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Do leaf lignin content or leaf mass-to-area bias the estimation of intrinsic water use efficiency from leaf bulk $\delta^{13}C$? A test with seedlings from five oak species



Hana Ghouil^{2,3}, Béatrice Richard¹, Pierre Montpied¹, Erwin Dreyer¹, and Oliver Brendel^{1*}

Abstract

Key Message Leaves of seedlings from five oak species (*Quercus robur* L.; *Q. pubescens* L.; *Q. suber* L.; *Q. afares* Pomel; *Q. ilex* L.) displayed large, mainly inter-specific, differences in leaf mass-to-area ratio (*LMA*) and lignin content, as well as in the ¹³C composition of bulk leaf biomass. The variation in leaf lignin content and *LMA* did not impact the offset between the ¹³C composition measured in bulk leaf material versus soluble sugars. This observation, as well as the similar correlations between intrinsic leaf water use efficiency and the ¹³C compositions of bulk material or soluble sugars extracted from leaves, confirms their reliable use as a proxy for the former even when there is a large variation in *LMA* or lignin among samples.

Context Carbon isotope composition (δ^{13} C) of bulk leaf biomass is frequently used as a proxy for intrinsic water use efficiency (*iWUE*) in large-scale intra- and inter-specific comparisons. However, post-photosynthetic ¹³C discrimination during the synthesis of lignin combined with differences in leaf mass-to-area ratio (*LMA*) may bias the relationship between δ^{13} C of bulk leaf matter and *iWUE* and thus its use as a proxy of *iWUE*.

Aims To quantify the impact of differences in lignin content and *LMA* on the relationship between $\delta^{13}C$ of bulk leaf biomass and *iWUE* over a large gradient of lignin contents across five oak species (deciduous: *Quercus robur, Q. pubescens, Q. afares* and evergreen: *Q. ilex* and *Q. suber*).

Methods We measured lignin content, *LMA*, and $\delta^{13}C$ of bulk leaf biomass and of soluble sugars extracted from the leaves, as well as intrinsic water use efficiency (derived from leaf gas exchange) in seedlings of the five oak species grown under common conditions in a greenhouse and measured in a climate chamber.

Results There was a large range (mainly across species) in lignin content (4 to 33%) and *LMA* (60–180 g m⁻²). $\delta^{13}C$ of bulk leaf biomass and soluble sugars were tightly correlated, showing a significant mean offset of – 0.4‰. This offset was stable across species and not correlated to the lignin content of the leaves. A very loose correlation was found between the offset and *LMA*, mainly due to one species.

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*Correspondence: Oliver Brendel oliver.brendel@inrae.fr Full list of author information is available at the end of the article



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Conclusion Our results are a demonstration that potential variations in leaf lignin content or *LMA* have no or only a little effect on the $\delta^{13}C$ of bulk leaf biomass. They are unlikely to cause a bias when using bulk leaf $\delta^{13}C$ as a proxy for variations in intrinsic water use efficiency among Mediterranean and temperate broad-leaf forest tree species.

Keywords *Quercus robur, Quercus ilex, Quercus suber, Quercus pubescens, Quercus afares,* Carbon isotope composition, Soluble sugars, Leaf mass-to-area ratio

1 Introduction

Leaf structure and chemical composition differ widely among species, across biomes (Wright et al. 2004) and among phylogenetically closely related species within a genus. Such differences have large consequences for the main functions/processes of leaves, like carbon assimilation through photosynthesis, water use through transpiration, and efficiency parameters like water use efficiency (Niinemets 1999; Mediavilla et al. 2020; Alonso-Forn et al. 2021; Solé-Medina et al. 2022).

Large variations of intrinsic water use efficiency (*iWUE*), defined as the ratio of net CO₂ assimilation rate (*A*) vs. stomatal conductance to water vapor (g_s) (Meinzer et al 1991), have been recorded within and among species (Lamont et al. 2002; Rumman et al. 2018). Due to its simplicity and ease of handling of large numbers of samples, the ¹³C composition of bulk leaf material ($\delta^{I3}C_{\rm lb}$), or more precisely, the isotopic discrimination ($\Delta^{I3}C_{\rm lb}$) between atmospheric CO₂ (usually close to -8%) and leaf carbon, is frequently used as a proxy for *iWUE* based on a model developed by Farquhar and Richards (1984):

$$iWUE = \frac{c_a}{1.6} \left(\frac{b - \Delta^{13} C_{lb}}{b - a} \right),$$

with c_a the atmospheric CO₂ concentration and *a* and *b* the fractionation factors related to diffusion in still air and to carboxylation reactions.

This relationship between *iWUE* and the isotopic composition of plant carbon is due to the isotopic discrimination against ¹³C that occurs during the diffusion of CO₂ from the atmosphere to the sub-stomatal cavities (in the gas phase), then from these cavities to the chloroplast stroma through cell walls and different membranes (in a gas phase followed by a liquid phase) and during the carboxylation of RuBP by Rubisco. This process was described in the discrimination models developed by Farquhar et al. (1982) and has been largely used since then to estimate *iWU*E of different plant species including crops and trees (Brendel 2021). Indeed, this approach was validated by the tight correlations observed between $\Delta^{13}C$ and direct estimates of *iWUE* at leaf level through gas exchange records (A/g_s) , or through monitoring of whole plant WUE estimated from the ratio biomass increment/transpiration (Farquhar and Richards 1984; Richards et al. 2002; Roussel et al. 2009; Rasheed et al. 2015 among others).

This approach helped screen *iWUE* in response to changing environments (for instance in tree rings, Brendel et al. 2002; Gessler et al. 2014; Rabarijaona et al. 2022 among many others) or across genotypes and populations within species grown under common conditions, including forest trees like *Pinus pinaster* Aiton, *Castanea sativa* Mill., *Quercus robur* L., *Faidherbia albida* Delile A. Chev., and *Populus* sp. (Brendel et al. 2002, 2008; Lauteri et al. 2004; Roupsard et al. 1998; Monclus et al. 2005).

However, the relationship between ${}^{13}C$ in bulk leaf material and *iWUE* needs to be used with caution, as has been underlined by Seibt et al. (2008), due to:

- 1. Variation in the biological processes related to the parameters in the Farquhar model, such as mesophyll conductance (i.e., the conductance between sub-sto-matal cavities and chloroplastic stroma; Flexas et al. 2012; Ma et al. 2021) or anaplerotic respiration (i.e., carbon fixation by PEPc; Wieloch et al. 2022), may impact directly the relationship between plant $\delta^{I3}C$ and *iWUE*.
- 2. Temporal changes in *iWUE* due to diel fluctuations in the micro-environment or due to seasonal changes in microclimate, particularly soil water availability, may result in a varying integration of the isotopic signal in the non-structural sugar pool versus the carbon in leaf structure, with an integration time of hours (for the former) and weeks during leaf expansion for the latter (Brugnoli et al 1988; Roussel et al. 2009); this requires in particular that comparisons are made under strictly similar environments in common garden plantations or in greenhouse or climate chamber conditions.
- 3. Variations in leaf structure and composition (and particularly the content in lignin) may result in significant deviation of the ¹³C signal due to postphotosynthetic discrimination during lipid synthesis (Bowling et al. 2008), impacting directly the relationship between $\delta^{I3}C_{\rm lb}$ and *iWUE*. Sugars, starch, and cellulose are usually ¹³C-enriched compared with bulk leaf matter, while lipids and lignin are ¹³C-depleted (Fernández et al. 2003; Badeck et al.

2005). In C₃ leaves, lignin may be depleted by 3-4% compared to bulk material (Benner et al. 1987).

It is commonly accepted that the isotopic composition of soluble sugars extracted from leaves $(\delta^{I3}C_{ss})$ provides rather reliable estimates of *iWUE* because of the following: (i) soluble sugars are direct products of photosynthesis not affected by post-photosynthetic discrimination and (ii) they display a high turnover rate at daily scale (Brugnoli et al. 1988). There is however still a need to investigate whether large differences in leaf structure (mainly *LMA*) and composition (mainly lignin content) may bias the relationship between $\delta^{I3}C_{lb}$ and *iWUE* when using $\delta^{I3}C_{lb}$ in large-scale inter-specific comparisons of species.

To obtain a large range of LMA and lignin content in leaves, we used 5 different oak species. Indeed, the genus Quercus comprises deciduous and evergreen species adapted to a wide range of climates and soil moisture availability (Escudero et al. 2017), from which we selected Q. ilex L. and Q. suber L. as evergreen, and Q. afares Pomel, Q. robur L., and Q. pubescens Willd. as deciduous species. Holm oak (Quercus ilex L.) is a sclerophyllous and drought-tolerant Mediterranean species, distributed in the central-western part of the Mediterranean basin (Gratani and Bonito 2009; Martín-Sánchez et al. 2022). Cork oak (Q. suber L.) is a widely distributed forest tree species in the western Mediterranean basin with sclerophyllous leaves and is characterized by an intermediate drought tolerance compared to other oaks (Gil-Pelegrín et al. 2017). Q. afares is an endemic oak species of North Africa (Mir et al. 2006). Pedunculate oak (Q. robur) and downy oak (Q. pubescens) are among the most abundant oak species in Western and Central Europe. Q. pubescens is distributed mainly in south-eastern Europe with the faculty to withstand moderate summer drought, while Q. robur is widespread in temperate Europe with a preference for deep and moist soils. Species from this genus are therefore an ideal material to test the stability of the relationship between $\delta^{13}C_{lb}$ and *iWUE* given a large variation in leaf density and lignin content.

We therefore based our investigation on the assumption that these five oak species would display a significant range of values of leaf mass-to-area ratio (*LMA*) and lignin content in order to test the following hypotheses:

- 1. There is a correlation between iWUE recorded directly from leaf gas exchange measurements and $\delta^{I3}C$ in soluble sugars ($\delta^{I3}C_{ss}$), across species.
- 2. There is an offset in $\delta^{I3}C$ between bulk leaf material $(\delta^{I3}C_{\rm lb})$ and soluble sugars $(\delta^{I3}C_{\rm ss})$, and this offset depends on the lignin content or *LMA* of the leaves.

The experiment was run in a climate chamber at Nancy-Champenoux with potted oak seedlings that had been grown under common greenhouse conditions. During the experiment, the seedlings were maintained under stable irradiance, temperature, and humidity, and wellwatered. Leaf gas exchange was recorded from repeated measurements during the daily cycle, and leaves were harvested for additional measurements (*LMA*, lignin content, $\delta^{I3}C_{\rm lb}$ and $\delta^{I3}C_{\rm ss}$) at the end of the experiment.

2 Material and methods

2.1 Plant material and growth conditions

Acorns of Q. suber and Q. afares were collected in northern Tunisia between Tabarka and Ain Drahem (36.77 N, 8.68 E). Acorns of Q. ilex were collected from Djebel Zaghouan, eastern Tunisia (36.37 N, 10.11 E). Seedlings of Q. robur and Q. pubescens were obtained from acorns collected in the domain forest of Compiègne, northern France (49.38 N, 2.89 E). The acorns were heat-treated (40 °C) to destroy fungi and were sown during May 2010, into 5-L pots filled with a 2:1 (v/v) sand:blond peat mixture. Twenty grams of slow-release fertilizer (Nutricote 100, N, P, K 13:13:13+oligo elements) were added to each pot. An automated drip-irrigation system provided water twice daily to each pot. Seedlings were placed in a greenhouse at Champenoux (Nancy, north-eastern France, 48.75 N, 6.34 E) under natural illumination. After 6 months, 10 individuals per species were transferred and randomly distributed into two climate chambers fitted with a rotating plate to homogenize the irradiance received by each individual.

Each climate chamber contained 25 plants (5 individuals per species). Microclimate in the chambers was programmed to the same environmental conditions: 12 h photoperiod (6:00–18:00); air temperature, 25 °C day/18 °C night; relative humidity, 85/45%, and a photosynthetic photon flux density (PPFD) of about $450 \pm 15 \ \mu mol \ m^{-2} \ s^{-1}$ at the top of the canopies.

2.2 Daily time courses of net CO₂ assimilation rate A, stomatal conductance g_s, and intrinsic water use efficiency *iWUE*

A and g_s were measured under ambient irradiance with a portable photosynthesis system (Li-6400) using a 6-cm² chamber covered with a transparent lid. Conditions in the chamber were as follows: PPFD, around 450 µmol m⁻² s⁻¹; C_{inlet} , 380 µmol mol⁻¹; VPD, 0.6–1.9 kPa; and leaf temperature, 25 °C. During each day, three measurements were made between 09:00 and 17:00. For an overall 10 days, each day, one individual from each species was measured resulting in 10×3 replicate measurements per species. Measurements were made on a pre-selected fully expanded, mature, and unshaded leaf. For each

individual, daily mean values of net CO_2 assimilation rate (*A*) and stomatal conductance to water vapor (g_s) were computed. Intrinsic water use efficiency was calculated as $iWUE = A/g_s$. As the integration of iWUE into the isotopic signal of plant carbon depends on the rate of carbon fixed, a daily mean value of iWUE weighted by *A* ($iWUE_w$) was computed by dividing iWUE for each individual at each time point by a weight representing the fraction of the corresponding *A* relative to the sum of *A* over the day:

$$iWUE_{\rm w} = \frac{\sum_{t=1}^{n} \frac{iWUE_t \times \sum_{t=1}^{n} A_t}{A_t}}{n}$$

The measured leaf was then harvested, frozen in liquid nitrogen, and stored at -80 °C. For *Q. ilex*, as the leaves were small, a second leaf was harvested just above or below the sample leaf. The sampled leaves were used for measurements of $\delta^{I3}C$, *C* and *N* mass percentage in bulk leaf material, $\delta^{I3}C$ of soluble sugars, *LMA*, and lignin content.

2.3 Leaf mass-to-area ratio (LMA)

LMA (g m⁻²) was estimated on fully expanded leaves of each individual, close to the one used for gas exchange. Leaf area was measured with an AM-100 Area Meter (Analytical Development Company, Herts, UK) on fresh leaves, and the leaves were then dried at 60 °C for 48 h. Dry mass and leaf area were measured without the petiole.

2.4 Extraction of leaf soluble sugars

The leaves used for gas exchange measurements were freeze-dried at - 80 °C, ground and milled for 1 min at 30 oscillations s^{-1} in a ball mill (Retsch MM301, Retsch GmbH, Haan, Germany), and 60 mg of leaf powder was weighed in 2 mL microtubes. The protocol for soluble sugar extraction was modified from the method described by Wanek et al. (2001). Methanol-chloroformwater (MCW, 12:5:3, v/v/v) (350 µL) was added. After shaking with a vortex mixer, the samples were placed into a water bath at 70 °C for 30 min. Immediately, the microtubes were centrifuged at 11,400g for 3 min. This step was repeated three times, and the supernatant was collected into a new 2 mL microtube. To induce phase separation, 200 µL chloroform and 500 µL deionized water were added to the supernatant and vigorously mixed. The samples were left for a few minutes, then centrifuged at 11,400g for 3 min. The aqueous phase was transferred to a new 2 mL microtube and complemented with 300 μ L freshly hydrated Dowex 1X8, 200-400 mesh [Cl]⁻ form resin, (Fluka, Sigma-Aldrich, Saint-Quentin-Fallavier, France) converted to [HCO₂]⁻ with sodium formate.

The samples were agitated for 2 h at room temperature. After centrifugation, the supernatant was transferred to a new 2 μ L microtube complemented with 300 μ L freshly hydrated Dowex 50W, 200–400 mesh [H]⁺ form resin (Sigma Aldrich, Saint-Quentin-Fallavier, France). Samples were again agitated for 2 h at room temperature. After centrifugation, the supernatant was transferred to a pre-weighed microtube and dried to complete dryness on a rotary evaporator (HETO, DK3450, Allerd, Denmark). An aliquot of the concentrated soluble sugars (1 mg) was diluted with 60 μ L water, transferred into a tin capsule, and freeze-dried (FreeZone, Labconco, Kansas City, MO, USA).

The quality of the sugar extraction was tested by recording C and N content (see below). C content was approximately 38%, which is close to the value expected for pure carbohydrates. N content was low (around 0.07%), which is close to the detection limit, showing that carbohydrates were largely dominant in this soluble fraction.

2.5 Measurement of lignin content in leaves

An aliquot of 0.1 g of each sample of freeze-dried and milled leaves was put into individual pouches made of PTFE pre-cut filter membranes with 1 μ m pores (Alltech, France). The pouches were closed with Teflon ribbon, and all pouches were placed in a Soxhlet reactor. They were extracted with an acetone:water (95:5 v:v) mixture for 7 h to eliminate extractives (waxes, oils, pigments, terpenoids, phenolic compounds) that could interfere with the gravimetric measurement of Klason lignin. The respective content was transferred from the pouches into glass tubes and dried at 60 °C for 36 h. The solvent extraction step yielded an extractive-free residue (cell wall residue, CWR).

Klason lignin is the acid-insoluble fraction of lignin, which is the major fraction in terms of quantity (ASTM standard D1106-96, 2007). A first hydrolysis was achieved in 72% sulfuric acid at 30 °C for 60 min in a rotary water bath. For 0.06 g of dry extractives-free (CWR) leaf sample, 1 mL of 72% sulfuric acid was added. The hydrolysis was completed in an autoclave (1 h, 1.5 bars, 121 °C) after being diluted to 3% sulfuric acid through the addition of water. The reaction mixture was filtered on fiberglass. The solid residues were dried at 105 °C overnight and weighed.

Ash content determination (ASTM standard E1755-01, 2015) in Klason lignin residue was performed by calcination of the residue in a muffle furnace with a ramp program until 525 °C for 2 h in a porcelain crucible.

The Klason lignin content was calculated as a mass percentage of the dried extractives-free leaf, subtracted from ashes (% of CWR-Ash), and reported as the average of nine independent determinations for each Quercus species.

2.6 Carbon isotope composition of bulk leaf matter ($\delta^{13}C_{lb}$) and of soluble sugars ($\delta^{13}C_{sc}$)

One milligram of dry leaf powder was weighed into tin capsules. Carbon isotope composition of bulk leaf ($\delta^{I3}C_{\rm lb}$) and leaf soluble sugars ($\delta^{I3}C_{\rm ss}$), as well as total carbon (%*C*) and nitrogen (%*N*) content, was analyzed with an elemental analyzer (Carlo Erba, NA 1500-NC, Milan, Italy) coupled to an isotope-ratio mass spectrometer (Finnigan, Delta-S, Bremen, Germany) with a precision of 0.1‰. The offset between the isotopic composition of bulk leaf matter and leaf-extracted soluble sugars was calculated for each leaf as follows: $\delta^{I3}C_{\rm o} = \delta^{I3}C_{\rm lb} - \delta^{I3}C_{\rm ss}$.

2.7 Statistical analyses

All statistical analyses were performed with R 3.4.3 (R Core Team 2024) on the data set (Brendel 2024).

The significance of correlations among variables was tested using the *lm* function, and the regression equations shown in the figures were estimated using the standard major axis method. The correlation matrix among all measured traits was estimated using the Pearson method. All tests and correlations were considered significant when P < 0.05. Means are expressed with their standard error (SE). $\delta^{I3}C_{\rm lb}$ and $\delta^{I3}C_{\rm ss}$ were compared using a paired *t*-test.

For all traits except gas exchange variables, a species effect was tested using a one-way ANOVA (*Im* function) with species as the main effect; species differences were tested using a Tukey HSD post hoc test (R package Agricolae, HSD test). For gas exchange variables (A, g_s , iWUE, $iWUE_w$), different ANOVA models were tested using climate chamber, day, and time as main factors in addition to species. These factors were introduced first into a SS type I model to take them into account before the species effect was introduced. Thus, the most complete model tested was:

$$Y = \text{chamber} + \text{day}(\text{chamber}) + \text{time} + \text{species} + \text{error}$$

No significant effect of climate chamber and day was detected for any gas exchange variable. To test if the time of the day had a significant effect, first, the following model was applied to gas exchange variables:

Y = time + species + interaction + error

As the time effect was of no interest to the objectives of this study, for coherence with the other traits studied, inter-specific differences for gas exchange variables were finally tested using only the *species* factor and the post hoc tests described above.

3 Results

3.1 Variability in leaf mass-to-area ratio (*LMA*), lignin, and C content in the leaves

A large gradient of leaf traits was observed across all individual leaves: 60 to 178 g m⁻² for *LMA* and 4.2 to 33.0%, for lignin (Fig. 1a). The variation was dominated by interspecific differences (Table 1).

LMA and %C were positively correlated to lignin content, but the correlation coefficient was low for *LMA* and higher for %C (R^2 =25 and 53%, respectively; Fig. 1a, b and Table 2). Similarly, *LMA* was positively but loosely correlated to %C (R^2 =18%, Table 2).

3.2 Leaf gas exchange (A and g_s), intrinsic water use efficiency (*iWUE*), and $\delta^{13}C$ in bulk leaf matter and in soluble sugars

Time of day had a significant effect on A and g_s , with decreasing values during the course of the day, despite constant climate chamber conditions. As a result, *iWUE* was significantly higher in the morning and afternoon compared to midday (Fig. 5 and Table 3 in Appendix).

 $\delta^{I3}C_{\rm lb}$ and $\delta^{I3}C_{\rm ss}$ were tightly inter-correlated $(R^2_{\rm adj}=55\%;$ Table 2 and Fig. 2), and $iWUE_{\rm w}$ was correlated to the two traits, with a higher $R^2_{\rm adj}$ for $\delta^{I3}C_{\rm ss}$ (0.28, Fig. 3, Table 2) compared to $\delta^{I3}C_{\rm lb}$ ($R^2_{\rm adj}=21\%; P<0.01;$ $\delta^{I3}C_{\rm lb}=-31.76+0.05\;iWUE)$. Q. ilex displayed the lowest and Q. robur the highest value of $iWUE_{\rm w}$. However, Q. suber displayed an ambiguous ranking as $\delta^{I3}C_{\rm ss}$ and $iWUE_{\rm w}$ displayed different rankings among the species (Table 1). The correlation between $\delta^{I3}C_{\rm lb}$ and $iWUE_{\rm w}$ was slightly weaker than that between $\delta^{I3}C_{\rm ss}$ and $iWUE_{\rm w}$ ($R^2_{\rm adj}=0.28$ and 0.22, respectively).

A paired *t*-test showed that $\delta^{I3}C_{ss}$ was significantly higher than $\delta^{I3}C_{lb}$ (-26.8 vs. -27.2‰) with a mean offset between the two parameters $\delta^{I3}C_o = -0.4$ ‰. The offset was independent of the leaf's lignin content, on a species mean level (Fig. 4a) as well as when using all data from individual leaves (*P*=0.93). There was a very loose correlation between *LMA* and the offset, but this was only due to the peculiar position of the individuals of *Q. ilex* as compared to all other ones (Fig. 4b).

4 Discussion

4.1 Variability of leaf structure and composition

As hypothesized, leaves from seedlings of the 5 *Quercus* species displayed a large range of *LMA* and lignin contents, from 60 to 178 g m⁻² and 4.2 to 33.0%, respectively. *LMA* values for temperate forest species (based on Wright et al. 2004 supplementary data for temperate forest trees 5 to 95% quantiles) range from 43 to 214 g m⁻². Leaves from different Mediterranean and temperate European *Quercus* species (Castro-Díez et al. 1997; Villar et al. 2006;



Fig. 1 a Correlation between the leaf mass-to-area ratio, *LMA* (g m⁻²) and the lignin content (*%lignin* of dry mass) and **b** between the carbon and lignin contents (*%C* and *%lignin* of dry mass) in leaves of seedlings of the five oak species: *Q. robur*, *Q. pubescens*, *Q. ilex*, *Q. suber*, and *Q. afares* grown under common conditions. The equations of the regression across the whole data set are provided. All regressions are significant at P < 0.001. Species mean values \pm 95% confidence intervals are superimposed to the individual data

Table 1 Species means (± SE of the mean) of traits recorded on 10 individuals in each of five *Quercus* species. One-way ANOVA to test for the effects of species (significance: *Sp. effect, in bold if P<0.05*; adjusted model R^2 : R^2adj). Different letters represent significant differences among species as tested by a post hoc Tukey test (*P* < 0.05). Abbreviations: *LMA*, leaf mass-to-area ratio; *%lignin*, lignin content in the leaves; *%C and %N*, carbon and nitrogen content in the leaf biomass; *A*, net CO₂ assimilation rate; g_s , stomatal conductance to water vapor; *iWUE*, mean daily intrinsic water use efficiency; *iWUE*_w, mean daily water use efficiency weighted by *A*; $\delta^{13}C_{\rm lot}$, $\sigma_{\rm r}$ offset between the two preceding values

	Q. robur	Q. pubescens	Q. afares	Q. suber	Q. ilex	Sp. effect	<i>R</i> ² adj
<i>LMA</i> (g m ⁻²)	78.5±3.41 d	96.1±2.57 c	104±3.57 bc	116.5±2.11 b	158±5.16 a	P<0.0001	0.859
%lignin	12.5±1.08 c	8.25±1.25 c	27.1 ± 1.19 a	27.3±1.10a	21.7±0.81 b	P<0.0001	0.843
%C	43.9±0.22 b	43.6±0.22 b	46.1±0.30 a	46.3±0.29 a	45.7±0.24 a	P<0.0001	0.675
%N	2.26±0.145 a	2.14±0.06 a	2.30±0.22 a	2.14±0.14 a	1.58±0.05 b	P<0.005	0.232
A (μmol m ⁻² s ⁻¹)	8.10 ± 0.459	7.06 ± 0.574	7.55 ± 0.730	7.06 ± 0.4332	9.06 ± 0.570	P=0.0595	0.017
$g_{\rm s} ({\rm mmol} \; {\rm m}^{-2} {\rm s}^{-1})$	86.0±6.42 b	82.7±8.99 b	86.4±7.29 b	68.1±5.04 b	119±10.3 a	P<0.001	0.167
<i>iWUE</i> (µmol mol ⁻¹)	102.5±6.38 a	93.3±5.47 ab	88.1±5.10 ab	107±4.069 a	81.1±3.37 b	P<0.01	0.103
<i>iWUE</i> _w (µmol mol ⁻¹)	106±8.08 ab	96.5±6.62 ab	90.5±6.05 ab	112±5.558 a	82.8±4.07 b	P<0.01	0.181
$\delta^{13}C_{\text{lb}}$ (‰)	- 25.5±0.24 a	−27.1±0.19 b	-27.6±0.27 bc	-27.7±0.26 bc	-28.2±0.23 c	P<0.0001	0.597
$\delta^{13}C_{ss}$ (‰)	−24.9±0.43 a	-26.7±0.23 b	-26.9±0.48 b	-27.2±0.37 bc	— 28.5 ± 0.17 с	P<0.0001	0.495
$\delta^{13}C_{o}$ (‰)	-0.54 ± 0.36	-0.45 ± 0.22	-0.73 ± 0.55	-0.43 ± 0.16	0.32 ± 0.26	P=0.279	0.028

Mediavilla et al. 2008) as well as of 9 European temperate broad-leaf species from different genera (*Acer, Carpinus, Corylus, Fagus, Fraxinus, Populus, Quercus, Tilia, Ulmus;* Hejcmanová et al. 2014) ranged overall from 8.5 to 24% leaf lignin content. This confirms that the leaves from the seedlings of the species sampled here actually cover a large fraction of the *LMA* and leaf lignin content spectrum for Mediterranean and temperate deciduous and evergreen species. Variations in *LMA* are usually attributed to two traits: (1) leaf thickness and (2) leaf density (Villar et al. 2013; Escudero et al. 2017). High *LMA* results usually from larger cell sizes, greater numbers of mesophyll cell layers,

Table 2 Pearson's correlation coefficients (lower diagonal) and P values (upper diagonal) for the traits measured on individual leave	S
during the experiment. Significant correlations with P <0.05 are in bold. The same symbols and units as in Table 1	

	g _s	Α	iWUE	<i>iWUE</i> w	LMA	Lignin	% N	% C	$\delta^{13}C_{lb}$	$\delta^{13}C_{ss}$
g _s		P<0.001	P<0.001	0.001	0.073	0.980	0.254	0.792	0.577	0.045
Α	0.813		0.89	0.92	0.395	0.895	0.556	0.678	0.458	0.787
iWUE	-0.476	0.0100			0.09	0.863	0.238	0.978	0.003	P<0.001
iWUE _w	-0.460	0.0143			0.021	0.716	0.139	0.900	0.001	P<0.001
LMA	0.261	0.125	-0.247	-0.331		0.002	0.003	0.002	P<0.001	P<0.001
Lignin	-0.00390	0.0205	-0.0267	-0.0565	0.456		0.585	P<0.0001	P<0.0001	-0.003
N _{lb}	-0.169	-0.0882	0.175	0.210	-0.424	-0.0845		0.559	0.021	0.074
$C_{\rm lb}$	-0.0396	-0.0622	-0.0041	-0.0123	0.445	0.790	-0.087		0.001	0.002
$\delta^{13}C_{\rm lb}$	-0.0834	0.110	0.418	0.470	-0.652	-0.575	0.336	- 0.478		P<0.001
$\delta^{13}C_{\rm ss}$	-0.296	-0.0409	0.504	0.542	-0.6935	-0.432	0.265	-0.438	0.748	



Fig. 2 Correlation between the carbon isotope composition of bulk leaf ($\delta^{13}C_{lb}$) and soluble sugars ($\delta^{13}C_{ss}$) in the leaves of the five oak species: *Q. robur, Q. pubescens, Q. ilex, Q. suber,* and *Q. afares* grown under common conditions in a greenhouse. The equations of the regression across the whole data set are provided. The regression is significant at *P* < 0.001. Species mean values ± 95% confidence intervals are superimposed to the individual data

and higher cell mass densities (including starch content) as described in the "EXACT" approach (John et al. 2017). Leaf carbon was tightly and positively correlated to lignin content as expected, while the correlations of the two parameters with *LMA* were much less tight, which suggests that

variation in *LMA* is controlled also by variation in tissue density independently of the chemical composition (San-cho-Knapik et al. 2021).

Differences in *LMA* values followed the expected trends, with the sclerophyllous leaves of *Q. ilex* and *Q. suber* displaying the highest *LMA* and lignin content of the 5 species.



Fig. 3 Correlation between the carbon isotope composition in leaf soluble sugars ($\delta^{13}C_{ss}$) and the weighted intrinsic leaf water use efficiency (*iWUE*_w) recorded from leaf gas exchange among five oak species: *Q. robur, Q. pubescens, Q. ilex, Q. suber,* and *Q. afares* grown under common conditions. The equations of the regression across the whole data set are provided. The regressions are significant at *P* < 0.001. Species mean values \pm 95% confidence intervals are superimposed to the individual data



Fig. 4 Offset $(\delta^{13}C_0)$ between the carbon isotope composition in the bulk leaf biomass $(\delta^{13}C_{1b})$ and in leaf soluble sugars $(\delta^{13}C_{s})$ as a function of **a** the *%lignin* in leaves and **b** the leaf mass-to-area ratio (*LMA*) for five oak species: *Q. robur, Q. pubescens, Q. ilex, Q. suber,* and *Q. afares* grown under common conditions. The horizontal line represents the 0 line. For **b**, the equation of the regression across the whole data set is provided; the regression is significant at *P* < 0.05. Species mean values ± 95% confidence intervals are superimposed to the individual data

Q. afares, a deciduous species, displayed nevertheless high *LMA* and lignin content.

We need to emphasize that the observed values were recorded on young seedlings exposed to rather moderate levels of irradiance during their growth and that these traits display important phenotypic plasticity induced by age and micro-environments (Escudero et al. 2017). Such a large variation confirms the validity of the species sample used here for analyzing the potential consequences of large variations in leaf lignin content and *LMA* when using $\delta^{I3}C_{\rm lb}$ as a proxy for *iWUE* in diverse species.

4.2 Variations of weighted intrinsic water use efficiency $(iWUE_w)$ and of ¹³C content in soluble sugars $(\delta^{13}C_{ss})$ among leaves from different *Quercus* species

A currently accepted hypothesis is that (i) the soluble sugar pools present in the leaves are to a large extent produced during the few days preceding the harvest, and (ii) their isotopic signature is largely influenced by the C-assimilated during periods of high assimilation rates. The latter effect may be quite large during a diurnal cycle with varying levels of irradiance, while it should be smaller in a climate chamber with stable irradiance, as used in this experiment. Nevertheless, given the diurnal changes observed in A (Fig. 5 in the Appendix), we used a diurnal mean value of *iWUE* weighted by the recorded assimilation rates A $(iWUE_w)$ for the comparisons with $\delta^{I3}C_{\rm ss}$. *iWUE*_w and $\delta^{I3}C_{\rm ss}$ correlated significantly. For $\delta^{I3}C_{ss}$, nearly all species had significantly different values, with *Q. ilex* at the lower end, and *Q. robur* at the higher; for *iWUE_w*, *Q. suber* actually showed the highest values, together with Q. robur. This might be related to differences in one of the parameters of the complete isotopic discrimination model (Farquhar and Richards 1984), in which variations in mesophyll conductance to CO_2 (g_m) have the largest impact. Actually, Peguero-Pina et al. (2017) estimated $g_{\rm m}$ for *Q. suber* to be 0.09 mol CO₂ m⁻² s^{-1} , while they estimated it to be 0.03 mol CO₂ m⁻² s⁻¹ for *Q. ilex*, with however a large within-species variation. Our observations suggest in concordance a relatively low mesophyll conductance of Q. ilex compared to the other oak species.

4.3 Comparison between δ^{13} C in bulk leaf material ($\delta^{13}C_{lb}$) and in soluble sugars ($\delta^{13}C_{cc}$) in the five species

The comparison was done by investigating the differences (offset) between $\delta^{I3}C$ in bulk leaf material ($\delta^{I3}C_{\rm lb}$) and in soluble sugars ($\delta^{I3}C_{\rm ss}$) and the correlation between them.

The very loose correlation between *LMA* and the offset was driven mainly by the peculiar position of leaves from *Q. ilex*, which displayed a positive offset. An effect of the mesophyll conductance, particularly small in this species as compared to all other ones, may be responsible for this, but we do not have enough information to actually demonstrate this hypothesis.

Despite the large range of LMA and in lignin content observed in our sample of leaves, did the offset not significantly differ among the five species and reached a mean value of -0.38% (with species extremes at + 0.32% and - 0.73%). Bulk leaf matter contains carbon fixed in the cell wall structure, which represents therefore, in terms of carbon assimilation and leaf functioning, the time period of leaf growth and maturation, but contains also carbon in rapid turnover molecules (starch, soluble sugars, organic and amino acids etc.), which represent leaf functioning from days (sugars) to weeks (starch) before the harvesting (Brugnoli et al 1988; Brugnoli and Farquhar 2000). $\delta^{13}C$ of bulk leaf matter ($\delta^{I3}C_{lb}$) represents therefore a weighted mean of the $\delta^{13}C$ of these different components, whereas $\delta^{13}C$ of soluble sugars ($\delta^{13}C_{ss}$) integrates mostly 1 day of photosynthetic discrimination and only the primary products of photosynthesis.

Cellulose and lignin are two major compounds of leaves. Sugars, starch, and cellulose are ¹³C-enriched compared to the bulk biomass of leaves, as cellulose is isotopically very close to the glucose produced during photosynthesis (Brugnoli et al. 1988). In a comparison of 9 Acacia species, Konate et al. (2016) found that cellulose extracted from the leaves was enriched by $\approx 1\%$ compared to bulk leaf material in all species. On the opposite, lipids and lignin are usually ¹³C-depleted with respect to bulk biomass (Benner et al. 1987; Brugnoli and Farquhar 2000). The amino acids phenylalanine and tyrosine are precursors for the lignin monomers (Benner et al. 1987). They both are about 10% depleted in ¹³C relative to glucose (Gleixner et al. 1998). Comparisons of $\delta^{13}C$ of lignin and of bulk leaf biomass or of cellulose extracted from leaves are scarce. A difference of 3.2 and 4.2‰ was detected between lignin and bulk leaf material for Juncus and Carex (Benner et al. 1987). Lignin from *Picea abies* L. needles was depleted in ¹³C by 1.3 to 2.4‰ compared to bulk leaf biomass (Gleixner et al. 1993). Results on wood are similar: lignin was depleted in ¹³C by 3.5‰ compared to cellulose (Wilson and Grinsted 1977), while a depletion of 3‰ was found

in lignin with respect to cellulose in *Q. robur* wood (Loader et al. 2003).

We thus might assume 3‰ as a reasonable difference between the ¹³C content of bulk leaf and lignin and use this value in a mass balance model to assess how the observed variation in leaf lignin content might impact $\delta^{I3}C_{\rm lb}$. Considering the observed mean $\delta^{I3}C_{\rm lb}$ value of -27.2%, we can calculate a mean $\delta^{I3}C_{\rm l}$ for lignin $(\delta^{I3}C_{\rm l})$ of -27.2% - 3% = -30.2%. Across all species, we measured a mean lignin fraction (*lf*) of 0.19. We then used these values to estimate the isotopic composition of the residual leaf biomass excluding lignin ($\delta^{I3}C_r$), to be:

$$\delta^{13}C_r = \left(\delta^{13}C_{1b} - \delta^{13}C_1 \times lf\right) / (1 - lf) = -26.5\%$$

We then can use the following equation to calculate the expected value of $\delta^{13}C_{lb}$, based on the calculated values for $\delta^{13}C_l$ and for $\delta^{13}C_r$, and depending on the lignin fraction *lf*, to be:

$$\delta^{13}C_{1b} = \delta^{13}C_1 \times lf + \delta^{13}C_r \times (1 - lf)$$

Using this latter equation and varying the lignin fraction (lf) from a minimum of 0.04 to a maximum of 0.33 (minimum and maximum values of leaf lignin fraction observed across all leaves analyzed of the 5 Quercus species), the resulting $\delta^{13}C_{\rm lb}$ varies by 0.9‰, where a higher lignin content results in more negative $\delta^{13}C_{\rm lb}$ values. Assuming a constant $\delta^{I3}C_{ss}$, this would result in a negative correlation between $\delta^{I3}C_o$ and lignin content. This largely contradicts our results, which did not reveal any correlation between $\delta^{13}C_0$ and lignin content, neither for species means (Fig. 4a), nor for individual leaf data (Table 2). One hypothesis to explain this lack of impact of the lignin content could be the pool effect proposed by Hobbie and Werner (2004). This effect is based on the hypothesis that, as lignin is synthesized, the remaining pool of sugars should be increasingly ¹³C enriched. They indeed observed a positive correlation between the offset $\delta^{I3}C_{\text{lignin}}$ - $\delta^{I3}C_{\text{cellulose}}$ and the lignin content over a range of species. The correlation observed by Hobbie and Werner (2004) was however based on a mix of C3 and C4 species including herbaceous and woody species, among which the monomer composition of lignin differs (Boerjan et al. 2003). The small and stable offset $\delta^{13}C_{lb}$ - $\delta^{13}C_{ss}$ among species in our study, independently of a large difference in lignin content, could confirm the pool effect on a basis of close species, for which we can hypothesize a more similar lignin monomer composition. However, any further discussion would need measured data on the ¹³C signatures of the different carbon pools in the leaves of a variety of Quercus species.

4.4 To what extent do the differences in leaf structure influence *iWUE* as recorded from gas exchange and $\delta^{13}C$?

 $\delta^{13}C_{\rm lb}$ and $\delta^{13}C_{\rm ss}$ were negatively related to LMA, whereas %C or lignin content was positively related to LMA. Two hypotheses can be evoked for such correlations. Leaf structure can directly impact gas exchange and thus *iWUE*, where a higher *iWUE* might result from a higher assimilation rate, due to a higher photosynthetic capacity per leaf surface, i.e., to a higher LMA. Such a hypothesis would suggest a positive correlation between ${}^{13}C$ and LMA, which we did not observe. But LMA may also impact the isotopic discrimination model linking *iWUE* with $\delta^{I3}C_{ss}$ (Farquhar et al. 1982) via differences in mesophyll conductance to CO_2 (g_m). Higher LMA is often positively associated with high tissue density and negatively with the fraction of intercellular air spaces (Villar et al. 2013; Escudero et al. 2017). A negative correlation between g_m and *LMA* was sometimes detected (Niinemets et al. 2005; Flexas et al. 2008; Sperlich et al. 2015), whereas data from Peguero-Pina et al. (2017) on different oak species, including Q. ilex and Q. suber, did not reveal any correlation. Furthermore, Piel et al. (2002) observed a positive correlation between *LMA* and g_m ; only this latter positive correlation would explain the observed negative correlation between $\delta^{13}C$ and *LMA*, involving an *LMA*-dependent variation of $g_{\rm m}$ among species.

5 Conclusion: is ¹³C in bulk leaf material ($\delta^{13}C_{lb}$) a reliable estimator of *iWUE* among *Quercus* species?

Contrary to our expectations, the quite large interspecific differences in LMA (60.0 to 178 g m⁻²) and in lignin content (4.2 to 33.0%) in leaves of young Quercus seedlings did not result in any visible impact on the difference in ¹³C composition between soluble sugars $(\delta^{13}C_{ss})$ and bulk leaf biomass $(\delta^{13}C_{lb})$. This suggests that the leaf composition in lignin versus non-lignin compounds and the large differences in LMA did not bias the isotopic signal in bulk leaf material and that therefore $\delta^{I3}C_{lb}$ may be used as a reliable proxy for inter-specific comparisons of *iWUE* among Mediterranean and temperate broad-leaf forest tree species. Our demonstration was based on a small sample of species with large variations in both LMA and lignin content. Further research would be needed to confirm our findings, and a larger effort to identify the ¹³C content in different leaf compartments (in particular lignin vs. cellulose vs starch) would still be needed to obtain a more reliable view of the significance of the ¹³C content in bulk leaf biomass as a proxy for *iWUE*.





Fig. 5 Time course of net CO_2 assimilation rate A (µmol m⁻² s⁻¹), stomatal conductance to water vapor g_s (mol m⁻² s⁻¹), and intrinsic water use efficiency A/g_s (µmol mol⁻¹), along a diurnal cycle. Five oak species were used: *Q. robur, Q. pubescens, Q. ilex, Q. suber*, and *Q. afares*. During each day, three measurements were made between 09:00 and 17:00. Each point represents the mean from 10 individuals per species

Table 3 Analysis of variance, using type III sum of squares for net CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s), and intrinsic water use efficiency calculated as $iWUE=A/g_{s'}$ factors are *species*, measurement *time* of the day, and the interaction (*int*); significances are shown as follows: "***" < 0.001 < "**" < 0.01 < "*" < 0.05; R^2_{adj} is the whole model coefficient of determination; differences between morning, midday, and afternoon measurement times were tested using the Tukey HSD test

Trait	Species	Time	ime Int R ² _{adj} HSD tes Morning Midday	HSD test	it		
					Morning	Midday	Afternoon
A	***	***	***	0.13	а	b	с
g s	***	***	***	0.21	а	b	С
iWUE	***	***	***	0.10	b	С	а

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Authors' contributions

Conception and design: OB, ED, and HG. Experimental setup: HG, PM, OB, ED. Chemical analyses: BR, HG. Data analysis: OB, PM, HG. Paper writing: OB, ED, HG. Paper revisions: all co-authors. The authors read and approved the final manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication

All authors gave their informed consent to this publication and its content.

Conflict of interest

E. Dreyer is the Editor-in-Chief of the Annals of Forest Science. The authors declare that they have no conflict of interest.

Author details

¹ Université de Lorraine, AgroParisTech, INRAE, Silva, Nancy, France. ²Laboratory of Forest Ecology, National Institute of Research in Rural Engineering, Water and Forests (INRGREF), University of Carthage, Hédi Elkarray Street, El Menzah IV, BP 10, Ariana 2080, Tunisia. ³Faculty of Sciences of Bizerte, University of Carthage, Jarzouna 7021, Tunisia.

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