el tallo siete veces menores que los presentados por *Q. robur* (sección *Quercus*), un congénere muy próximo filogenéticamente que habita climas templados (Peguero-Pina *et al*., 2015).

En este sentido, se explicaría el beneficio directo de la reducción de la superficie foliar en cuanto a la mejora de la capacidad de suministrar agua a las hojas transpirantes en *Q. faginea*, compensando la notable diferencia encontrada en la conductividad hidráulica entre ambas especies. Otra diferencia entre *Q. faginea* y *Q. robur* es su resistencia a la embolia. De este modo, la especie mediterránea soporta niveles muy superiores de tensión en el xilema sin sufrir pérdida en su conductividad por cavitación. Esta cualidad de los robles propios de clima mediterráneo se presenta en un buen número de especies (Gil-Pelegrín *et al*., 2017), aunque no es una condición general dentro de este conjunto de especies (Martín-Sánchez *et al*., 2022).

Sin embargo, estos dos tipos de robles presentan un gran número de rasgos funcionales que los diferencian. Estos rasgos funcionales agrupan las características morfo-fisio-fenológicas que influyen en su adecuación al ambiente (Violle *et al*., 2007).

Atributos morfológicos

Entre las características diferenciadoras de los dos tipos de robles mediterráneos destaca la textura foliar. Así, se diferencian el tipo no esclerófilo o malacófilo (*e.g.*, *Q. faginea*) y el tipo esclerófilo (*e.g.*, *Q. ilex* subsp. *rotundifolia*).

Aunque no se pudo definir un solo factor que explicara la esclerofilia, se evidenció un efecto sinérgico de todos ellos en cuanto al desarrollo de hojas esclerófilas en el género *Quercus* (**capítulo II**). Por consiguiente, en aquellos hábitats en los que convergen varios de esos factores la esclerofilia es más frecuente. Este es el caso de *Q. ilex* subsp. *rotundifolia* que habita suelos degradados con escasez de agua, falta de nutrientes, altas temperaturas y presenta hojas con alta longevidad.

En el **capítulo II**, se concluyó que, desde un punto de vista funcional en las especies del género *Quercus*, el hábito foliar modula en gran medida las implicaciones fisiológicas de la esclerofilia. Se destacó que, entre las especies de hoja perenne, la esclerofilia tiene un coste no solo en términos de inversión de carbono, sino que también limita la capacidad fotosintética, principalmente a través de limitaciones de difusión (Wright *et al*., 2004; Flexas *et al*., 2008; Hassitou *et al*., 2009; Niinemets *et al*., 2009; Onoda *et al*., 2017; Peguero-Pina *et al*., 2017a, b). Por el contrario, el aumento de LMA entre los robles de hoja caduca se asocia con una mayor capacidad fotosintética (Takashima *et al*., 2004; Peguero-Pina *et al*., 2015, 2016; Gil-Pelegrín *et al*., 2017). Esto se refleja en la estrategia de los robles de hoja caduca mediterráneos para maximizar la ganancia de carbono durante los períodos óptimos, como una adaptación a la fragmentación del periodo vegetativo.

Cuando se analizaron los rasgos morfológicos, anatómicos y composicionales de las hojas de 85 especies de *Quercus*, las especies caducifolias y perennifolias presentaron diferencias en varios de sus atributos. Entre estos atributos destacaron la masa seca foliar por área (LMA), el espesor de la hoja (LT) y el área foliar, confirmando lo observado previamente en otros estudios (Corcuera *et al*., 2002; Escudero *et al*., 2017). Sin embargo, los dos tipos de robles no difirieron en la densidad foliar (LD) a pesar de sus diferencias en rasgos foliares relacionados con la densidad como la concentración de celulosa, la distancia entre extensiones de las vainas del haz y su anchura (**capítulo III**).

El escenario 1 del **capítulo III** contemplaba que un aumento en LMA se lograría con hojas más gruesas y aumentaría la tasa instantánea de asimilación de carbono por unidad de área (A_{area}) lo que compensaría temporadas favorables más cortas en ambientes climáticos estresantes. Sin embargo, las especies caducifolias con alto LMA debido a un mayor LT habitaron climas con periodos cortos de frío, aunque con periodos largos de aridez intensa. Este periodo más largo de aridez podría contrarrestar el efecto de un periodo corto de frío acortando así la temporada favorable (Peguero-Pina *et al.*, 2016), lo que señalaría la aridez como el factor climático que promueve el aumento de LT en los robles caducifolios. En el caso de las perennifolias, aquellas que viven en ambientes con periodos de crecimiento cortos (*i.e.*, periodos largos de frío) y/o con periodos de aridez intensa, incrementaron su LMA al aumentar LT (**capítulo III**).

En el escenario 2 se consideró que un aumento en LMA estaría asociado con hojas más densas para incrementar su resistencia en condiciones climáticas estresantes. La aridez intensa en las especies caducifolias estuvo directamente relacionada con un incremento de LMA a través del aumento de LT, pero con una disminución de LD. Esto sugirió que una mayor aridez promovió hojas más gruesas y menos densas, lo que pudo mejorar A_{area} al incrementar la conductancia del mesófilo (g_{m}) (Niinemets *et al.*, 2009). Por otra parte, las perennifolias incrementaron LMA al aumentar su LD en aquellos ambientes que presentaron aridez intensa como es el caso de las especies mediterráneas *Q. calliprinos* y *Q. coccifera* (**capítulo III**). Además, las perennifolias que habitan climás fríos presentaron LT mayores debido a un mesófilo lagunar más espeso que protege a las hojas del daño por congelación (Wyka y Oleksyn, 2014).

Aunque la mayoría de los estudios ecofisiológicos usan LMA como valor proxy para cuantificar la esclerofilia (*e.g.*, Niinemets, 2001; Wright *et al.*, 2004), se requieren medidas directas de las propiedades mecánicas para cuantificar la esclerofilia (Aranwela *et al.*, 1999; Read y Sandon, 2003; Onoda *et al.*, 2011). Es por eso por lo que en el **capítulo IV** se profundizó aún más en el estudio de la esclerofilia usando métodos directos para su estimación como el "*punch and die*", que mide la resistencia mecánica foliar. Esto permitió conocer la importancia relativa de cada rasgo anatómico y composicional de las hojas sobre las propiedades mecánicas. En este sentido, el espesor de capa más externa de la epidermis y el contenido de celulosa destacaron por su fuerte contribución a la dureza foliar. El primero permitió obtener hojas más duras sin grandes cambios en la masa acumulada por superficie de hoja ni grandes costes añadidos en la formación de la hoja (Villar y Merino, 2001). El segundo, se relacionó con el mayor espesor de las vainas del haz y de la pared celular externa de la epidermis.

Las características foliares permitieron separar los robles caducifolios de los perennifolios surgiendo algunas observaciones interesantes (**capítulo IV**). Por una parte, las especies de la sección *Ilex* mostraron rasgos foliares similares independientemente del clima en el que viven. Esto podría explicarse por su origen filogenético común en la Flora Paleotropical en el este de Asia (Jing *et al.*, 2019) que ya presentaba rasgos xeromórficos (Zhou *et al.*, 2007; He *et al.*, 2014). Por otra parte, las especies perennifolias mediterráneas, independientemente de su origen filogenético, presentaron rasgos foliares comunes. Por eso, parece probable la existencia de una convergencia evolutiva en esos rasgos foliares de las especies esclerófilas en el clima mediterráneo.

Otro atributo anatómico diferenciador es la disposición de los vasos del xilema y su distribución entre las células del parénquima. Este rasgo permite una distinción de los dos tipos de roble en especies de poro en anillo difuso y de poro difuso. En las especies de poro en anillo, como es el caso de *Q. faginea*, los vasos se localizan agrupados en dos tipos de madera a lo largo del año: la madera de primavera y la de verano. La madera de primavera presenta vasos grandes mientras que la de verano tiene vasos de menor tamaño. En el caso de las especies de poro difuso, como *Q. ilex* subsp. *rotundifolia*, solo se encuentran vasos pequeños. Este atributo favorece ciertas diferencias fisiológicas que se discutirán más abajo.

Atributos fenológicos

Las limitaciones impuestas por el clima mediterráneo y la fragmentación del periodo vegetativo dificultan la existencia de un ajuste fenológico preciso con la estacionalidad del clima a diferencia de lo que ocurre en los climas templados (Lieth, 1974). Además, la variabilidad interanual de este clima limita la regularidad y duración de los períodos favorables, aumentando todavía más la dificultad del mencionado ajuste fenológico (Mooney y Dunn, 1970). Estas limitaciones posiblemente facilitan la coexistencia de los dos tipos fenológicos en muchas comunidades de plantas leñosas mediterráneas (Montserrat-Martí *et al.*, 2004). En este sentido, varios autores han reportado su coexistencia (Tognetti *et al.*, 1998; Nardini *et al.*, 1999; Montserrat-Martí *et al.*, 2009) que está considerada como una peculiaridad del bioma mediterráneo (Baldocchi *et al.*, 2010). Un buen ejemplo de esta situación serían los bosques mixtos de *Q. faginea* y *Q. ilex* subsp. *rotundifolia* en las zonas interiores de la península ibérica (Montserrat-Martí *et al.*, 2009; Forner *et al.*, 2018). Mientras que *Q. faginea* es un caducifolio de invierno con cierto grado de marcescencia, *Q. ilex* subsp. *rotundifolia* es un perennifolio que mantiene varias cohortes de hojas que pueden durar algunos años. Retener las hojas viejas durante varios años permitiría una mayor asimilación neta de CO_2 en todo el árbol (Escudero y Mediavilla, 2003) ya que las hojas viejas, aunque con tasas bajas de asimilación, pueden contribuir significativamente a la fotosíntesis de la copa (Niinemets *et al.*, 2005; Peguero-Pina *et al.*, 2017).

Pese a los beneficios de mantener las hojas durante varios años, se ha observado una disminución importante en la actividad fotosintética de las diferentes cohortes de *Q. ilex* subsp. *rotundifolia* (Escudero y Mediavilla, 2003; Niinemets *et al.*, 2005, 2006). Los resultados obtenidos en el **capítulo V** sostienen que la fotosíntesis neta (A_N) estuvo limitada principalmente por la conductancia del mesófilo (g_m) y que la importancia relativa de la limitación del mesófilo (l_m) incrementó con la edad de la hoja. La reducción de g_m y A_N en las hojas viejas estuvo claramente inducida por una reducción de la superficie de los cloroplastos expuesta a espacios intercelulares por unidad de áres (S_c/S) y, en menor medida, por el aumento del espesor de la pared celular (T_{cw}). Además, se observó una reducción de la concentración de nitrógeno, principalmente en el nitrógeno del citoplasma de las hojas más viejas (**capítulo V**). Por lo tanto, cabría esperar que la cantidad de Rubisco también se viera afectada por el desmantelamiento del aparato fotosintético. En este sentido, la reducción de S_c/S y de la concentración de nitrógeno citoplasmático en las hojas más viejas podrían estar relacionadas con el desmantelamiento progresivo del aparato fotosintético asociado al envejecimiento foliar (Niinemets *et al.*, 2009, 2012).

Atributos fisiológicos

Uno de los atributos morfológicos mencionados anteriormente es la distinción entre especies de poro en anillo y de poro difuso. *Quercus faginea* presenta madera de primavera con vasos grandes que contribuyen a la mayor parte del flujo de savia y madera de verano con vasos de menor tamaño que representan una parte muy pequeña de la conductividad hidráulica (Corcuera *et al.*, 2005). Por el contrario, *Q. ilex* subsp. *rotundifolia* es un ejemplo de especie de poro difuso, ya que solo presenta vasos pequeños, pero menos vulnerables a la cavitación por congelación o estrés hídrico (Tyree *et al.*, 1994; Corcuera *et al.*, 2005). Estas diferencias en la disposición, distribución y tamaño de los vasos del xilema se traducen en un desempeño fisiológico distinto frente a las condiciones impuestas por el clima mediterráneo.

Q. faginea mostró un mejor desempeño en cuanto a los atributos fisiológicos respecto de *Q. ilex* subsp. *rotundifolia* en condiciones de riego normal (**capítulo VI**). En este sentido, la especie caducifolia tuvo valores más altos (entre 34% y 45%) en A_N, en su conductancia estomática (g_s), g_m y en la conductancia del brote (K_{shoot}) sugiriendo una coordinación entre el intercambio de gases y la hidráulica de la hoja (Brodribb *et al*., 2007; Boyer, 2015; Scoffoni *et al*., 2016; Xiong y Nadal, 2020 y referencias incluidas). Esta coordinación explicaría la mayor capacidad para asimilar carbono de *Q. faginea* en absencia de estrés hídrico, a costa de un mayor consumo de agua cuando hay demanda evaporativa atmosférica (Peguero-Pina *et al*., 2016). Bajo condiciones de sequía, *Q. faginea* mostró una alta sensibilidad estomática a la a falta de agua en el suelo respecto de *Q. ilex* subsp. *rotundifolia*, lo que minimizó las pérdidas de agua a costa de una reducción de A_N (**capítulo VI**). Sin embargo, es realmente destacable que, en este estudio, la fotosíntesis se vio limitada bajo condiciones de estrés hídrico por factores no estomáticos. Este es el caso de la g_m, el principal factor que explicaría las diferencias entre las dos especies en cuanto a la respuesta fotosintética cuando condiciones fueron desfavorables (**capítulo VI**).

En cuanto a los rasgos hidráulicos, *Q. faginea* mostró una disminución más temprana y rápida de K_{shoot} en respuesta a la sequía que *Q. ilex* subsp. *rotundifolia*. Sin embargo, ambas especies mostraron valores similares de conductividad hidráulica (K_{stem}) durante la progresión del ciclo de sequía (**capítulo VI**). Lo cual pone de manifiesto la capacidad de *Q. faginea* para proteger el tallo y usar la hoja como "fusible hidráulico" para reducir las pérdidas de agua por transpiración, asegurando la supervivencia de las yemas para el desarrollo futuro de nuevas hojas (Hinckley *et al*., 1983; Peguero-Pina *et al*., 2015; Skelton *et al*., 2018). Esta capacidad se conoce como "segmentación de la vulnerabilidad" y se relaciona con el embolismo foliar como estrategia para evitar el subsiguiente embolismo xilemático del tallo (Hochberg *et al*., 2017). Por su parte, *Q. ilex* subsp. *rotundifolia* no mostró evidencias de una "segmentación de la vulnerabilidad" y mantuvo las hojas verdes durante casi todo el ciclo de sequía. En este sentido, se ha sugerido que esta estrategia se relaciona con su mayor longevidad foliar y su habilidad para mantener la capacidad fotosintética durante más de un periodo vegetativo (Corcuera *et al*., 2005; Peguero-Pina *et al*., 2015).

Por lo tanto, la coexistencia de ambas especies en estas áreas de transición de la cuenca mediterránea occidental podría estar asociada a condiciones edáficas locales que se traducen en una menor capacidad de almacenamiento de agua como consecuencia de la degradación del suelo (Corcuera *et al*., 2005). Esta situación se agudizaría en el contexto del cambio climático, que prevé un aumento de la temperatura y la aridez en la cuenca mediterránea (Klausmeyer y Shaw, 2009; Ruiz-Labourdette *et al*., 2012). Por ello, existen algunos modelos de predicción que indican una notable reducción del rango de distribución potencial de las especies de *Quercus* caducifolios mediterráneos (*e.g.*, *Q. faginea*) y su sustitución por especies perennifolias mediterráneas como *Q. ilex* (Sánchez de Dios *et al*., 2009).

7.2. Conclusions

The conclusions related to each specific objective of the thesis are presented below.

i) *Sclerophylly in the genus* Quercus *from the information available in the literature and public databases: factors that promote it, its functional role and consequences for the leaf in the context of the "*Leaf Economics Spectrum*".*

1. The wide variability found in terms of sclerophylly in the genus *Quercus* could not be satisfactorily explained by each of the ecological factors (water, nutrients, herbivory, temperature or leaf longevity) when they are considered independently. However, there is evidence of an effect of every one of them in terms of the development of sclerophyllous leaves in the genus. In addition, habitats that combine several ecological constraints are those in which sclerophylly is more frequent, as would be the case of Mediterranean evergreens.

2. From a functional point of view, leaf habit greatly modulated the physiological implications of sclerophylly in the genus. Among evergreen species, sclerophylly has a cost not only in terms of carbon investment, but also limits photosynthetic capacity, mainly through limitations in CO_2 diffusion. In contrast, the increase in LMA in deciduous species was associated with increased photosynthetic capacity, reflecting the Mediterranean deciduous oak strategy to maximize carbon gain during optimal periods, as an adaptation to growing fragmentation.

ii) *Quantification and study of the diversification in the morphology, anatomy and chemical composition of the leaves of deciduous and evergreen oaks, analyzing the role of climate in the differential diversification of LMA within both types of oaks.*

3. Deciduous and evergreen species showed morphological differences that revealed a contrasting adaptive response. Regardless of phylogeny or natural habitat, species differed in leaf anatomical attributes, such as LMA, LT, or leaf area. In contrast, they did not differ in LD despite their significant differences in density-related leaf characteristics such as cellulose concentration, IVD, and width of bundle sheath extensions.

4. Deciduous species with shorter growth periods showed a tendency to develop leaves with lower AML and thickness than those with longer growth periods, while evergreens showed the opposite pattern. Taking into account both habits, LMA and LT increased in arid environments. The increase in LMA and LT was associated with a higher LD in evergreens and, counterintuitively, with a lower LD in deciduous ones.

5. LMA in deciduous species appears to have diversified in coordination with LT, mainly modulated by aridity. On the contrary, the diversification of this parameter in evergreens was partly influenced by phylogeny. Regarding the environmental factors that promote sclerophylly in evergreens, interspecific variability in LT was modulated by both aridity and cold, while LD diversification was only related to aridity.

iii) *Study of foliar mechanical resistance in deciduous and evergreen species of the genus* Quercus*: determination of the anatomical and compositional features that explain the variability within the genus.*

6. The leaves of sclerophyllous species had higher hardness values, as revealed by mechanical tests (punch and die) due to differences in structural attributes of the epidermis, with the development of external cell walls and thicker cuticles. This fact does not imply large changes in the accumulated mass per unit of leaf surface or higher construction costs. At the compositional level, the cellulose concentration in cell walls played a critical role in the increase in leaf mechanical resistance, closely associated with the greater thickness of the bundle sheaths and the external cell wall of the epidermis, both structures very rich in cellulose.

7. Regardless of their phylogenetic origin, Mediterranean evergreens showed similar leaf traits, suggesting an evolutionary convergence of sclerophylly in the Mediterranean type climates.

8. The species of the *Ilex* section, despite occupying habitats with contrasting climatic conditions (from tropical to continental Mediterranean or arid) share the same structural and compositional features. The paleotropical origin of this section would justify these results.

iv) *Evaluation of changes in morphological, anatomical, chemical and photosynthetic traits related to leaf age in an evergreen Mediterranean oak with high leaf longevity.*

9. A decrease in photosynthetic capacity associated with leaf aging was observed. This decrease was driven by anatomical changes that decreased mesophyll conductance, by a sharp decrease in S_c/S, and by biochemical changes through a decrease in the amount of nitrogen available to the photosynthetic machinery. These changes reflected a gradual physiological deterioration related to the dismantling of the photosynthetic apparatus.

v) *Analysis of the different responses to drought in photosynthetic, photochemical and hydraulic traits in different organs of two Mediterranean species with different leaf habits (*Q. faginea *and* Q. ilex *subsp.* rotundifolia*).*

10. On the one hand, *Q. faginea*, compensated for the shorter leaf life span with a higher carbon assimilation capacity through a higher g_m, V_{cmax} and K_{shoot}, although at the expense of higher water consumption under favorable conditions. As the water deficit in the soil increased, there was a decrease in g_m and an increase in leaf embolism, with an absence of effective photoprotective mechanisms. On the contrary, *Q. faginea* stems were highly resistant to xylem embolism, allowing the plant to develop new leaves after the effects of drought.

11. On the other hand, *Q. ilex* subsp. *rotundifolia* showed a conservative strategy, characterized by a high resistance to cavitation induced by drought both in the leaf and in the stems, as well as effective photoprotective mechanisms at the leaf scale. The survival of the leaves, even overcoming conditions of intense drought, would be associated with their capacity for the functional maintenance of the leaves throughout more than one period of stress.

7.3. Conclusiones

A continuación, se exponen las conclusiones relacionadas con cada objetivo específico de la tesis.

i) *La esclerofilia en el género* Quercus *a partir de la información disponible en la literatura y bases de datos públicas: factores que la promueven, su papel funcional y las consecuencias para la hoja en el contexto del* "Leaf Economics Spectrum".

1. La amplia variabilidad encontrada en términos de esclerofilia en el género *Quercus* no pudo ser explicada satisfactoriamente teniendo en cuenta cada uno de los factores ecológicos (escasez de agua, nutrientes, herbivoría, temperatura o longevidad foliar) cuando son considerados independientemente. No obstante, se evidencia un efecto de todos y cada uno de ellos en cuanto al desarrollo de hojas esclerófilas en el género. Además, los hábitats que combinan varias limitaciones ecológicas son aquellos en los que la esclerofilia es más frecuente, como sería el caso de los perennifolios Mediterráneos.

2. Desde un punto de vista funcional, el hábito foliar moduló en gran medida las implicaciones fisiológicas de la esclerofilia en el género. Entre las especies perennifolias, la esclerofilia tiene un coste no solo en términos de inversión de carbono, sino que también limita la capacidad fotosintética, principalmente a través de limitaciones en la difusión del CO_2. Por el contrario, el aumento de LMA en las especies caducifolias se asoció con una mayor capacidad fotosintética, lo que refleja la estrategia de los robles caducifolios mediterráneos para maximizar la ganancia de carbono durante los períodos óptimos, como una adaptación a la fragmentación del periodo vegetativo.

ii) *Cuantificación y estudio de la diversificación en la morfología, anatomía y composición química de las hojas de robles caducifolios y perennifolios, analizando el papel del clima en la diversificación diferencial de LMA dentro de ambos tipos de robles.*

3. Las especies caducifolias y perennifolias mostraron diferencias morfológicas foliares que revelaron una respuesta adaptativa contrastada. Independientemente de la filogenia o del hábitat natural, las especies difirieron

en atributos anatómicos de las hojas, como LMA, LT o área foliar. Por el contrario, no difirieron en LD a pesar de sus diferencias significativas en las características de la hoja relacionadas con la densidad, como la concentración de celulosa, IVD y el ancho de las extensiones de las vainas del haz.

4. Las especies caducifolias con periodos de crecimiento más corto mostraron una tendencia a desarrollar hojas con menor LMA y espesor que aquellos con periodos de crecimiento más largo, mientras que las perennifolias presentaron el patrón opuesto. Teniendo en cuenta ambos hábitos, LMA y LT aumentaron en ambientes áridos. El aumento en LMA y LT se asoció con una mayor LD en perennifolias y, contraintuitivamente, con una menor LD en las caducifolias.

5. LMA en especies caducifolias parece haberse diversificado en coordinación con LT, modulado principalmente por la aridez. Por el contrario, la diversificación de este parámetro en perennifolias se vio en parte influenciada por la filogenia. Respecto a los factores ambientales que promueven la esclerofilia en perennifolias, la variabilidad interespecífica en LT estuvo modulada tanto por la aridez como por el frío, mientras que la diversificación en LD presentó relación tan sólo con la aridez.

iii) *Estudio de la resistencia mecánica foliar en especies caducifolias y perennifolias del género* Quercus: *determinación de los rasgos anatómicos y composicionales que explican la variabilidad dentro del género.*

6. Las hojas de las especies esclerófilas presentaron valores más altos de dureza, según revelaron ensayos mecánicos (“*punch and die*”) debido a diferencias en atributos estructurales de la epidermis, con el desarrollo de paredes celulares externas y cutículas más gruesas. Este hecho no supone grandes cambios en la masa acumulada por unidad de superficie foliar ni mayores costes de construcción. A nivel composicional, la concentración de celulosa en las paredes celulares tuvo un papel crítico en el aumento de la resistencia mecánica foliar, estrechamente asociada al mayor espesor de las vainas del haz y de la pared celular externa de la epidermis, ambas estructuras muy ricas en celulosa.

7. Independientemente de su origen filogenético, las especies perennifolias mediterráneos mostraron rasgos foliares similares, lo que pone de manifiesto una convergencia evolutiva de la esclerofilia en el clima mediterráneo.

8. Las especies de la sección *Ilex*, pese a ocupar hábitats de condiciones climáticas contrastadas (desde tropicales a mediterráneos continentales o áridos) comparten unos mismos rasgos estructurales y composicionales. El origen paleotropical de esta sección justificaría estos resultados.

iv) *Evaluación de los cambios en los rasgos morfológicos, anatómicos, químicos y fotosintéticos relacionados con la edad de las hojas en un roble mediterráneo perennifolio con una alta longevidad foliar.*

9. Se observó una disminución en la capacidad fotosintética asociada con el envejecimiento de la hoja. Esta disminución fue impulsada por cambios anatómicos que disminuyeron la conductancia del mesófilo, por una fuerte disminución en S_c/S, y por cambios bioquímicos, a través de un descenso en la cantidad de nitrógeno disponible para la maquinaria fotosintética. Estos cambios reflejaron un deterioro fisiológico paulatino relacionado con el desmantelamiento del aparato fotosintético.

v) *Análisis de la diferente respuesta a la sequía en los rasgos fotosintéticos, fotoquímicos e hidráulicos en diferentes órganos de dos especies mediterráneas con diferente habito foliar (*Q. faginea *y* Q. ilex *subsp.* rotundifolia*).*

10. Por una parte, *Q. faginea,* compensó la menor vida útil de las hojas con una mayor capacidad de asimilación de carbono por medio de una mayor g_m, V_{cmax} y K_{shoot}, aunque a expensas de un mayor consumo de agua en condiciones favorables. A medida que se incrementó el déficit de agua en el suelo, se registró una disminución en la g_m y un aumento de la embolia foliar, con ausencia de mecanismos fotoprotectores efectivos. Por el contrario, los tallos de *Q. faginea* se mostraron altamente resistentes a la embolia del xilema, permitiendo al vegetal el desarrollo de nuevas hojas tras los efectos de la sequía.

11. Por otra parte, *Q. ilex* subsp. *rotundifolia* mostró una estrategia foliar conservadora, caracterizada por una alta resistencia a la cavitación inducida por la sequía tanto en la hoja como en los tallos, así como mecanismos fotoprotectores efectivos a escala foliar. La supervivencia de las hojas, superando incluso condiciones de sequía intensa, se asociaría a su capacidad para el mantenimiento funcional de las hojas a lo largo de más de un periodo de estrés.

7.4. Bibliografía

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Apéndice

A continuación, se detallan las publicaciones derivadas de esta tesis que ya han sido publicadas junto con el factor de impacto de la revista, su área temática y la contribución del doctorando.

Alonso-Forn D, Sancho-Knapik D, Ferrio JP, Peguero-Pina JJ, Bueno A, Onoda Y, Cavender-Bares J, Niinemets Ü, Jansen S, Riederer M, Cornelissen JHC, Chai Y and Gil-Pelegrín E. 2020. Revisiting the Functional Basis of Sclerophylly Within the Leaf Economics Spectrum of Oaks: Different Roads to Rome. **Current Forestry Reports** 6: 260–281. doi: 10.1007/s40725-020-00122-7

Factor de impacto: 6.656
Área temática: *Forestry*
Contribución: el doctorado participó en el planteamiento del estudio y en la redacción del manuscrito, así como en la edición y la organización de las contribuciones de los otros autores.

Sancho-Knapik D, Escudero A, Mediavilla S, Scoffoni C, Zailaa J, Cavender-Bares J, Álvarez-Arenas TG, Molins A, **Alonso-Forn D**, Ferrio JP, Peguero-Pina JJ and Gil-Pelegrín E. 2021. Deciduous and evergreen oaks show contrasting adaptive responses in leaf mass per area across environments. **New Phytologist** 230: 521-534. doi: 10.1111/nph.17151

Factor de impacto: 10.323
Área temática: *Plant Sciences*
Contribución: el doctorando participó en las medidas y el análisis de los datos climáticos y filogenéticos. Además, contribuyó a la revisión y edición del manuscrito.

Alonso-Forn D, Sancho-Knapik D, Fariñas MD, Nadal M, Martín-Sánchez R, Ferrio JP, Resco de Dios V, Peguero-Pina JJ, Onoda Y, Cavender-Bares J, Álvarez Arenas T, Gil-Pelegrín E. Disentangling leaf structural and material properties in relation to their anatomical and chemical compositional traits in oaks (*Quercus* L.). **En revisión** y publicado como *preprint* en bioRxiv. doi: 10.1101/2022.10.21.513225

Contribución: el doctorando participó en el planteamiento y diseño del estudio, realizó las medidas y analizó los resultados. Así mismo, escribió y editó el manuscrito.

Alonso-Forn D, Peguero-Pina JJ, Ferrio JP, García-Plazaola JI, Martín-Sánchez R, Niinemets Ü, Sancho-Knapik D, Gil-Pelegrín E. 2022. Cell-level anatomy explains leaf age-dependent declines in mesophyll conductance and photosynthetic capacity in the evergreen Mediterranean oak *Quercus ilex* subsp. *rotundifolia*. **Tree Physiology** 42: 1988–2002. doi: 10.1093/treephys/tpac049

Factor de impacto: 4.561
Área temática: *Forestry*
Contribución: el doctorando participó en el diseño experimental, realizó las medidas de las que derivan los resultados y participó en la redacción del manuscrito.

Alonso-Forn D, Peguero-Pina JJ, Ferrio JP, Mencuccini M, Mendoza-Herrer Ó, Sancho-Knapik D, Gil-Pelegrín E. 2021. Contrasting functional strategies following severe drought in two Mediterranean oaks with different leaf habit: *Quercus faginea* and *Quercus ilex* subsp. *rotundifolia*. **Tree Physiology** 41: 371–387. doi: 10.1093/treephys/tpaa135213

Factor de impacto: 4.561
Área temática: *Forestry*
Contribución: el doctorando participó en el planteamiento y diseño del experimento, realizó las medidas, analizó parte de los resultados y participó en la escritura del manuscrito.