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Research paper

Cuticular wax coverage and its transpiration barrier properties in *Quercus coccifera* L. leaves: does the environment matter?

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Plants prevent uncontrolled water loss by synthesizing, depositing and maintaining a hydrophobic layer over their primary aerial organs—the plant cuticle. *Quercus coccifera* L. can plastically respond to environmental conditions at the cuticular level. When exposed to hot summer conditions with high vapour-pressure deficit (VPD) and intense solar radiation (Mediterranean atmospheric conditions; MED), this plant species accumulates leaf cuticular waxes even over the stomata, thereby decreasing transpirational water loss. However, under mild summer conditions with moderate VPD and regular solar radiation (temperate atmospheric conditions; TEM), this effect is sharply reduced. Despite the ecophysiological importance of the cuticular waxes of *Q. coccifera*, the wax composition and its contribution to avoiding uncontrolled dehydration remain unknown. Thus, we determined several leaf traits for plants exposed to both MED and TEM conditions. Further, we qualitatively and quantitatively investigated the cuticular lipid composition by gas chromatography. Finally, we measured the minimum leaf conductance (g_{min}) as an indicator of the efficacy of the cuticular transpiration barrier. The MED leaves were smaller, stiffer and contained a higher load of cuticular lipids than TEM leaves. The amounts of leaf cutin and cuticular waxes of MED plants were 1.4 times and 2.6 times higher than that found for TEM plants, respectively. In detail, MED plants produced higher amounts of all compound classes of cuticular waxes, except for the equivalence of alkanoic acids. Although MED leaves contained higher cutin and cuticular wax loads, the g_{min} was not different between the two habitats. Our findings suggest that the qualitative accumulation of equivalent cuticular waxes might compensate for the higher wax amount of MED plants, thereby contributing equally to the efficacy of the cuticular transpirational barrier of *Q. coccifera*. In conclusion, we showed that atmospheric conditions profoundly affect the cuticular lipid composition of *Q. coccifera* leaves, but do not alter its transpiration barrier properties.

Keywords: cuticular lipids, dehydration tolerance, environmental change, leaf area reduction, minimum leaf conductance.

Introduction

Plant transition from an exclusively aquatic to a terrestrial environment happened ~450 million years ago. Besides providing important advantages, the new environment was the cause of a set of challenges like imminent desiccation, increased temperature and exposure to ultraviolet radiation (Waters 2003, Leliaert et al. 2011, Yeats and Rose 2013). Since this time, plants have evolved a multitude of morphological and physiological features that allow them to cope with these new challenges. However, the capacity to synthesize, deposit and maintain a hydrophobic surface layer, named the cuticle, on the outside of primary aerial organs has been claimed to be one of the most critical adaptive trait for plant survival in the highly dehydrating terrestrial environment (Yeats and Rose 2013).

The primary function of the plant cuticle is avoiding uncontrolled water loss (Riederer and Schreiber 2001). The plant

cuticle consists of a cutin matrix impregnated and coated with cuticular waxes. The biopolymer cutin is mainly composed of C_{16} to C_{18} hydroxy alkanoic acids and their derivatives, which are esterified within a complex network (Pollard et al. 2008). The cutin polyester is non-extractable but hydrolysable, whereas cuticular waxes are solvent-extractable, complex mixtures typically comprising homologous series of very-long-chain aliphatic and, additionally, in some plant species pentacyclic compounds (Jetter et al. 2006). The cutin matrix is involved in waterproofing, but it mainly contributes to the mechanical integrity of the plant cuticle (Khanal and Knoche 2017). So far, the functional barrier against water diffusion through the cuticle has been attributed to the very-long-chain aliphatic waxes (Riederer and Schreiber 1995, Jetter and Riederer 2016), whereas the pentacyclic components have been associated with protection against herbivory and with stabilization of heat-stressed cuticles (Reichardt et al. 1984, Oliveira and Salatino 2000, Schuster et al. 2016).

Besides avoiding dehydration, plants also depend on acquiring carbon dioxide for photosynthesis. The balance between stomatal transpiration and carbon dioxide uptake is essential for the life of terrestrial plants. While the stomata are open to carbon dioxide uptake, plants inevitably lose water to the surrounding atmosphere. Under unfavourable conditions, plants close their stomata and, therefore, the remaining water loss only occurs through the cuticle. It has been proposed that excessive cuticular water loss and high leaf-to-atmosphere vapour pressure may lead to sudden xylem cavitation during heatwaves (Cochard 2019). Thus, the efficient control of cuticular water loss is of fundamental importance for maintaining xylem hydraulic safety and, thereby, ensuring plant fitness and survival.

Elevated temperature, high vapour-pressure deficit (VPD) and a high number of sunshine hours are among the major limiting factors for reproduction, growth, development and geographical distribution of plants in Mediterranean ecosystems. *Quercus coccifera* L. is a sclerophyllous evergreen shrub, which can withstand prolonged periods of abiotic stress (Vilagrosa et al. 2003, Peguero-Pina et al. 2008). This plant species is one of the most representative constituents of the shrub-land flora in the arid regions of the Iberian Peninsula (Peguero-Pina et al. 2008), but its distribution even reaches temperate oceanic conditions in the Iberian Atlantic coast (Castro-Díez and Navarro 2007). *Quercus coccifera* is capable of plastically responding to environment variations, allowing this plant species to occur in these contrasting habitats (Rubio de Casas et al. 2007). Roth-Nebelsick et al. (2013) demonstrated that *Q. coccifera* is capable of developing cuticular wax structures to reduce the stomatal conductance when growing under Mediterranean atmospheric conditions (MED): elevated summer temperatures, high VPD and intense solar radiation. The cuticular waxes reduce the stomatal pore area from 32 to 5 μm^2. Moreover, Peguero-Pina et al. (2015) showed that this phenomenon is strongly reduced when *Q. coccifera* grows under temperate atmospheric conditions (TEM): mild temperatures, moderate VPD and regular solar radiation. The authors attributed this fact to the plasticity of stomatal protection by cuticular waxes in response to contrasting climatic conditions. Previous studies have also reported that environmental factors like low water availability, high temperatures, excessive light exposure and high VPD lead to a higher cuticular wax accumulation (Shepherd and Griffiths 2006). Despite the physiological and ecological importance of the cuticular waxes, the relationship between the wax composition and its transpirational barrier properties in *Q. coccifera* leaves remains unknown.

This study aims to investigate the effect of the atmospheric conditions on the leaf cuticular lipids and the efficacy of the cuticular transpiration barrier of *Q. coccifera*. We hypothesize that in plants living under MED conditions compared with those under TEM ones, (i) the accumulation of cuticular lipids increases and their qualitative composition widely differ and (ii) the cuticle is more efficient to avoid water loss. From these hypotheses, we predicted that MED plants (i) possess higher amounts of cutin monomers and cuticular waxes and (ii) have a lower cuticular permeability than TEM plants due to the higher accumulation of very-long-chain aliphatic compounds. We tested these hypotheses (i) by qualitatively and quantitatively determining the chemical composition of the leaf cuticle and (ii) by measuring the minimum leaf conductance (g_{min}) at 25 °C for plants of *Q. coccifera* grown either at the MED or TEM sites.

Materials and methods

Plant material and growing conditions

Seeds of *Q. coccifera* L. (Fagaceae) were harvested from a natural population growing near Zaragoza, Spain. The seeds were germinated in a mixture of 80% compost (Neuhaus Humin Substrate N6; Klasman-Deilmann GmbH; Geeste, Germany) and 20% perlite under greenhouse conditions. After the first vegetative period, plants were cultivated outside at CITA de Aragón (41°39′N, 0°52′W, Zaragoza, Spain) under MED (Figure 1). Finally, 2-year-old plants were randomly selected and transplanted into the Jardín Botánico de Iturrarán (43°13′N, 02°01′W, Gipuzkoa, Spain), which features TEM. Plants in both MED and TEM sites were watered as needed and fertilized with Osmocote Plus (Sierra Chemical). Measurements were conducted using 1-year-old fully developed leaves of 8-year-old plants.

Scanning electron microscopy

Small air-dried leaf sections were mounted on aluminium holders using double-sided adhesive tape (Plannet Plano) and sputter-coated with ~15 nm of gold:palladium (150 s, 25 mA, partial argon pressure 0.05 mbar, SCD005 sputter coater, Bal-Tec). Afterwards, the samples were examined with a field-emission scanning electron microscope (JEOL JSM-7500F)

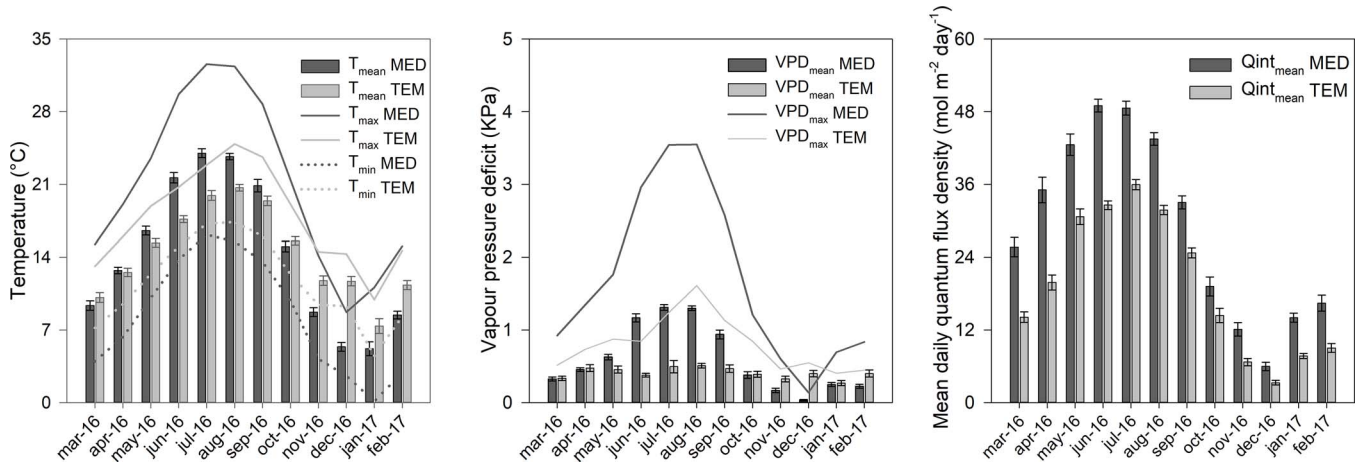

Figure 1. Mean (T_{mean}), maximum (T_{max}) and minimum (T_{min}) daily temperature (°C), mean diurnal (VPD_{mean}) VPD (kPa) - from dawn to sunset - and maximum diurnal VPD (VPD_{max}) and mean daily quantum flux density ($Qint_{mean}$, mol m^{-2} day^{-1}) for the MED and TEM sites during the growing season of 2016–17 (from March to February). Bars represent mean ± SE. Lines stand for single values.

using a 5-kV acceleration voltage and a 10 mm working distance. Micrographs were taken from both adaxial and abaxial leaf surfaces. The processed micrographs were used for determining the stomatal density.

Leaf traits

Overnight, leaves were full hydrated in a humid chamber before the measurements. The water-saturated fresh weight (FW) of leaves was determined using an analytical balance (MC-1 AC210S, Sartorius; precision 0.1 mg) and the dry weight (DW) was obtained after oven drying the leaves at 90 °C for 24 h. The actual fresh weights (FW_{actual}) during leaf-drying experiments were used to calculate the relative water deficit (RWD) according to the following:

$$RWD = 1 - \frac{FW_{actual} - DW}{FW - DW}.$$

Leaves were scanned at high resolution using a flatbed scanner, and the leaf area (LA) was measured from the scanned leaf image using the Adobe Photoshop software. Leaf mass per area (LMA) was obtained by dividing the DW by the LA. The leaf water content (LWC) was calculated by subtracting the DW from the FW and, subsequently, dividing the result by the FW.

Minimum leaf conductance

Minimum leaf conductance (g_{min}) was determined gravimetrically from the consecutive weight loss of desiccating leaves in darkness and at low atmospheric humidity. It corresponds to the lowest conductance a leaf can reach when stomata are maximally closed as a consequence of desiccation. Cut petioles of water-saturated leaves were sealed with high melting paraffin wax (Fluka). Subsequently, the sealed leaves were placed in an incubator at 25 °C (IPP 110, Memmert). The air temperature and humidity were monitored using a digital thermo-hygrometer (Testoterm 6,010, Testo). Silica gel (Applichem) was used to control the moisture in the incubator. The weight of desiccating leaves was determined as a function of desiccation time using an analytical balance (MC-1 AC210S, Sartorius; precision 0.1 mg). The transpiration rate (J) was calculated from the change in fresh weight (ΔFW) with time (t) divided by the dual projected leaf area (A):

$$J = \frac{\Delta FW}{\Delta t \times A}.$$

The cuticular water conductance (g) was calculated from the transpiration rate (J) divided by the driving force for water loss from the outer epidermal cell wall to the surrounding atmosphere. The driving force for the vapour-based conductance corresponds to the difference between the saturation concentrations of water vapour at the temperature of the leaf ($C_{wv\ sat\ leaf}$) and the surrounding atmosphere ($C_{wv\ sat\ air}$) multiplied by the water activity in the epidermal apoplast (α_{apo}) and the atmosphere (α_{air}):

$$g = \frac{J}{\alpha_{apo} \times C_{wv\ sat\ leaf} - \alpha_{air} \times C_{wv\ sat\ air}}.$$

The water activity of the atmosphere (α_{air}) over silica gel is nearly zero. The water activity in the apoplast adjacent to the inner side of the cuticle (α_{apo}) is assumed to be close to one. Thus, the active driving force for cuticular transpiration in the setup used here is the saturation concentration of water vapour at actual leaf temperature ($C_{wv\ sat\ leaf}$). Leaf temperature was measured using an infrared laser thermometer (Harbor Freight Tools, one-point measurements), and the corresponding water vapour saturation concentrations at leaf temperature were derived from tabulated values (Nobel 2009). The cuticular water conductance at a given dehydration point was plotted versus the respective RWD.

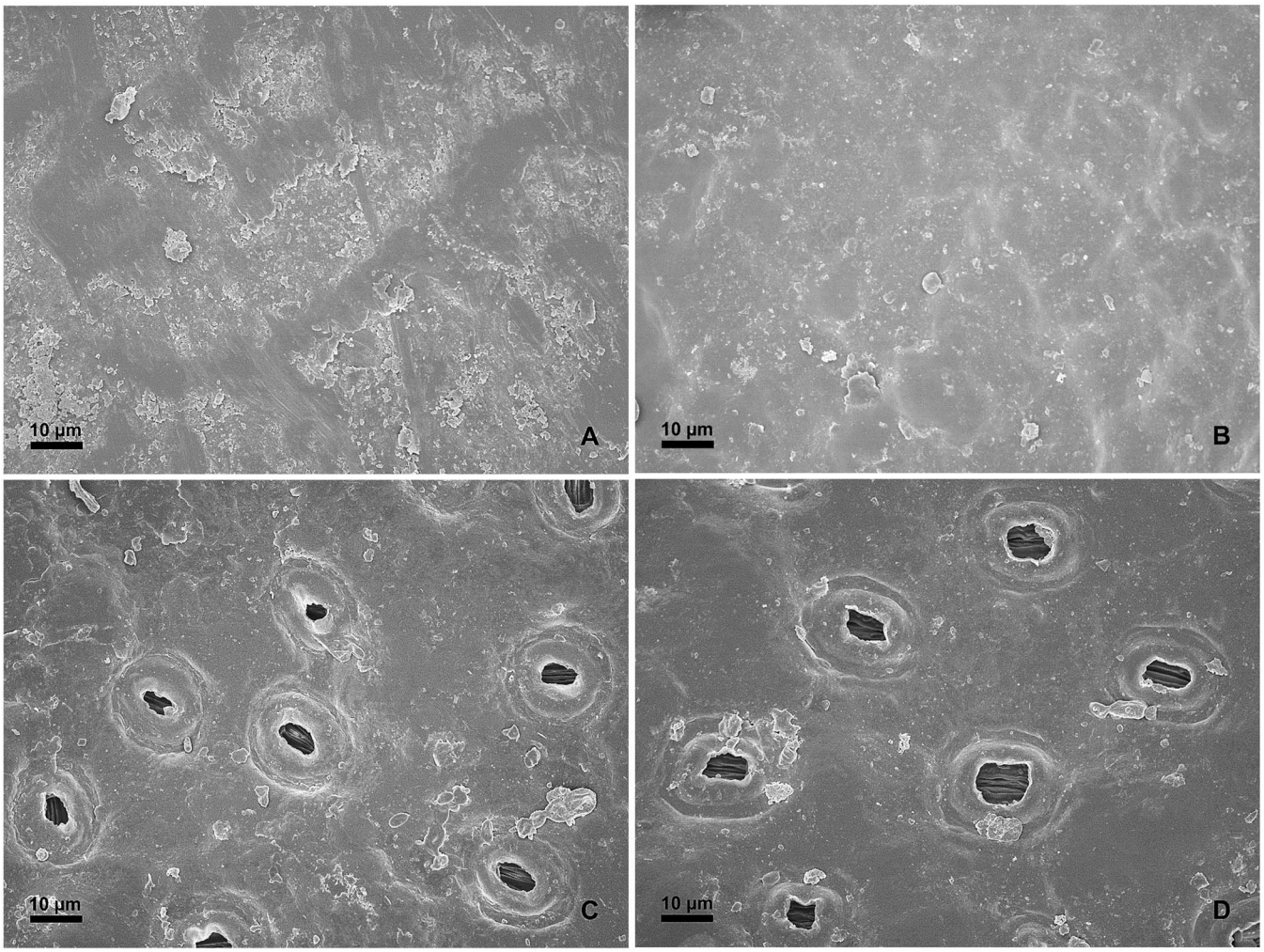

Figure 2. Scanning electron micrographs of the adaxial and abaxial surfaces of *Q. coccifera* leaves from the MED site (A, C) and TEM site (B, D). The stomatal density of MED leaves (445 ± 61 stomata mm^{-2}) was slightly higher than that of TEM leaves (401 ± 51 stomata mm^{-2}), but there was no difference at $P < 0.05$ between sites. Each value represents mean ± SD ($n = 10$).

Chemical analyses of cuticular waxes

Cuticular waxes were extracted by dipping the whole leaf (except the wounds of cut petioles) twice into trichloromethane (≥99.8%, Roth) at room temperature for 1.5 min. *N*-tetracosane (C_{24}; ≥99.5%, Sigma-Aldrich) was added as an internal standard, and the solutions were reduced to dryness under a gentle flow of nitrogen. Dry cuticular wax samples were derivatized with *N*,*O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA, Marchery-Nagel) in dry pyridine (≥99.5%, Roth) at 70 °C for 30 min. Quantification of cuticular wax compounds was performed with a gas chromatograph equipped with a flame ionization detector and an on-column injector (7890A, Agilent Technologies). Separation of compounds was carried out on a fused-silica capillary column (DB1-ms; 30 m length × 0.32 mm inner diameter, 0.1 µm film thickness, Agilent Technologies) with hydrogen as a carrier gas. The temperature program consisted of injection at 50 °C for 2 min, raised by 40 °C min^{-1} to 200 °C, held at 200 °C for 2 min, and then raised by 3 °C min^{-1} to 320 °C and held at 320 °C for 30 min. Qualitative analysis was carried out using a gas chromatograph equipped with a mass spectrometric detector (5975 iMSD, Agilent Technologies) following the same gas chromatographic conditions but using helium as the carrier gas. Cuticular wax compounds were identified comparing a query mass spectrum with reference mass spectra in a library via spectrum matching and quantitated against the internal standard.

The weighted median carbon-chain-lengths (MCLs) for cuticular waxes at both the MED site and the TEM site were calculated. Each compound had its molar coverage calculated from the gas chromatographic data and summed up according to carbon-chain-lengths. For each chain-length (N_i), the mol fraction (W_i) was determined and used as a weight for calculating the MCL. For *n* distinct ordered chain-lengths N_1, N_2, N_3

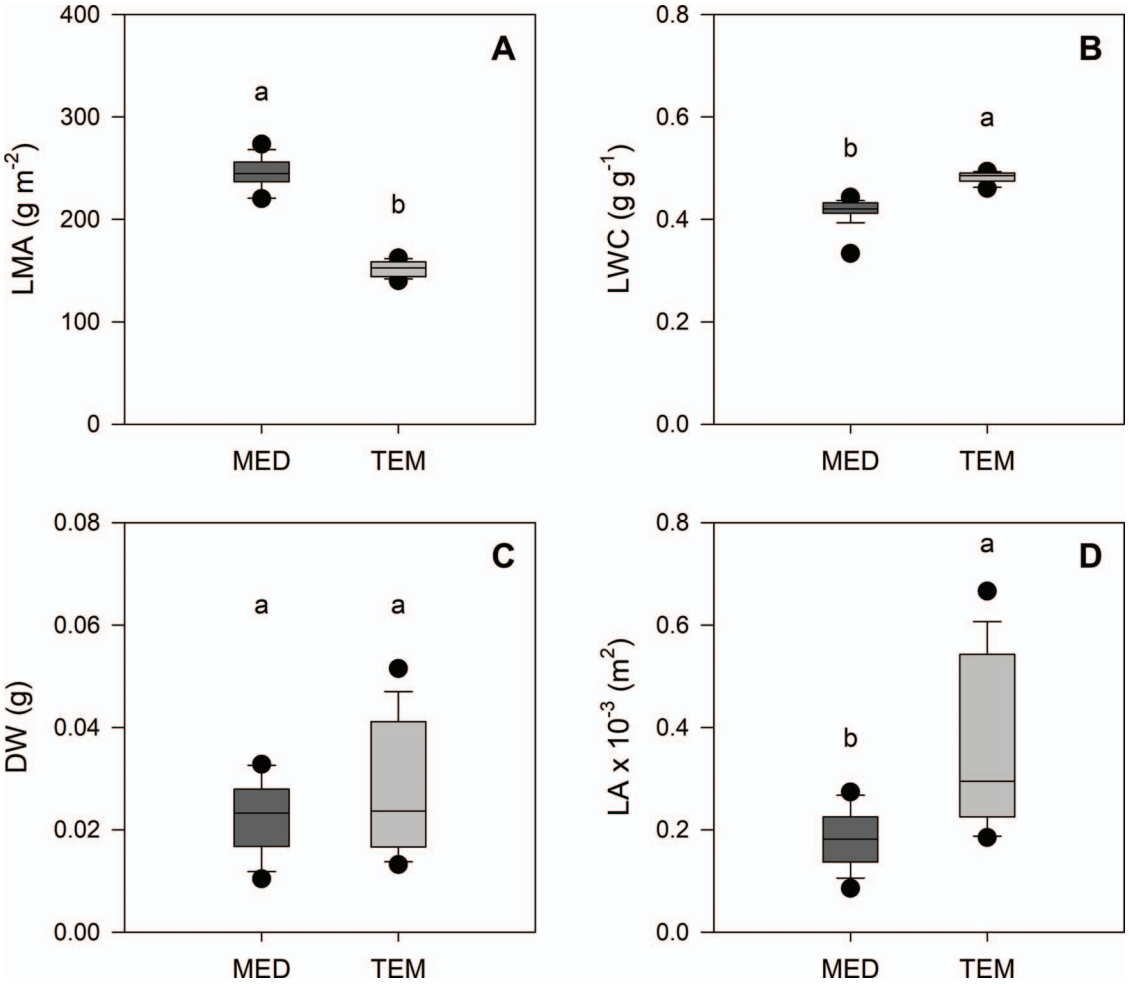

Figure 3. Leaf traits of *Q. coccifera* grown at the MED and TEM sites, respectively ($n \geq 16$). The LMA (A), LWC (B), leaf DW (C) and the dual projected LA (D) were determined for plants grown under the two conditions. Different letters indicate significant differences at $P < 0.05$ between the two sites.

. . . , N_n with weights W_1, W_2, W_3 . . . , W_n, the MCL is the chain-length N_k satisfying:

$$\sum_{i=1}^{k-1} w_i \leq \frac{1}{2} \text{ and } \sum_{i=k+1}^{n} w_i \leq \frac{1}{2}.$$

Chemical analysis of the cutin matrix

For cutin depolymerization, completely delipidated leaves were transesterified with boron trifluoride in methanol (Fluka) at 70 °C overnight. After cooling down, a saturated aqueous solution of sodium chloride (AppliChem), trichloromethane and *n*-dotriacontane (C_{32}; Sigma-Aldrich) as an internal standard were added to the reaction mixtures. From this two-phase system, the deesterified cutin monomers were extracted three times with trichloromethane. The combined organic phases were dried over anhydrous sodium sulfate (AppliChem). All extracts were filtered, and the organic solvent was evaporated under a gentle flow of nitrogen. Derivatization with *N*,*O*-bis-trimethylsilyl-trifluoroacetamide in pyridine was performed at 60 °C for 60 min. Analysis of cutin monomers was performed similarly to the gas chromatographic analysis of cuticular waxes. Separation of cutin mixtures was carried out at 50 kPa for 60 min, 10 kPa min^{-1} to 150 kPa and at 150 kPa for 30 min using a temperature program of 50 °C for 1 min, raised by 10 °C min^{-1} to 150 °C, held at 150 °C for 2 min, and then raised by 3 °C min^{-1} to 320 °C and held at 320 °C for 30 min. Qualitative and quantitative composition of the mixtures was studied using capillary gas chromatography with mass spectrometric and flame ionization detection under the same chromatographic conditions. Single cutin monomers were identified based on the electron ionization mass spectra using authentic standards, the Wiley 10th/NIST 2014 mass spectral library (W10N14, John Wiley & Sons) or by interpretation of the spectra, by the retention times and/or by comparison with literature data and quantitated against the internal standard.

Statistical analyses

Data were tested for normality by Shapiro–Wilk test. Afterwards, comparisons between leaves of MED and TEM sites were investigated using the *t*-test for normally distributed data and the Mann–Whitney U test for those non-normally distributed.

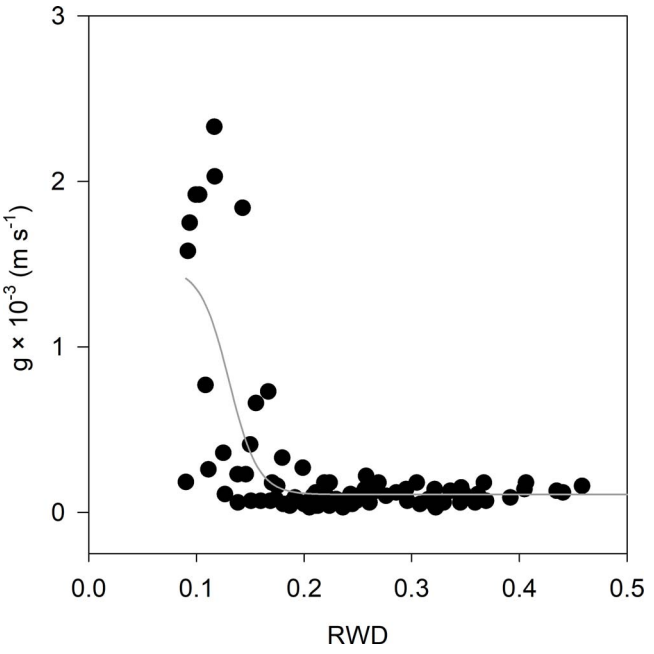

Figure 4. Cuticular water conductance (g) as a function of the RWD of *Q. coccifera* grown at the MED site. Each point represents a single measurement obtained from the leaf drying curves of nine leaves at 25 °C. A sigmoidal four-parameter curve is fitted to guide the eye. The transition between the declining stage and the plateau stage of leaf conductance represents stomatal closure. After maximum stomatal closure, leaf conductance remains constant representing the minimum leaf conductance (g_{min}).

Statistical analyses were performed using the SPSS Statistics software version 23.0 (IBM Corporation).

Results

Leaf surface properties

Leaves of *Q. coccifera* were analysed with scanning electron microscopy to examine the morphology of the leaf surface. Trichomes, stomata and epicuticular wax structures were the principal features. Leaves from the MED and the TEM sites were similar as they presented only a few trichomes on both adaxial and abaxial leaf surfaces and possessed stomata exclusively on the abaxial surface (hypostomatic). The stomata distribution occurs without any distinct pattern across the leaf surface. The adaxial leaf surface showed a continuous smooth cuticular wax layer with the presence of few epicuticular wax granules (Figure 2A, and B). On the abaxial leaf surface, the epicuticular wax granules were more abundant, and the continuous cuticular wax layer projected over the stomata, thus partially covering the stomatal opening (Figure 2C, and D).

Leaf traits

Leaf traits of *Q. coccifera* were calculated (Figure 3). The LMA at the MED site amounted to 245.7 ± 14.4 g m^{-2} (mean ± SD), which was 1.6 times higher ($P < 0.05$) compared with the TEM site (152.0 ± 7.1 g m^{-2}). The LWC was lower ($P < 0.05$) in MED plants (0.42 ± 0.02 g g^{-1}) in comparison with TEM plants (0.48 ± 0.01 g g^{-1}). Leaf DW did not show significant differences at $P < 0.05$ between both sites (0.02 ± 0.01 and 0.03 ± 0.01 g for MED and TEM sites, respectively). Leaf size, accessed as dual projected LA, was the half ($P < 0.05$) at the MED site (0.18 ± 0.05 × 10^{-3} m^2) compared with the TEM site (0.37 ± 0.16 × 10^{-3} m^2).

Minimum leaf conductance

Minimum leaf conductance (g_{min}) at maximal stomatal closure was determined at 25 °C from leaf drying curves. The first stage of drying curves was characterized by high leaf conductance (g) that decreases with leaf dehydration until reaching a plateau of constant leaf conductance values when stomata maximally close (Figure 4). The continuous low leaf conductance corresponds to the g_{min} and results of the maximum stomatal closure. Minimum leaf conductance of *Q. coccifera* was 12.0 ± 3.7 × 10^{-5} m s^{-1} and 12.4 ± 4.1 × 10^{-5} m s^{-1} for MED and TEM sites, respectively (Figure 5). Significant differences between both growing conditions were not found ($P < 0.05$).

Chemical composition of leaf cuticular waxes

The cuticular waxes of *Q. coccifera* were analysed qualitatively and quantitatively using gas chromatography to investigate the potential effect of the atmospheric conditions on the leaf cuticular wax coverage. The amount of cuticular waxes was 2.6 times higher for plants grown at the MED

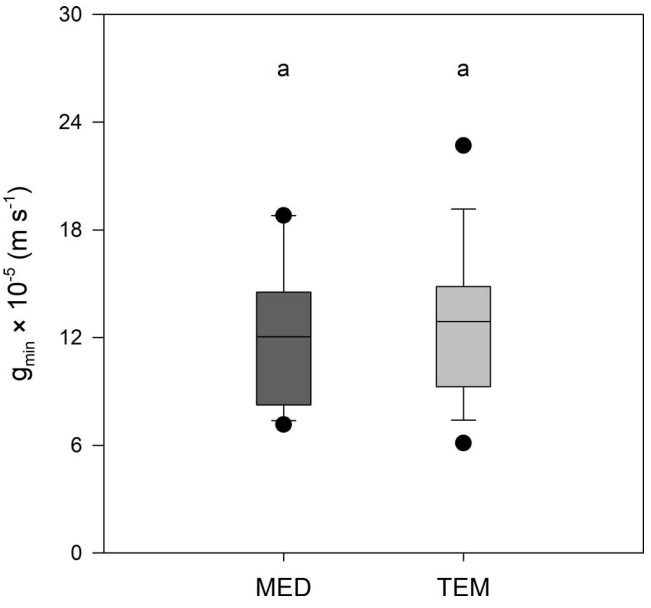

Figure 5. Minimum leaf conductance (g_{min}) of *Q. coccifera* grown at the MED and TEM sites, obtained from drying curves at 25 °C ($n \geq 16$). The g_{min} did not differ between plants from the MED and TEM sites (t (31) = −0.33; $P = 0.74$).

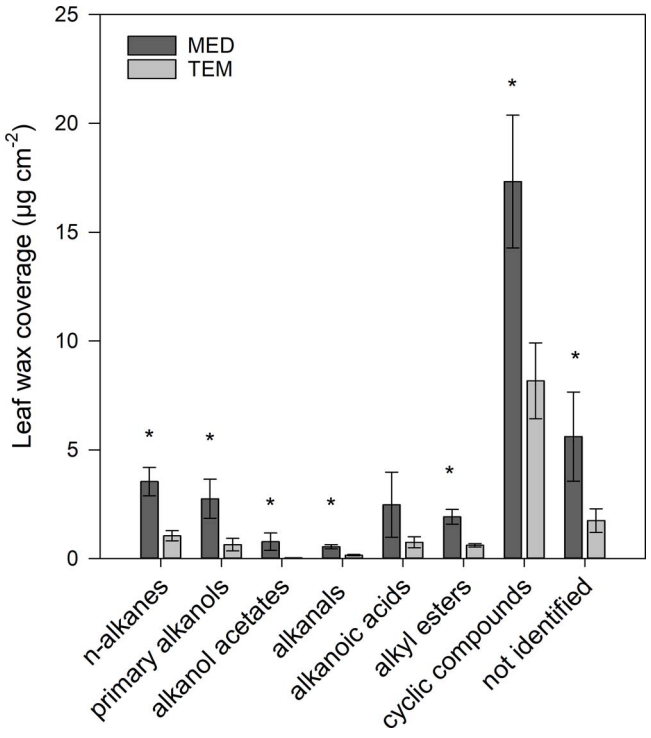

Figure 6. Cuticular wax coverage of *Q. coccifera* leaves grown at the MED and TEM sites, arranged by compound class. Each value represents the mean value ± SD ($n = 4$). Asterisks indicate significant difference at $P < 0.05$ between the MED and the TEM sites.

site (34.9 ± 6.6 µg cm^{-2}) compared with the TEM site (13.0 ± 2.8 µg cm^{-2}). Except for the similar amounts of alkanoic acids, all the compound classes increased at the MED site. However, the qualitative composition of the cuticular waxes was not different (Figure 6). Pentacyclic triterpenoids were the most abundant class of cuticular wax constituents of both MED (17.1 ± 3.0 µg cm^{-2}, 49% of the total cuticular waxes) and TEM (7.9 ± 1.7 µg cm^{-2}, 61%) leaves. The major cuticular wax constituents were germanicol (10.1 ± 2.3 µg cm^{-2}, 29% at MED site; 3.4 ± 0.6 µg cm^{-2}, 26% at TEM site) and lupeol (2.9 ± 1.4 µg cm^{-2}, 8% at MED site; 1.6 ± 0.4 µg cm^{-2}, 12% at TEM site). Very-long-chain aliphatic compounds with carbon-chain-lengths ranging from C_{20} to C_{51} amounted to 12.0 ± 2.1 µg cm^{-2} (34%) at the MED site and 3.5 ± 0.6 µg cm^{-2} (25%) at the TEM site (Figure 7). Within the aliphatic cuticular wax fraction, *n*-alkanes were the main compound class (3.5 ± 0.7 µg cm^{-2}, 10% at MED site; 1.1 ± 0.2 µg cm^{-2}, 8% at TEM site). The *n*-alkane fraction comprised a homologous series from C_{25} to C_{32} *n*-alkanes with odd-numbered *n*-alkanes dominating above even-numbered *n*-alkanes, and *n*-nonacosane (C_{29}) was the major constituent (1.6 ± 0.7 µg cm^{-2}, 5% at MED site; 0.4 ± 0.1 µg cm^{-2}, 3% at TEM site). Primary alkanols, alkanol acetates, alkanals, alkanoic acids and alkyl esters were also identified in the *Q. coccifera* cuticular waxes of the two sites (Table 1).

Chemical composition of the leaf cutin matrix

The cutin monomeric composition was analysed using gas chromatography with flame ionization and mass spectrometry detection after depolymerization of the cutin polyester. The

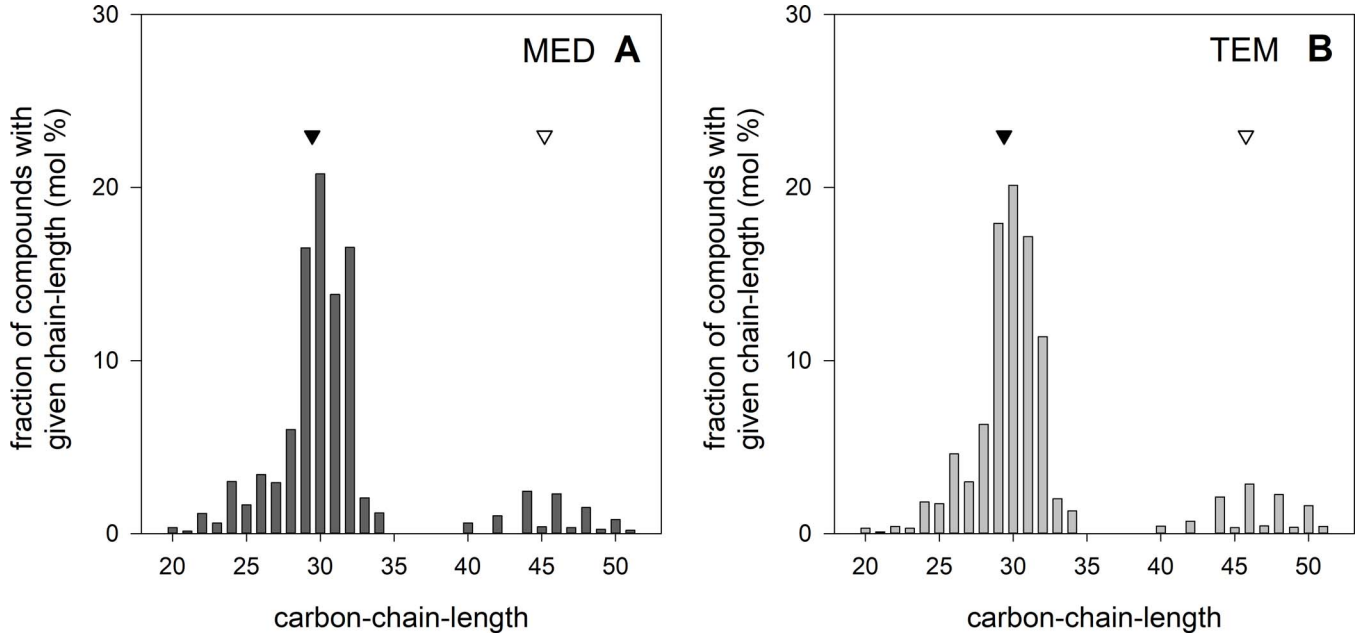

Figure 7. Chain-length distributions of the very-long-chain aliphatic fraction of the cuticular waxes of *Q. coccifera* leaves grown at the MED (A) and TEM (B) sites ($n = 4$). Bars stand for the mole-based contribution of a single chain-length to the total very-long-chain aliphatic wax coverage. Triangles denote the weighted MCL of the very-long-chain aliphatic compounds with chain-lengths <40 (closed symbol) and ≥40 (open symbol) carbon atoms. The 50% weighted percentile of the chain lengths corresponds to the MCL.

Table 1. Chemical composition of leaf cuticular waxes of *Q. coccifera* grown at the MED and TEM sites. Each value represents the mean value ± SD ($n = 4$)

Compound class	Carbon-chain-length	Coverage (μg cm^{-2})	
		MED	TEM
n-alkanes	25	0.07 ± 0.01	0.02 ± 0.01
	26	0.06 ± 0.01	0.01 ± 0.00
	27	0.20 ± 0.05	0.05 ± 0.02
	28	0.20 ± 0.02	0.03 ± 0.02
	29	1.61 ± 0.75	0.43 ± 0.13
	30	0.21 ± 0.02	0.11 ± 0.05
	31	0.98 ± 0.22	0.37 ± 0.04
	32	0.21 ± 0.06	0.04 ± 0.02
Total *n*-alkanes		3.54 ± 0.65	1.06 ± 0.24
Primary alkanols	22	0.01 ± 0.00	0.00 ± 0.00
	23	0.02 ± 0.00	0.00 ± 0.00
	24	0.14 ± 0.06	0.02 ± 0.01
	25	0.03 ± 0.01	0.01 ± 0.00
	26	0.10 ± 0.01	0.08 ± 0.02
	27	0.04 ± 0.02	0.01 ± 0.01
	28	0.07 ± 0.01	0.02 ± 0.02
	30	0.54 ± 0.27	0.10 ± 0.04
	31	0.38 ± 0.08	0.09 ± 0.07
	32	1.01 ± 0.56	0.19 ± 0.12
	33	0.22 ± 0.01	0.05 ± 0.03
	34	0.20 ± 0.02	0.06 ± 0.04
Total primary alkanols		*2.75* ± 0.90	0.65 ± 0.29
Alkanol acetates	26	0.02 ± 0.01	-
	27	0.03 ± 0.02	-
	28	0.13 ± 0.11	-
	29	0.06 ± 0.03	0.01 ± 0.01
	30	0.43 ± 0.25	0.03 ± 0.02
	31	0.11 ± 0.04	-
Total alkanol acetates		0.78 ± 0.40	0.04 ± 0.01
Alkanals	28	0.03 ± 0.02	0.02 ± 0.01
	30	0.20 ± 0.08	0.09 ± 0.02
	32	0.31 ± 0.02	0.07 ± 0.04
Total alkanals		0.55 ± 0.09	0.17 ± 0.03
Alkanoic acids	20	0.02 ± 0.01	0.01 ± 0.00
	21	0.01 ± 0.00	0.00 ± 0.00
	22	0.08 ± 0.07	0.00 ± 0.00
	23	0.03 ± 0.01	0.00 ± 0.00
	24	0.12 ± 0.10	0.02 ± 0.01
	25	0.04 ± 0.03	0.01 ± 0.00
	26	0.11 ± 0.07	0.03 ± 0.02
	27	0.04 ± 0.02	0.02 ± 0.01
	28	0.17 ± 0.12	0.10 ± 0.03
	29	0.18 ± 0.08	0.10 ± 0.07
	30	0.85 ± 0.55	0.25 ± 0.14
	31	0.22 ± 0.06	0.10 ± 0.01
	32	0.52 ± 0.47	0.08 ± 0.06
	33	0.08 ± 0.03	0.03 ± 0.01

(Continued)

Table 1. Continued.

Compound class	Carbon-chain-length	Coverage (μg cm^{-2})	
		MED	TEM
Total alkanoic acids		2.48 ± 1.50	0.75 ± 0.25
Alkyl esters	40	0.11 ± 0.03	0.02 ± 0.01
	42	0.19 ± 0.05	0.04 ± 0.01
	44	0.47 ± 0.12	0.11 ± 0.03
	45	0.07 ± 0.01	0.02 ± 0.00
	46	0.45 ± 0.08	0.15 ± 0.03
	47	0.07 ± 0.02	0.02 ± 0.00
	48	0.31 ± 0.04	0.12 ± 0.03
	49	0.05 ± 0.01	0.02 ± 0.01
	50	0.17 ± 0.01	0.09 ± 0.02
	51	0.04 ± 0.01	0.02 ± 0.00
Total alkyl esters		1.93 ± 0.34	0.62 ± 0.08
Total very-long-chain aliphatic compounds		12.04 ± 2.09	3.46 ± 0.62
α-amyrin		0.24 ± 0.09	0.17 ± 0.05
β-amyrin		0.98 ± 0.10	0.29 ± 0.07
Betulin		0.34 ± 0.05	0.20 ± 0.07
Betulinic acid		0.59 ± 0.15	0.26 ± 0.20
Erythrodiol		-	0.11 ± 0.02
Fridelin		0.23 ± 0.08	0.05 ± 0.04
Fridelinol		0.32 ± 0.17	0.11 ± 0.05
Germanicol		10.11 ± 2.28	3.45 ± 0.55
Germanicone		0.13 ± 0.01	0.03 ± 0.01
Lupeol		2.88 ± 1.39	1.60 ± 0.36
Oleanoic acid		0.23 ± 0.04	0.17 ± 0.10
Ursolic acid		0.21 ± 0.08	0.08 ± 0.05
Uvaol		0.28 ± 0.04	0.21 ± 0.07
Unknown triterpenoid 1		0.06 ± 0.02	-
Unknown triterpenoid 2		0.27 ± 0.07	-
β-sisterol		0.24 ± 0.06	1.13 ± 0.81
Total cyclic compounds		17.10 ± 3.03	7.86 ± 1.67
β-tocopherol		0.07 ± 0.03	0.11 ± 0.08
δ-tocopherol		0.04 ± 0.01	0.03 ± 0.02
Total phenolic compounds		0.12 ± 0.04	0.14 ± 0.09
Not identified		5.61 ± 2.05	1.75 ± 0.55
Total cuticular waxes		34.87 ± 6.64	13.03 ± 2.77

amount of the cutin monomers of *Q. coccifera* leaves was 255.2 ± 22.9 μg cm^{-2} at the MED site and 178.8 ± 4.4 μg cm^{-2} at the TEM site (Table 2). The leaf cutin matrix was composed of 88% aliphatic and 14% phenolic cutin monomers for the MED site and 91% aliphatic and 9% phenolic cutin monomers for the TEM site, respectively. For both sites, 9,10-epoxy 18-hydroxy alkanoic acid averaging out at 67.3 μg cm^{-2} was the predominant cutin monomer (27% of total cutin monomers for MED and 37% for TEM). Additionally, 9/10,16-dihydroxy hexadecanoic acid (63.3 μg cm^{-2}), 18-hydroxy octadec-9-enoic acid (27.0 μg cm^{-2}), 9,10,18-trihydroxy octadecanoic acid (23.1 μg cm^{-2}) and 4-hydroxy cinnamic acid (*para*-coumaric acid; 16.3 μg cm^{-2}) were detected in high quantities in the cutin matrix of MED plants. In particular, distinctly lower amounts of 9/10,16-dihydroxy hexadecanoic acid (1.9 times), 9,10,18-trihydroxy octadecanoic acid (5.3 times) and 4-hydroxy cinnamic acid (2.5 times) in TEM plants accounted for the 1.4 times difference in the total cutin monomeric quantity when comparing both MED and TEM sites (Figure 8). Due to the different monomeric composition, the degree of epoxylated and unsaturated alkanoic acids was lower for the leaf cutin of the MED site (0.27 and 0.13) compared with the TEM site

Table 2. Chemical composition of the leaf cutin matrix of *Q. coccifera* grown at the MED and TEM sites. Each value represents the mean value ± SD ($n = 4$)

Compound class	Carbon-chain-length	Cutin coverage (μg cm^{-2})	
		MED	TEM
Alkanoic acid	16	8.62 ± 0.44	5.16 ± 0.22
Alka-9,12-dienoic acid	18:2	2.50 ± 0.55	1.02 ± 0.15
Alk-9-enoic acid	18:1	1.42 ± 0.38	0.90 ± 0.30
Alkanoic acid	18	3.37 ± 1.21	1.16 ± 0.22
Alkanoic acid	20	1.52 ± 0.15	0.32 ± 0.10
Alkanoic acid	22	0.64 ± 0.09	0.55 ± 0.13
Alkanoic acid	24	2.70 ± 0.46	1.83 ± 0.63
Alkanoic acid	30	0.43 ± 0.08	0.10 ± 0.06
Alkane-1,16-dioic acid	16	0.52 ± 0.07	0.26 ± 0.04
Alkane-1,18-dioic acid	18	1.00 ± 0.06	0.39 ± 0.17
Primary alkanol	16	0.01 ± 0.02	0.01 ± 0.02
Primary alkanol	18	0.94 ± 0.39	0.32 ± 0.10
Primary alkanol	20	0.83 ± 0.33	0.29 ± 0.09
9/10-Hydroxy alkane-1,16-dioic acid	16	0.21 ± 0.09	0.69 ± 0.19
16-Hydroxy alk-9-enoic acid	16:1	2.34 ± 0.50	6.24 ± 2.25
16-Hydroxy alkanoic acid	16	2.61 ± 0.20	2.65 ± 0.23
18-Hydroxy alk-9-enoic acid	18:1	27.02 ± 1.42	31.48 ± 2.08
9/10,16-Dihydroxy alkanoic acid	16	63.25 ± 5.93	33.72 ± 1.86
9/10,18-Dihydroxy alkanoic acid	18	2.82 ± 0.13	2.92 ± 0.18
9,10-Epoxy 18-hydroxy alkanoic acid	18	68.98 ± 9.15	65.66 ± 4.26
9,10,18-Trihydroxy alkanoic acid	18	23.11 ± 3.10	4.38 ± 0.77
2-Hydroxy alkanoic acid	16	0.58 ± 0.16	0.44 ± 0.05
2-Hydroxy alkanoic acid	20	0.03 ± 0.04	0.02 ± 0.05
2-Hydroxy alkanoic acid	22	1.26 ± 0.18	1.40 ± 0.36
2-Hydroxy alkanoic acid	23	0.77 ± 0.38	0.52 ± 0.10
2-Hydroxy alkanoic acid	24	0.88 ± 0.12	0.28 ± 0.11
2-Hydroxy alkanoic acid	26	0.74 ± 0.21	0.26 ± 0.08
3,4-Dihydroxy benzoic acid		2.07 ± 0.22	1.20 ± 0.26
3-Methoxy 4-hydoxy benzoic acid		2.26 ± 0.44	1.59 ± 0.54
4-Hydroxy benzoic acid		0.57 ± 0.09	0.37 ± 0.08
3,4-Dihydroxy cinnamic acid		0.83 ± 0.10	0.18 ± 0.03
4-Hydroxy cinnamic acid		16.26 ± 1.15	6.63 ± 1.33
4-Hydroxy cinnamic acid derivatives		14.10 ± 1.71	5.83 ± 0.33
Total cutin monomers		255.22 ± 22.93	178.77 ± 4.36

(0.37 and 0.22). Furthermore, the ratio of predominate C_{16} and C_{18} aliphatic cutin acids differed between 1:1.7 for the MED site and 1:2.2 for the TEM site.

Discussion

Elevated temperatures often co-occur with high VPD and intense solar radiation during the summer season in Mediterranean ecosystems. Leaf size plays a vital role in water and leaf energy balance, especially under dry and hot atmospheric conditions. The LMA reflects the intrinsic relation between carbon gain and longevity (Díaz et al. 2016), while LWC roughly indicates leaf density (Garnier and Laurent 1994) and may prolong the leaf survival time after stomatal closure. *Quercus coccifera* responds to MED conditions by reducing leaf size (2.0 times in comparison with leaves grown at the TEM site) and increasing LMA (1.6 times). The small leaves with high LMA might be associated with the exposure to the increased light and VPD at the MED site.

In summer, it is common in MED environments that the temperature rises while the water vapour density stays constant, resulting in considerably reduced atmospheric humidity. This combination leads to a rise in the driving force for water loss by transpiration. Although *Q. coccifera* has its main distribution area in the MED zone, this plant species also occurs at the Iberian Atlantic coast under constantly humid conditions. It is

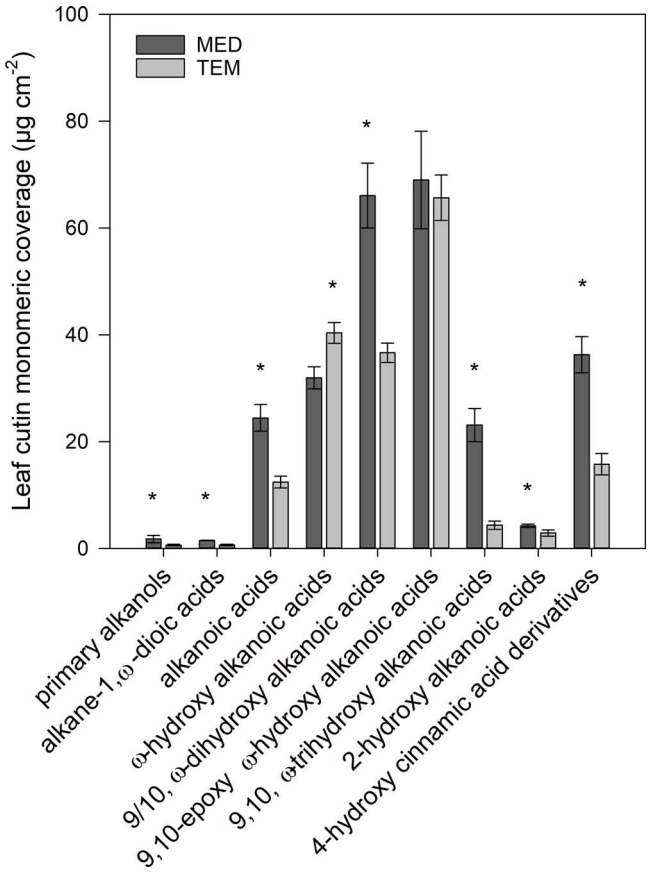

Figure 8. Cutin monomer coverage of *Q. coccifera* leaves grown at the MED and TEM sites. Each value represents the mean ± SD ($n = 4$). Asterisks indicate significant difference at $P < 0.05$ between the MED and the TEM sites.

generally assumed that plants adapted to a high driving force for water loss have a very robust and efficient cuticle. Therefore, one may intuitively expect the minimum leaf conductance (g_{min}) of *Q. coccifera* to be lower when grown at the MED site due to phenotypic modifications to avoid cuticular water loss. However, our findings do not support this hypothesis since g_{min} of *Q. coccifera* remained unaffected by the contrasting atmospheric conditions in the growth sites.

In line with our findings, the water permeability of the astomatous isolated leaf cuticle of the evergreen tree *Citrus aurantium* L. grown at a temperature ranging from 15 °C to 35 °C and relative humidity of 50% or 90% remained unaffected by the different conditions (Geyer and Schönherr 1990). Schuster et al. (2016) reported that the leaf cuticular permeability of the evergreen desert shrub *Rhazya stricta* Decne. is comparable to those of woody plant species from various habitats, including humid ones. Similarly, the leaf cuticular permeability of the summer-green desert vine *Citrullus colocynthis* (L.) Schrad. is close to that of non-evergreen forbs from TEM climates (Bueno et al. 2019). These authors also showed that the leaf cuticular permeability of the evergreen desert tree *Phoenix dactylifera* L. is still equivalent to the permeability reported for evergreen woody plants from other climates. Therefore, the common assumption that the plant cuticle either genetically or phenotypically adapted to high transpirational demand should have a more efficient barrier to avoid water loss is not supported by experimental evidence.

Some studies have suggested that cuticular water permeability is mainly determined by genetic control and not, or only slightly, subject to environmental influence. Gil-Pelegrín et al. (2017) investigated 11 *Quercus* species growing in a common garden and found out that g_{min} of typical evergreen MED *Quercus* species was slightly lower compared with deciduous TEM ones. However, g_{min} of deciduous MED and TEM *Quercus* species did not differ. Moreover, the leaf cuticular permeability from 160 plant species extracted from the literature was summarized and revealed that only in two particular cases, epiphytes and climbers/lianas, the cuticular permeability was exceptionally low (Schuster et al. 2017). Therefore, cuticular water permeability might be related to the plant life strategy to deal with environmental constraints, as suggested by Bueno et al. (2019).

The plant cuticle acts as a protective barrier against a wide range of biotic and abiotic stresses and might respond to environmental changes. Our findings showed that cuticular wax and cutin coverages increased for MED leaves, corroborating our hypothesis that *Q. coccifera* shrubs grown under MED conditions accumulate more cuticular lipids than those in TEM conditions. However, the assumption that the qualitative chemical composition would also be affected by the atmospheric conditions was rejected. Although the quantitative variations were detected for leaf cutin monomers between the MED and TEM sites, the cutin composition was similar for leaves of both habitats, and the main component was in both cases the 9,10-epoxy 18-hydroxy alkanoic acid. Previous studies have shown that the mechanical strength of the cutin matrix, especially under conditions of high temperature or protracted exposure to sun, plays a pivotal role in maintaining the barrier function of the plant cuticle in particular and the physiological plant integrity in general (Heredia 2003, Bargel et al. 2006, Khanal and Knoche 2017).

Similarly, MED leaves had almost three times more cuticular waxes than TEM leaves, but the relative composition of the cuticular waxes was not different between both sites. All the identified wax compound classes (pentacyclic triterpenoids, *n*-alkanes, primary alkanols, alkanol acetates, alkanals, alkanoic acids and alkyl esters) were found in both MED and TEM leaves. Pentacyclic triterpenoids correspond to about half of the total cuticular waxes of *Q. coccifera* leaves regardless of the habitat. The potential triterpenoid functions are protecting plants against herbivory and stabilizing the cuticle of heat-stressed plants (Reichardt et al. 1984, Oliveira and Salatino 2000, Schuster et al. 2016). However, the contribution of pentacyclic triterpenoids to avoid uncontrolled water loss has

been considered small or absent (Leide et al. 2007, 2011 Buschhaus and Jetter 2012, Jetter and Riederer 2016, Schuster et al. 2016). Therefore, the efficacy of the cuticular transpiration barrier has been attributed to the very-long-chain aliphatic compounds (Jetter and Riederer 2016). These findings are in line with the molecular structure model of cuticular waxes proposed by Riederer and Schreiber (1995). According to these authors, the cuticular waxes are multiphase systems made up of mobile amorphous zones within highly structured crystalline domains. This model predicts that the very-long-chain aliphatic compounds build up impermeable crystalline domains, and the amorphous zones incorporate the chain ends and pentacyclic molecules. Hence, the model assumes that the very-long-chain aliphatic compounds constitute the cuticular transpiration barrier in plants. In our study, these compounds increased by 3.5 times in leaves of *Q. coccifera* grown at the MED site, but the efficacy of the cuticular transpiration barrier remained unaltered. In line with our findings, studies on several plant species have shown that cuticular water permeability does not correlate with the amount of cuticular waxes or cuticle thickness (Schreiber and Riederer 1996, Riederer and Schreiber 2001, Schuster 2016, Bueno, 2018). Several plant species under water stress had increased the production of cuticular waxes, including the herbaceous model plant *Arabidopsis thaliana* (L.) Heynh. (Cameron et al. 2006, Kim et al. 2007, Kosma et al. 2009, Le Provost et al. 2013). Indeed, water limitation caused by either soil drought or low atmospheric humidity affects the cuticular wax deposition. However, in this specific case, an effect of the soil water status can be excluded because the plants were watered as over the 8 years' cultivation.

Here arises the question: what are the environmental drivers of the increased leaf cuticular wax coverage of *Q. coccifera* at the MED site? The principal environmental differences between the two growth sites during the experimental year were summer temperature, solar radiation and VPD. Although the mean annual temperature differs only by 1 °C between both sites, the mean and maximum monthly temperatures during the summer at the MED site were up to 4 °C and 10 °C higher than at the TEM site, respectively. *Quercus coccifera* grown at the MED site also experienced higher solar radiation in comparison with TEM site during the whole year. Finally, yet importantly, the mean VPD during the summer months was higher at the MED site than at the TEM site. This scenario is even more evident when comparing the maximum monthly VPD, which was up to 3.5 times higher at the MED site.

Plants under stress conditions often exhibit changes in the amount and composition of cuticular waxes (Shepherd and Griffiths 2006). Studies about the effect of temperature on the cuticular wax composition are scarce, and the few studies available are contradictory (Shepherd and Griffiths 2006). Some studies have shown that a lower temperature stimulated high cuticular wax production in leaves of *Brassica* species (Whitecross 1963, Whitecross and Armstrong 1972, Baker 1974). In opposition to this, Riederer and Schneider (1990) reported that increasing the day temperature from 25 °C to 30 °C leads to an increase of about two times in cuticular waxes of *C. aurantium* leaves. Further, Reed and Tukey (1982) found that a higher amount of leaf cuticular waxes was produced at either lower for example in herbaceous *Brassica oleracea* L. or higher temperature for example in evergreen MED herb *Dianthus caryophyllus* L. In contrast to temperature, it has been widely accepted that high solar radiation leads to an increase in cuticular wax coverage (Baker 1974, Giese 1975, Reed and Tukey 1982, Shepherd et al. 1995). Another important environmental factor is atmospheric humidity. High relative humidity tends to reduce the evaporative demand by decreasing the VPD. Koch et al. (2006) showed that the cuticular wax accumulation of *B. oleracea* leaves strongly declines in response to a low VPD. Lihavainen et al. (2017) proposed that plants under low VPD transpire less, improving their water status and, thereby, reducing the demand for cuticular waxes. Therefore, one may assume that the main drivers of the increased cuticular wax coverage of *Q. coccifera* are the intense solar radiation, the high VPD and, potentially, the high temperatures at the MED site, especially in the summer. The high accumulation of cuticular lipids may confer higher resistance to the intense light exposure and high summer temperatures at the MED site. However, further studies on wild plants, especially in their natural ecosystems, are needed to trace potential clues of cuticular adaptation to cope with inherent environmental constraints.

In conclusion, we showed that the cuticle of *Q. coccifera* leaves plastically responds to the harsh MED conditions, which leads to high cuticular wax and cutin loads. However, the cuticular lipids at both MED and TEM sites are qualitatively very similar; i.e. the relative contribution of each component class and the carbon-chain-length of homologous compounds. Although, it is often stated that a thicker cuticle is a barrier with a higher efficiency against passive water loss than thinner ones with a lower amount of cuticular waxes (Purves et al. 2004, Lüttge 2007, Poorter and Garnier 2007, De Micco and Aronne 2012, Smith et al. 2012, Jones 2013), our findings suggest that the accumulation of functional equivalent cuticular waxes might compensate for the quantitative plasticity of the cuticular deposition of *Q. coccifera.* thereby conferring equal cuticular transpiration properties. Further, we stress that high cuticular wax loads do not increase the efficacy of the cuticular transpiration barrier and, therefore, might not extend the safety margin between stomatal closure and xylem hydraulic failure.

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