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

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12 **Abstract**

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

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

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37 **Introduction**

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

47 The primary function of the plant cuticle is avoiding uncontrolled water loss (Riederer and Schreiber
48 2001). The plant cuticle consists of a cutin matrix impregnated and coated with cuticular waxes. The
49 biopolymer cutin is mainly composed of C_{16} to C_{18} hydroxy alkanoic acids and their derivatives, which
50 are esterified within a complex network (Pollard et al. 2008). The cutin polyester is non-extractable but
51 hydrolysable, whereas cuticular waxes are solvent-extractable, complex mixtures typically comprising
52 homologous series of very-long-chain aliphatic and, additionally, in some plant species pentacyclic
53 compounds (Jetter et al. 2006). The cutin matrix is involved in waterproofing, but it mainly contributes
54 to the mechanical integrity of the plant cuticle (Khanal and Knoche 2017). So far, the functional barrier
55 against water diffusion through the cuticle has been attributed to the very-long-chain aliphatic waxes
56 (Riederer and Schreiber 1995; Jetter and Riederer 2016), whereas the pentacyclic components have
57 been associated with protection against herbivory and with stabilisation of the heat-stressed cuticle
58 (Reichardt et al. 1984; Oliveira and Salatino 2000; Schuster et al. 2016).

59 Besides avoiding dehydration, plants also depend on acquiring carbon dioxide for photosynthesis. The
60 balance between stomatal transpiration and carbon dioxide uptake is essential for the life of terrestrial
61 plants. While the stomata are open to carbon dioxide uptake, plants inevitably lose water to the
62 surrounding atmosphere. Under unfavourable conditions, plants close their stomata and, therefore, the
63 remaining water loss only occurs through the cuticle. It has been proposed that excessive cuticular
64 water loss and high leaf-to-atmosphere vapour pressure may lead to sudden xylem cavitation during

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65 heatwaves (Cochard 2019). Thus, the efficient control of cuticular water loss is of fundamental
66 importance for maintaining xylem hydraulic safety and, thereby, ensuring plant fitness and survival.

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

88 This study aims to investigate the effect of the atmospheric conditions on the leaf cuticular lipids and
89 the efficacy of the cuticular transpiration barrier of *Q. coccifera*. We hypothesise that in plants living
90 under MED conditions compared with those under TEM ones: (i) the accumulation of cuticular lipids
91 increases and their qualitative composition widely differ and (ii) the cuticle is more efficient to avoid
92 water loss. From these hypotheses, we predicted that MED plants (i) possess higher amounts of cutin
93 monomers and cuticular waxes and (ii) have a lower cuticular permeability than TEM plants due to the
94 higher accumulation of very-long-chain aliphatic compounds. We tested these hypotheses (i) by

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95 qualitatively and quantitatively determining the chemical composition of the leaf cuticle and (ii) by
96 measuring the minimum leaf conductance (g_{min}) at 25°C for plants of *Q. coccifera* grown either at the
97 MED or TEM sites.

98 **Material and Methods**

99 *Plant material and grown conditions*

100 Seeds of *Quercus coccifera* L. (Fagaceae) were harvested from a natural population growing near
101 Zaragoza, Spain. The seeds were germinated in a mixture of 80% compost (Neuhaus Humin
102 Substrate N6; Klasman-Deilmann GmbH) and 20% perlite under greenhouse conditions. After the first
103 vegetative period, plants were cultivated outside at CITA de Aragón (Zaragoza, Spain) under
104 Mediterranean atmospheric conditions (MED; Figure 1). Finally, two-year-old plants were randomly
105 selected and transplanted into the Jardín Botánico de Iturrarán (Gipuzkoa, Spain), which features
106 temperate atmospheric conditions (TEM). Plants in both MED and TEM sites were watered as needed
107 and fertilised with Osmocote Plus (Sierra Chemical). Measurements were conducted using one-year-
108 old fully developed leaves of eight-year-old plants.

109 *Scanning electron microscopy*

110 Small air-dried leaf sections were mounted on aluminium holders using double-sided adhesive tape
111 (Plannet Plano) and sputter-coated with approximately 15 nm gold:palladium (150 s, 25 mA, partial
112 argon pressure 0.05 mbar, SCD005 sputter coater, Bal-Tec). Afterwards, the samples were examined
113 with a field-emission scanning electron microscope (JEOL JSM-7500F) using a 5-kV acceleration
114 voltage and a 10 mm working distance. Micrographs were taken from both adaxial and abaxial leaf
115 surfaces. The processed micrographs were used for determining the stomatal density.

116 *Leaf traits*

117 Overnight, leaves were full hydrated in a humid chamber before the measurements. The water-
118 saturated fresh weight (FW) of leaves was determined using an analytical balance (MC-1 AC210S,
119 Sartorius; precision 0.1 mg) and the dry weight (DW) was obtained after oven drying the leaves at
120 90°C for 24 h. The actual fresh weights (FW_{actual}) during leaf drying experiments were used to
121 calculate the relative water deficit (RWD) according to:

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122 $$RWD = 1 - \frac{FWactual - DW}{FW - DW}.$$

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

127 *Minimum leaf conductance*

128 Minimum leaf conductance (g_{min}) was determined gravimetrically from the consecutive weight loss of
129 desiccating leaves in darkness and at low atmospheric humidity. It corresponds to the lowest
130 conductance a leaf can reach when stomata are maximally closed as a consequence of desiccation.
131 Cut petioles of water-saturated leaves were sealed with high melting paraffin wax (Fluka).
132 Subsequently, the sealed leaves were placed in an incubator at 25°C (IPP 110, Memmert). The air
133 temperature and humidity were monitored using a digital thermo-hygrometer (Testoterm 6010, Testo).
134 Silica gel (Applichem) was used to control the moisture in the incubator. The weight of desiccating
135 leaves was determined as a function of desiccation time using an analytical balance (MC-1 AC210S,
136 Sartorius; precision 0.1 mg). The transpiration rate (J) was calculated from the change in fresh weight
137 (ΔFW) with time (t) divided by the dual projected leaf area (A):

138 $$J = \frac{\Delta FW}{\Delta t \text{ x } A}.$$

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

145 $$g = \frac{J}{\alpha_{apo} \times C_{wv\ sat\ leaf} - \alpha_{air} \times C_{wv\ sat\ air}}.$$

146 The water activity of the atmosphere (α_{air}) over silica gel is nearly zero. The water activity in the
147 apoplast adjacent to the inner side of the cuticle (α_{apo}) is assumed to be close to one. Thus, the active
148 driving force for cuticular transpiration in the setup used here is the saturation concentration of water

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149 vapour at actual leaf temperature ($C_{wv\ sat\ leaf}$). Leaf temperature was measured using an infrared laser
150 thermometer (Harbor Freight Tools, one point measurements), and the corresponding water vapour
151 saturation concentrations at leaf temperature were derived from tabulated values (Nobel 2009). The
152 cuticular water conductance at a given dehydration point was plotted versus the respective relative
153 water deficit (RWD).

154 *Chemical analyses of cuticular waxes*

155 Cuticular waxes were extracted by dipping the whole leaf (except the wounds of cut petioles) twice into
156 trichloromethane (≥ 99.8%, Roth) at room temperature for 1.5 min. *N*-tetracosane (C_{24}; ≥ 99.5%,
157 Sigma-Aldrich) was added as an internal standard and the solutions were reduced to dryness under a
158 gentle flow of nitrogen. Dry cuticular wax samples were derivatised with *N*,*O*-
159 bis(trimethylsilyl)trifluoroacetamide (BSTFA, Marchery-Nagel) in dry pyridine (≥ 99.5%, Roth) at 70°C
160 for 30 min. Quantification of cuticular wax compounds was performed with a gas chromatograph
161 equipped with a flame ionisation detector and an on-column injector (7890A, Agilent Technologies).
162 Separation of compounds was carried out on a fused-silica capillary column (DB1-ms, 30 m length ×
163 0.32 mm inner diameter, 0.1 µm film thickness, Agilent Technologies) with hydrogen as a carrier gas.
164 The temperature program consisted of injection at 50°C for 2 min, raised by 40°C min^{-1} to 200°C, held
165 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
166 analysis was carried out using a gas chromatograph equipped with a mass spectrometric detector
167 (5975 iMSD, Agilent Technologies) following the same gas chromatographic conditions but using
168 helium as the carrier gas. Cuticular wax compounds were identified comparing a query mass spectrum
169 with reference mass spectra in a library *via* spectrum matching and quantitated against the internal
170 standard.

171 The weighted median carbon-chain-lengths (MCL) for cuticular waxes at both the MED site and the
172 TEM site were calculated. Each compound had its molar coverage calculated from the gas
173 chromatographic data and summed up according to carbon-chain-lengths. For each chain-length ($N_{i,}$),
174 the mol fraction (W_i) was determined and used as a weight for calculating the MCL. For n distinct
175 ordered chain-lengths N_1, N_2, N_3 …, N_n with weights W_1, W_2, W_3 …, W_n, the MCL is the chain-length
176 N_k satisfying:

177 $$\sum_{i=1}^{k-1} w_i \leq \frac{1}{2} \text{ and } \sum_{i=k+1}^{n} w_{i\leq} \frac{1}{2}.$$

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178 *Chemical analysis of the cutin matrix*

179 For cutin depolymerisation, completely delipidated leaves were transesterified with boron trifluoride in
180 methanol (Fluka) at 70°C overnight. After cooling down, a saturated aqueous solution of sodium
181 chloride (AppliChem), trichloromethane and *n*-dotriacontane (C_{32}; Sigma-Aldrich) as an internal
182 standard were added to the reaction mixtures. From this two-phase system, the deesterified cutin
183 monomers were extracted three times with trichloromethane. The combined organic phases were
184 dried over anhydrous sodium sulphate (AppliChem). All extracts were filtered, and the organic solvent
185 was evaporated under a gentle flow of nitrogen. Derivatisation with *N*,*O*-bis-trimethylsilyl-
186 trifluoroacetamide in pyridine was performed at 60°C for 60 min. Analysis of cutin monomers was
187 performed similarly to the gas chromatographic analysis of cuticular waxes. Separation of cutin
188 mixtures was carried out at 50 kPa for 60 min, 10 kPa min^{-1} to 150 kPa and at 150 kPa for 30 min
189 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
190 min, and then raised by 3°C min^{-1} to 320°C and held at 320°C for 30 min. Qualitative and quantitative
191 composition of the mixtures was studied using capillary gas chromatography with mass spectrometric
192 and flame ionisation detection under the same chromatographic conditions. Single cutin monomers
193 were identified based on the electron ionization mass spectra using authentic standards, the Wiley
194 10th/NIST 2014 mass spectral library (W10N14, John Wiley & Sons) or by interpretation of the
195 spectra, by the retention times and/or by comparison with literature data and quantitated against the
196 internal standard.

197 *Statistical analyses*

198 Data were tested for normality by Shapiro-Wilk test. Afterwards, comparisons between leaves of MED
199 and TEM sites were investigated using the *t*-test for normally distributed data and the *Mann-Whitney-U*
200 test for those non-normal distributed. Statistical analyses were performed using the SPSS Statistics
201 software version 23.0 (IBM Corporation).

202 **Results**

203 *Leaf surface properties*

204 Leaves of *Q. coccifera* were analysed with scanning electron microscopy to examine the morphology
205 of the leaf surface. Trichomes, stomata and epicuticular wax structures were the principal features.

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206 Leaves from the MED and the TEM sites were similar as they presented only a few trichomes on both
207 adaxial and abaxial leaf surfaces and possessed stomata exclusively on the abaxial surface
208 (hypostomatic). The stomata distribution occurs without any distinct pattern across the leaf surface.
209 The adaxial leaf surface showed a continuous smooth cuticular wax layer with the presence of few
210 epicuticular wax granules (Figure 2, A and B). On the abaxial leaf surface, the epicuticular wax
211 granules were more abundant, and the continuous cuticular wax layer projected over the stomata, thus
212 partially covering the stomatal opening (Figure 2, C and D).

213 *Leaf traits*

214 Leaf traits of *Q. coccifera* were calculated (Figure 3, A to D). The leaf mass per area (LMA) at the
215 MED site amounted to 245.7 ± 14.4 g m^{-2} (mean ± SD), which was 1.6 times higher ($p < 0.05$)
216 compared to the TEM site (152.0 ± 7.1 g m^{-2}). Leaf water content (LWC) was lower ($p < 0.05$) in MED
217 plants (0.42 ± 0.02 g g^{-1}) in comparison to TEM plants (0.48 ± 0.01 g g^{-1}). Leaf dry weight (DW) did not
218 show significant differences at $p < 0.05$ between both sites (0.02 ± 0.01 and 0.03 ± 0.01 g for MED
219 and TEM sites, respectively). Leaf size, accessed as dual projected leaf area (LA), was the half ($p <$
220 0.05) at the MED site (0.18 ± 0.05 × 10^{-3} m^2) when compared to the TEM site (0.37 ± 0.16 × 10^{-3} m^2).

221 *Minimum leaf conductance*

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

229 *Chemical composition of leaf cuticular waxes*

230 The cuticular waxes of *Q. coccifera* were analysed qualitatively and quantitatively using gas
231 chromatography to investigate the potential effect of the atmospheric conditions on the leaf cuticular
232 wax coverage. The amount of cuticular waxes was 2.6 times higher for plants grown at the MED site
233 (34.9 ± 6.6 μg cm^{-2}) compared to the TEM site (13.0 ± 2.8 μg cm^{-2}). Except for the similar amounts of

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234 alkanoic acids, all the compound classes increased at the MED site. However, the qualitative
235 composition of the cuticular waxes was not different (Figure 6). Pentacyclic triterpenoids were the
236 most abundant class of cuticular wax constituents of both MED (17.2 ± 3.1 µg cm^{-2}, 49% of the total
237 cuticular waxes) and TEM (8.0 ± 1.8 µg cm^{-2}, 61%) leaves. The major cuticular wax constituents were
238 germanicol (10.1 ± 2.3 µg cm^{-2}, 29% at MED site; and 3.4 ± 0.6 µg cm^{-2}, 26% at TEM site) and lupeol
239 (2.9 ± 1.4 µg cm^{-2}, 8% at MED site; and 1.6 ± 0.4 µg cm^{-2}, 12% at TEM site). Very-long-chain aliphatic
240 compounds with carbon-chain-lengths ranging from C_{20} to C_{51} amounted to 12.0 ± 2.1 µg cm^{-2} (34%)
241 at the MED site and 3.3 ± 0.6 µg cm^{-2} (25%) at the TEM site (Figure 7). Within the aliphatic cuticular
242 wax fraction, *n*-alkanes were the main compound class (3.5 ± 0.7 µg cm^{-2}, 10% at MED site; and 1.1 ±
243 0.2 µg cm^{-2}, 8% at TEM site). The *n*-alkane fraction comprised a homologous series from C_{25} to C_{32} *n*-
244 alkanes with odd-numbered *n*-alkanes dominating above even-numbered, and *n*-nonacosane (C_{29})
245 was the major constituent (1.6 ± 0.7 µg cm^{-2}, 5% at MED site; and 0.4 ± 0.1 µg cm^{-2}, 3% at TEM site).
246 Primary alkanols, alkanol acetates, alkanals, alkanoic acids, and alkyl esters were also identified in the
247 *Q. coccifera* cuticular waxes of the two sites (Table 1).

248 *Chemical composition of the leaf cutin matrix*

249 The cutin monomeric composition was analysed using gas chromatography with flame ionisation and
250 mass spectrometry detection after depolymerisation of the cutin polyester. The amount of the cutin
251 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
252 the TEM site (Table 2). The leaf cutin matrix was composed of 88% aliphatic and 14% phenolic cutin
253 monomers for the MED site and 91% aliphatic and 9% phenolic cutin monomers for the TEM site,
254 respectively. For both sites, 9,10-epoxy 18-hydroxy alkanoic acid averaging out at 67.3 µg cm^{-2} was
255 the predominant cutin monomer (27% of total cutin monomers for MED and 37% for TEM).
256 Additionally, 9/10,16-dihydroxy hexadecanoic acid (63.3 µg cm^{-2}), 18-hydroxy octadec-9-enoic acid
257 (27.0 µg cm^{-2}), 9,10,18-trihydroxy octadecanoic acid (23.1 µg cm^{-2}) and 4-hydroxy cinnamic acid
258 (*para*-coumaric acid; 16.3 µg cm^{-2}) were detected in high quantities in the cutin matrix of MED plants.
259 In particular, distinctly lower amounts of 9/10,16-dihydroxy hexadecanoic acid (1.9 times), 9,10,18-
260 trihydroxy octadecanoic acid (5.3 times) and 4-hydroxy cinnamic acid (2.5 times) in TEM plants
261 accounted for the 1.4 times difference in the total cutin monomeric quantity when comparing both MED
262 and TEM sites (Figure 8). Due to the different monomeric composition, the degree of epoxylated and
263 unsaturated alkanoic acids was lower for the leaf cutin of the MED site (0.27 and 0.13) compared to

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

266 **Discussion**

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

276 In summer, it is common in MED environments that the temperature rises while the water vapour
277 density stays constant, resulting in considerably reduced atmospheric humidity. This combination
278 leads to a rise in the driving force for water loss by transpiration. Although *Q. coccifera* has its main
279 distribution area in the MED zone, this plant species also occurs at the Iberian Atlantic coast under
280 constantly humid conditions. It is generally assumed that plants adapted to a high driving force for
281 water loss have a very robust and efficient cuticle. Therefore, one may intuitively expect the minimum
282 leaf conductance (g_{min}) of *Q. coccifera* to be lower when grown at the MED site due to phenotypic
283 modifications to avoid cuticular water loss. However, our findings do not support this hypothesis since
284 g_{min} of *Q. coccifera* remained unaffected by the contrasting atmospheric conditions in the growth sites.

285 In line with our findings, the water permeability of the astomatous isolated leaf cuticle of the evergreen
286 tree *Citrus aurantium* L. grown at a temperature ranging from 15°C to 35°C and relative humidity of
287 50% or 90% remained unaffected by the different conditions (Geyer and Schönherr 1990). Schuster et
288 al. (2016) reported that the leaf cuticular permeability of the evergreen desert shrub *Rhazya stricta*
289 Decne. is comparable to those of woody plant species from various habitats, including humid ones.
290 Similarly, the leaf cuticular permeability of the summer-green desert vine *Citrullus colocynthis* (L.)
291 Schrad. is close to that of non-evergreen forbs from TEM climates (Bueno et al. 2019). These authors
292 also showed that the leaf cuticular permeability of the evergreen desert tree *Phoenix dactylifera* L. is

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293 still equivalent to the permeability reported for evergreen woody plants from other climates. Therefore,
294 the common assumption that the plant cuticle either genetically or phenotypically adapted to high
295 transpirational demand should have a more efficient barrier to avoid water loss is not supported by
296 experimental evidence.

297 Some studies have pointed out evidence that cuticular water permeability is mainly determined by
298 genetic control, and not or only slightly being subject to environmental influence. Gil-Pelegrín et al.
299 (2017) investigated eleven *Quercus* species growing in a common garden and found out that g_{min} of
300 typical evergreen MED *Quercus* species was slightly lower compared to deciduous TEM ones.
301 However, g_{min} of deciduous MED and TEM *Quercus* species did not differ. Moreover, the leaf cuticular
302 permeability from 160 plant species extracted from the literature was summarised and revealed that
303 only in two particular cases, epiphytes and climbers/lianas, the cuticular permeability was
304 exceptionally low (Schuster et al. 2017). Therefore, cuticular water permeability might be related to the
305 plant life strategy to deal with environmental constraints, as suggested by Bueno et al. (2019).

306 The plant cuticle acts as a protective barrier against a wide range of biotic and abiotic stresses and
307 might respond to environmental changes. Our findings showed that cuticular wax and cutin coverages
308 increased for MED leaves, corroborating our hypothesis that *Q. coccifera* shrubs grown under MED
309 conditions accumulate more cuticular lipids than those in TEM conditions. However, the assumption
310 that the qualitative chemical composition would also be affected by the atmospheric conditions was
311 rejected. Although the quantitative variations were detected for leaf cutin monomers between the MED
312 and TEM sites, the cutin composition was similar for leaves of both habitats, and the main component
313 was in both cases the 9,10-epoxy 18-hydroxy alkanoic acid. Previous studies have shown that the
314 mechanical strength of the cutin matrix, especially under conditions of high temperature or protracted
315 exposure to sun, plays a pivotal role in maintaining the barrier function of the plant cuticle in particular
316 and the physiological plant integrity in general (Heredia 2003; Bargel et al. 2006; Khanal and Knoche
317 2017).

318 Similarly, MED leaves had almost three times more cuticular waxes than TEM leaves, but the relative
319 composition of the cuticular waxes was not different between both sites. All the identified wax
320 compound classes (pentacyclic triterpenoids, *n*-alkanes, primary alkanols, alkanol acetates, alkanals,
321 alkanoic acids, and alkyl esters) were found in both MED and TEM leaves. Pentacyclic triterpenoids

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322 correspond to about half of the total cuticular waxes of *Q. coccifera* leaves regardless of the habitat.
323 The potential triterpenoid functions are protecting plants against herbivory and stabilising the cuticle of
324 heat-stressed plants (Reichardt et al. 1984; Oliveira and Salatino 2000; Schuster et al. 2016).
325 However, the contribution of pentacyclic triterpenoids to avoid uncontrolled water loss has been
326 considered small or absent (Leide et al. 2007, 2011; Buschhaus and Jetter 2012; Jetter and Riederer
327 2016; Schuster et al. 2016). Therefore, the efficacy of the cuticular transpiration barrier has been
328 attributed to the very-long-chain aliphatic compounds (Jetter and Riederer 2016). These findings are in
329 line with the molecular structure model of cuticular waxes proposed by Riederer and Schreiber (1995).
330 According to these authors, the cuticular waxes are multiphase systems made up of mobile
331 amorphous zones within highly structured crystalline domains. This model predicts that the very-long-
332 chain aliphatic compounds build up impermeable crystalline domains, and the amorphous zones
333 incorporate the chain ends and pentacyclic molecules. Hence, the model assumes that the very-long-
334 chain aliphatic compounds constitute the cuticular transpiration barrier in plants. In our study, these
335 compounds increased by 3.5 times in leaves of *Q. coccifera* grown at the MED site, but the efficacy of
336 the cuticular transpiration barrier remained unaltered. In line with our findings, studies on several plant
337 species have shown that cuticular water permeability does not correlate with the amount of cuticular
338 waxes or cuticle thickness (Schreiber and Riederer 1996; Riederer and Schreiber 2001; Schuster
339 2016; Bueno 2018). Several plant species under water stress had increased the production of
340 cuticular waxes, including the herbaceous model plant *Arabidopsis thaliana* (L.) Heynh. (Cameron et
341 al. 2006; Kim et al. 2007; Kosma et al. 2009; Le Provost et al. 2013). Indeed, water limitation caused
342 by either soil drought or low atmospheric humidity affects the cuticular wax deposition. However, in
343 this specific case, an effect of the soil water status can be excluded because the plants were watered
344 as needed over the eight years' cultivation.

345 Here arises the question: what are the environmental drivers of the increased leaf cuticular wax
346 coverage of *Q. coccifera* at the MED site? The principal environmental differences between the two
347 growth sites during the experimental year 2016 were summer temperature, solar radiation and vapour-
348 pressure deficit. Although the mean annual temperature differs only by 1°C between both sites, the
349 mean and maximum monthly temperatures during the summer at the MED site were up to 4°C and
350 10°C higher than at the TEM site, respectively. *Q. coccifera* grown at the MED site also experienced
351 higher solar radiation in comparison with TEM site during the whole year. Finally, yet importantly, the
352 mean vapour-pressure deficit during the summer months was higher at the MED site that at the TEM

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353 site. This scenario is even more evident when comparing the maximum monthly vapour-pressure
354 deficit, which was up to 3.5 times higher at the MED site.

355 Plants under stress conditions often exhibit changes in the amount and composition of cuticular waxes
356 (Shepherd and Griffiths 2006). Studies on the effect of temperature on the cuticular wax composition
357 are scarce, and the few studies available are contradictory (Shepherd and Griffiths 2006). Some
358 studies have shown that a lower temperature stimulated high cuticular wax production in leaves of
359 *Brassica* species (Whitecross 1963; Whitecross and Armstrong 1972; Baker 1974). In opposite,
360 Riederer and Schneider (1990) reported that increasing the day temperature from 25°C to 30°C lead
361 to an increase of about two times in cuticular waxes of *C. aurantium* leaves. Further, Reed and Tukey
362 (1982) found that a higher amount of leaf cuticular waxes was produced at either lower for example in
363 herbaceous *Brassica oleracea* L. or higher temperature for example in evergreen MED herb *Dianthus*
364 *caryophyllus* L. In contrast to temperature, it has been widely accepted that high solar radiation leads
365 to an increase in cuticular wax coverage (Baker 1974; Giese 1975; Reed and Tukey 1982; Shepherd
366 et al. 1995). Another important environmental factor is atmospheric humidity. High relative humidity
367 tends to reduce the evaporative demand by decreasing the vapour-pressure deficit. Koch et al. (2006)
368 showed that the cuticular wax accumulation of *B. oleracea* leaves strongly declines in response to a
369 low vapour-pressure deficit. Lihavainen et al. (2017) proposed that plants under low vapour-pressure
370 deficits transpire less, improving their water status and, thereby, reducing the demand for cuticular
371 waxes. Therefore, one may assume that the main drivers of the increased cuticular wax coverage of
372 *Q. coccifera* are the intense solar radiation, the high vapour-pressure deficits and, potentially, the high
373 temperatures at the MED site, especially in the summer. The high accumulation of cuticular lipids may
374 confer higher resistance to the intense light exposure and high vapour-pressure deficit at the MED
375 site. However, further studies on wild plants, especially in their natural ecosystems, are needed to
376 trace potential clues of cuticular adaptation to cope with inherent environmental constraints.

377 In conclusion, we showed that the cuticle of *Q. coccifera* leaves plastically responds to the harsh MED
378 conditions, which leads to high cuticular wax and cutin loads. However, the cuticular lipids at both
379 MED and TEM sites are qualitatively very similar; i.e. the relative contribution of each component
380 class, and the carbon-chain-length of homologous compounds. Although, it is often stated that a
381 thicker cuticle is a barrier with a higher efficiency against passive water loss than thinner ones with a
382 lower amount of cuticular waxes (Purves et al. 2004; Poorter and Garnier 2007; Lüttge 2007; De Micco

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383 and Aronne 2012; Smith et al. 2012; Jones 2013), our findings suggest that the accumulation of
384 functional equivalent cuticular waxes might compensate for the quantitative plasticity of the cuticular
385 deposition of *Q. coccifera* and, thereby, conferring equal cuticular transpiration properties. Further, we
386 stress that high cuticular wax loads do not increase the efficacy of the cuticular transpiration barrier
387 and, therefore, might not extend the safety margin between stomatal closure and xylem hydraulic
388 failure.

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Figure 1. Mean (T_{mean}), maximum (T_{max}), and minimum (T_{min}) daily temperature (°C), mean diurnal (from dawn to sunset) vapour-pressure deficit (VPD, kPa) and maximum diurnal vapour-pressure deficit (VPD_{max}), mean daily quantum flux density (Qint, mol m^{-2} day^{-1}), for the Mediterranean (MED) and temperate (TEM) sites during the growing season of 2016 to 2017 (from March to February). Bars represent mean ± SE. Lines stand for single values.

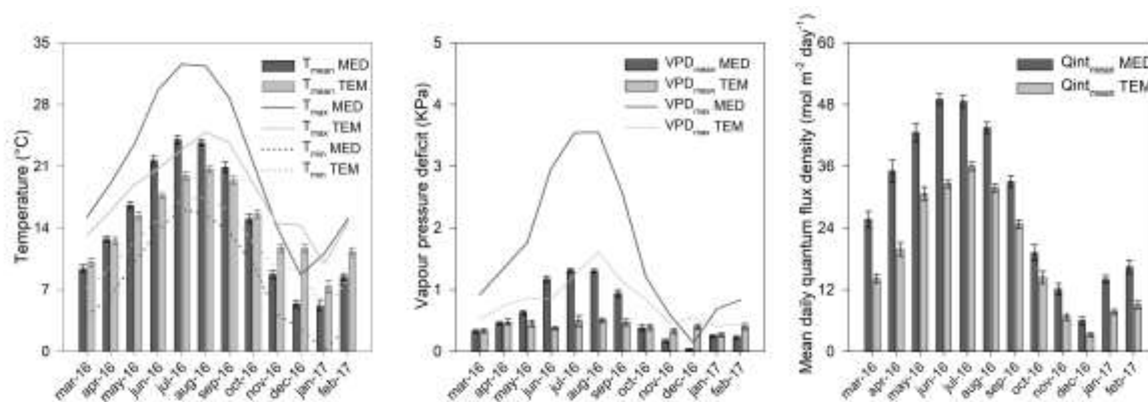

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Figure 2. Scanning electron micrographs of the adaxial and abaxial surfaces of *Quercus coccifera* leaves from Mediterranean (MED; A and C) and temperate sites (TEM, B and D). The stomatal density of MED leaves (445 ± 61 stomata mm^{-2}) was slightly 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).

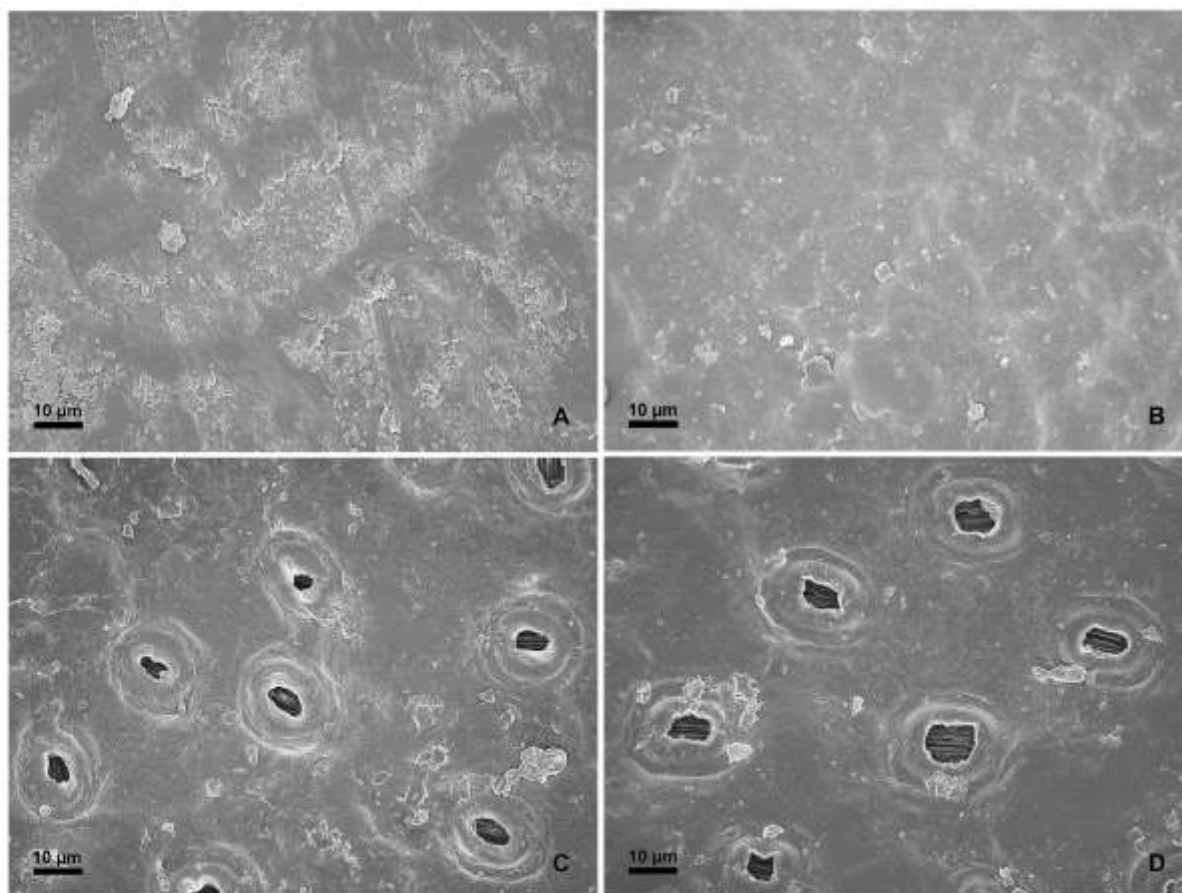

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Figure 3. Leaf traits of *Quercus coccifera* grown at Mediterranean (MED) and temperate (TEM) sites, respectively (n ≥ 16). Leaf mass per area (LMA; A), leaf water content (LWC; B), leaf dry weight (DW; C) and the dual projected leaf area (LA; D) were determined for plants grown under the two conditions. Different letters indicate significant differences at $p < 0.05$ between the two sites.

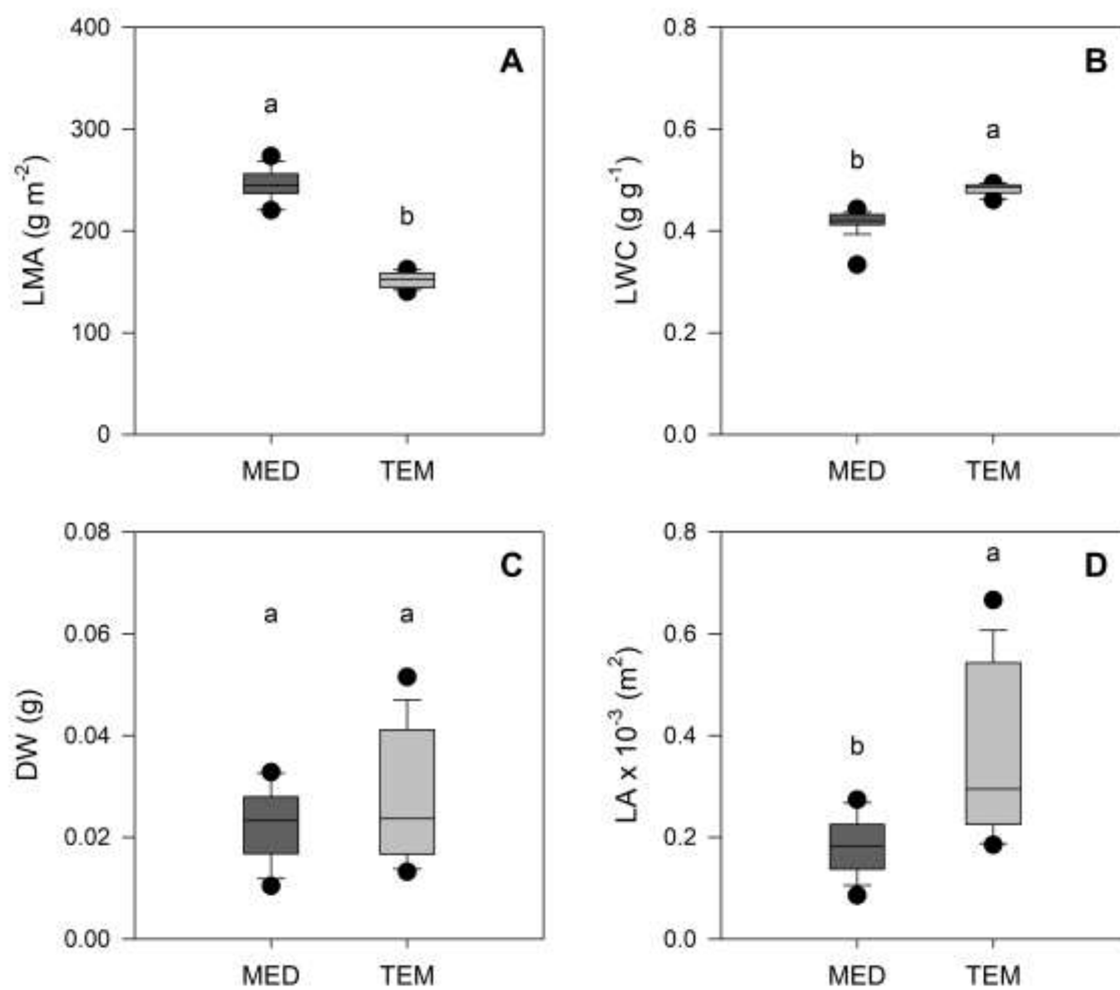

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Figure 4. Cuticular water conductance (g) as a function of the relative water deficit (RWD) of *Quercus coccifera* grown at the Mediterranean site. Each point represents a single measurement obtained from the leaf drying curves of nine leaves at 25°C. 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}).

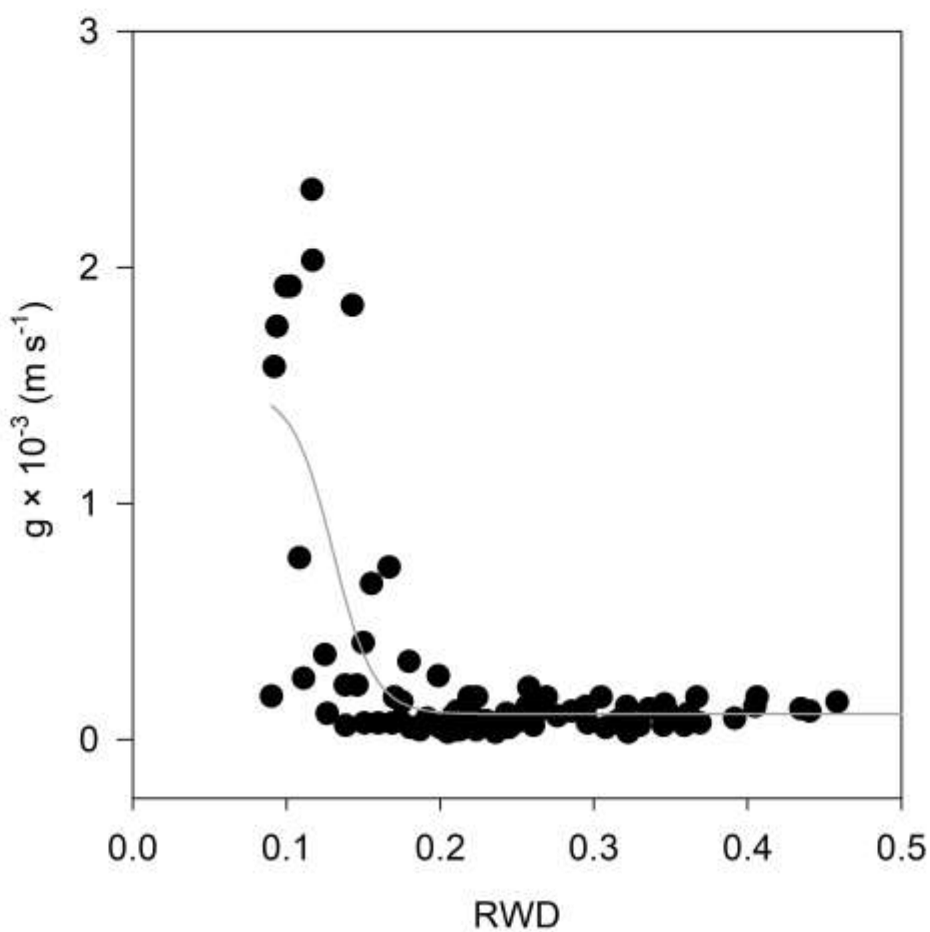

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Figure 5. Minimum leaf conductance (g_{min}) of *Quercus coccifera* grown at Mediterranean (MED) and temperate (TEM) sites, obtained from drying curves at 25°C ($n \geq 16$). The g_{min} did not differ between plants from the MED and the TEM sites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

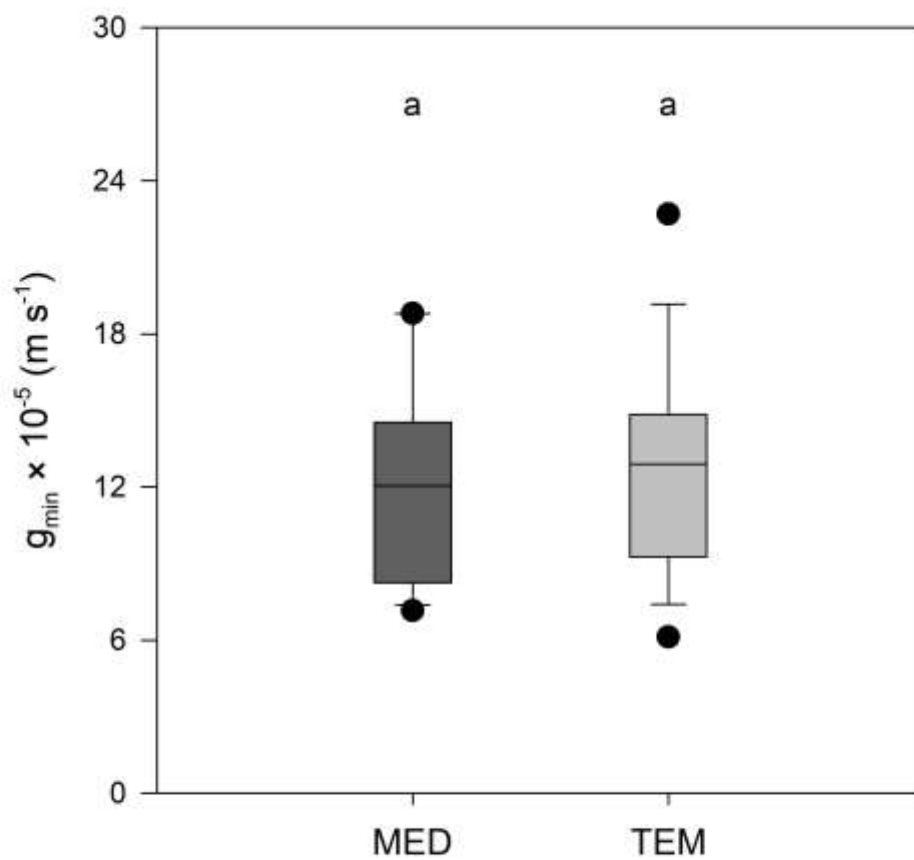

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Figure 6. Chain-length distributions of the very-long-chain aliphatic fraction of cuticular waxes of *Quercus coccifera* leaves grown at Mediterranean (MED; A) and temperate (TEM; B) sites (n = 4). Bars stand for the mole-based contribution of a single chain-length to the total very-long-chain aliphatic wax coverage. Triangles denote the weighted median chain-lengths (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.

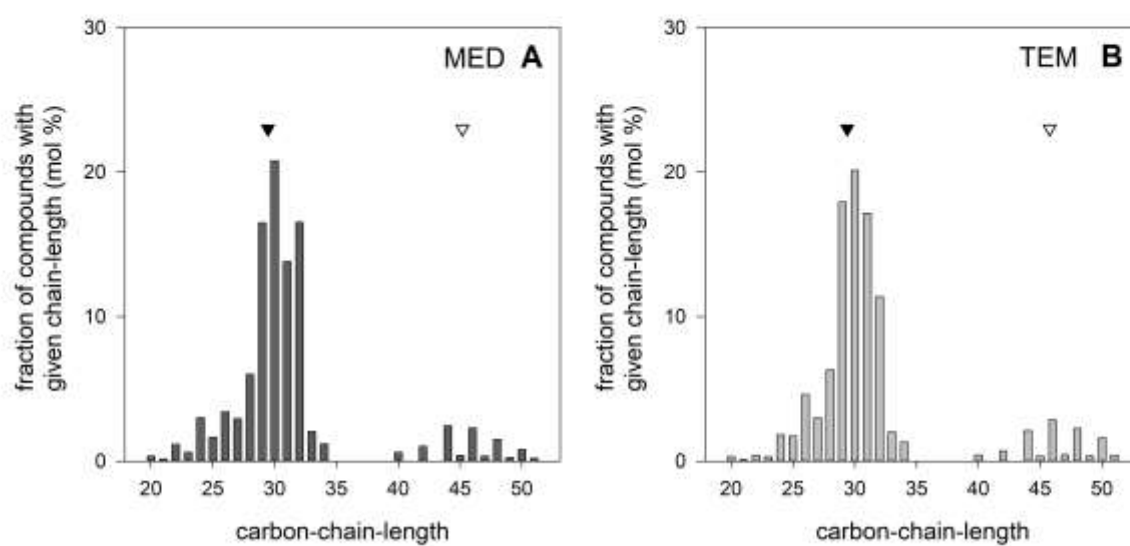

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Figure 7. Cuticular wax coverage of *Quercus coccifera* leaves grown at Mediterranean (MED) and temperate (TEM) sites, arranged by compound class. Each value represents the mean value ± SD (n = 4). Asterisk indicates significant difference at $p < 0.05$ between the MED and the TEM sites.

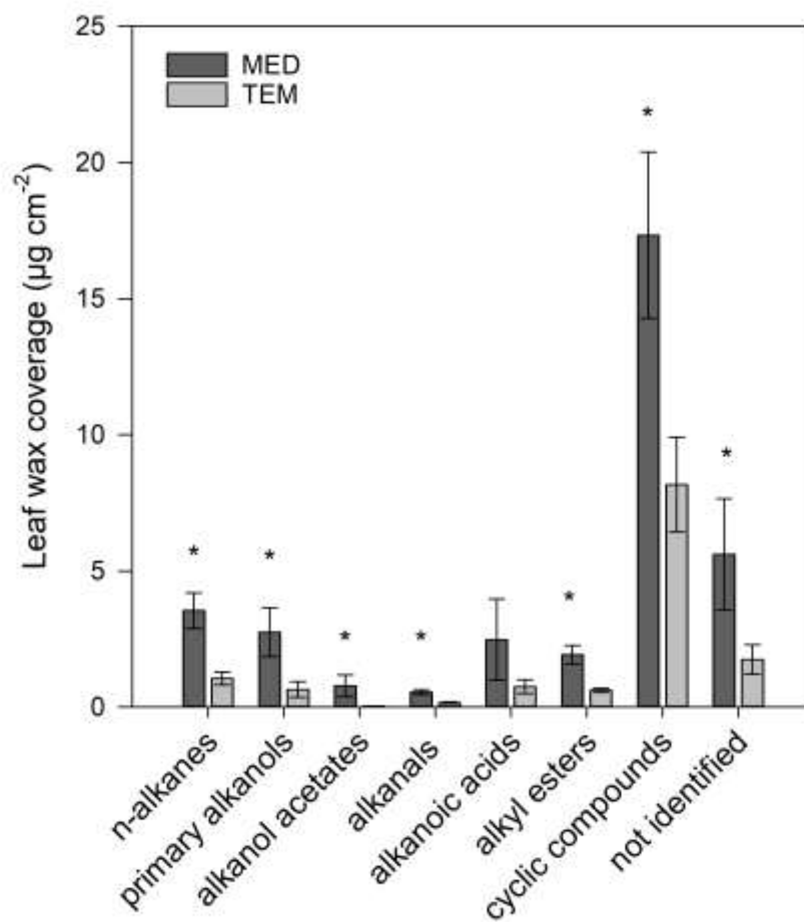

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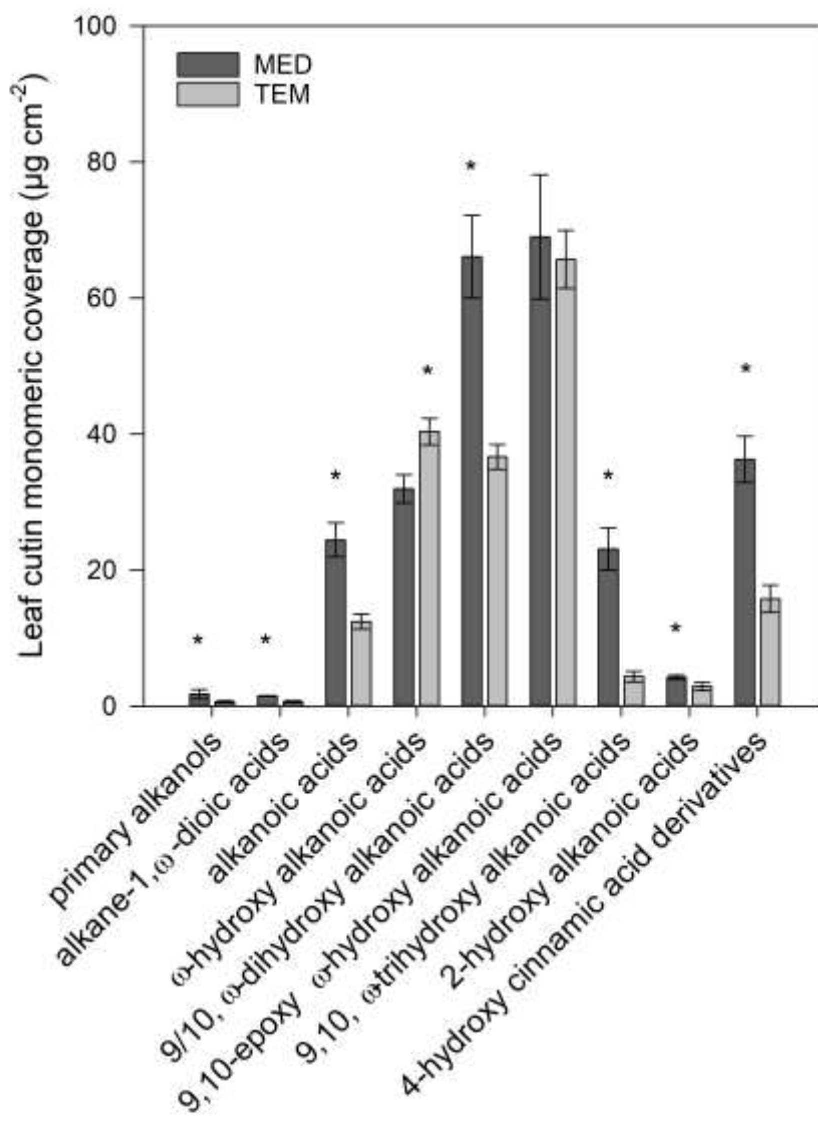
Figure 8. Cutin monomer coverage of *Quercus coccifera* leaves grown at a Mediterranean (MED) and a temperate (TEM) site, respectively. Each value represents the mean ± SD (n = 4). Asterisk indicates significant difference at $p < 0.05$ between the MED and the TEM sites.



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Tables

Table 1. Chemical composition of leaf cuticular waxes of *Quercus coccifera* grown at a Mediterranean and a temperate site, respectively. Each value represents the mean value ± SD (n = 4).

Compound class	Carbon-chain-length	Coverage (µg cm^{-2})					
		Mediterranean site			Temperate site		
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

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	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
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*

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Table 2. Chemical composition of the leaf cutin matrix of *Quercus coccifera* grown at a Mediterranean and a temperate site, respectively. Each value represents the mean value ± SD (n = 4).

Compound	Carbon-chain-length	Cutin coverage (µg cm^{-2})	
		Mediterranean site	Temperate site
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*

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