forests

MDPI

Article

Variation in Leaf Cutin Content and Chemical Composition along One Annual Cycle in the Mediterranean Cork Oak (*Quercus suber* L.)

Rita Simões, Isabel Miranda * and Helena Pereira

Centro de Estudos Florestais (CEF), Associate Laboratory TERRA, Instituto Superior de Agronomia, Universidade de Lisboa, 1349-017 Lisboa, Portugal
* Correspondence: imiranda@isa.ulisboa.pt

Abstract: Cork oak (*Quercus suber* L.) has high economic value given by its sustainable production of cork, and ecological importance in the Mediterranean region. The species is well adapted to the dry climate, namely through the sclerophyllous nature of its leaves with a well-developed cuticle, including cutin and cuticular waxes that contribute to protection against drought. Leaves of cork oaks were collected along one annual cycle, starting from the young leaves in May to the one-year-old leaves in March. Leaf cutin content and chemical composition were determined by transesterification subsequently to the determination of cuticular waxes, and leaf features, and were analyzed along the leaf cycle. Cutin is a major component of the cuticle, representing on average 72.4% of the cutin and cuticular waxes. Cutin amounted to 71.0 g/1000 g of dry leaves, without significant seasonal mass proportion variation, while cutin coverage increased from May to December (429.7 μg/cm^2 and 575.4 μg/cm^2, respectively). In contrast, a clear seasonality was found in cuticular wax mass proportion and coverage (18.4 g/1000 g of dry leaves and 113.5 μg/cm^2 in May, and 28.5 g/1000 g and 235.2 μg/cm^2 in September). Cutin is a glyceridic polyester composed by long-chain acids, mainly ω-hydroxyacids, followed by fatty acids with a few ω-diacids and alcohols, and by a substantial proportion of aromatics. Cutin composition varied along time with a proportional increase in ω-hydroxyacids (45.8% in May; 50.8% in December), and a significant decrease in aromatics (24.2% in May and 8.5% in March). The cuticle seasonal development in the cork oak contributes to protect the leaves and the trees from the dry summer conditions.

Keywords: cuticular; cuticular waxes; leaf sclerophylly; glyceridic polyester; specific leaf area

Citation: Simões, R.; Miranda, I.; Pereira, H. Variation in Leaf Cutin Content and Chemical Composition along One Annual Cycle in the Mediterranean Cork Oak (*Quercus suber* L.). *Forests* **2023**, *14*, 334. https://doi.org/10.3390/f14020334

Academic Editor: František Kačík

Received: 13 December 2022
Revised: 2 February 2023
Accepted: 3 February 2023
Published: 7 February 2023

1. Introduction

Plant leaves have one important protection barrier made up by a cuticle that covers their external surface and builds up a continuous extracellular, mostly hydrophobic, membrane on the epidermis that provides protection from biotic or abiotic stresses, namely from climatic harsh conditions, e.g., by limiting water loss and in heat insulation [1,2]. The cuticle includes a polymeric cutin matrix and cuticular waxes, linked to the epidermal cell wall by polysaccharides, also considered part of the cuticle as a cutin–polysaccharide matrix. Cutin is a polyester formed predominantly by cross-linking through ester bonds of C_{16} and C_{18} hydroxy fatty acids and glycerol, while cuticular waxes comprise very long-chain fatty acids and derivatives, alcohols, and alkanes as well as terpenes, sterols, and aromatics, and are soluble in non-polar solvents [3–6]. Cuticular waxes are located in the cuticle within the cutin matrix as an intracuticular layer, and on the outer surface of the cutin as an epicuticular layer [3]. Some cuticles also contain a resistant aliphatic polymer that is not depolymerized by ester-bond hydrolysis, the cutan, that has only a small proportion of aromatic moieties [7,8].

The functional properties of the cuticle are given by the structural and chemical features of the cuticular wax and cutin. The barrier against water diffusion established by the cuticle is preferentially given by the intracuticular wax since the epicuticular wax does not function as a transpiration barrier [9]. Water loss is limited mainly by the very long-chain aliphatic fraction [10–12], whereas the triterpenoids give mechanical and thermal stability to the cuticle [13,14]. Cutin provides strength and rigidity to the epidermal cells, contributing to the nanostructure of the surface, and may be important for the interaction with pathogenic fungi [15,16].

Several reviews have been published on cutin, often associated with suberin which is a chemically similar polyester and the main cell wall structural polymer of cork, although specifics on its protective functions and detailed macromolecular composition and assembly are lacking [7,17]. Knowledge on variability aspects of cutin is particularly scarce, e.g., related to species, environmental conditions, and seasonality, albeit its importance for the potential protection role of the leaf cuticle under changing climatic conditions. This aspect is particularly relevant in plants growing in desert or drought conditions, more so under the context of climatic changes in regions where intensification of dryness is expected to occur. This is the case of the Mediterranean basin for which climate changes of increasing temperature and decreasing rainfall are foreseen [18].

Cork oak (*Quercus suber* L.) is one important species in the western Mediterranean basin, and a considerable economic asset due to cork production that feeds an important industrial chain [17]. The trees are well adapted to the regional prevailing conditions with long dry summers, high solar irradiances, air temperatures and vapor pressure deficits, and very low rainfall while winters are moderately cold. However, the foreseen harshening of climatic conditions has brought concerns about the future sustainability of cork oak ecosystems. While cork oak research has been intense regarding cork biology and properties, as well as on cork processing and applications (as reviewed in Pereira [17]), comparatively little is known on cork oak leaves and on the characteristics of their cuticle. Cork oaks develop the new foliage beginning in April and terminating by June, with a leaf duration of up to approximately 14 months, with most of the older (1-year-old) leaves falling in spring when shoot growth starts and new leaves appear [19–22].

The leaves are sclerophilic, covered by a substantial cuticle with high amounts of cutin (corresponding to 518 $\mu g/cm^2$ of leaf area) and of cuticular waxes (154.3–235.1 $\mu g/cm^2$) that contributed to the building up of a nearly impermeable membrane [23,24]. The proportion and composition of cutin and cuticular waxes were shown to be independent of tree seed provenance for trees growing in the same environment [23,24].

The seasonal variation in the cork oak leaf cuticle along the leaf cycle will help to understand the role of the cuticular components on their protective function in association along leaf development, thereby contributing to design potential adaptation measures for climate change scenarios. The results obtained for the seasonal variation in the cuticular waxes from the young leaves in spring to one-year leaves before falling were already studied and published by our group, showing that the chemical variation in cuticular waxes along the leaf cycle gives support to the role taken by the intracuticular waxes and the long-chain lipids as a transpiration barrier during summer droughts [25]. The present paper addresses the seasonal dynamics of cutin deposition and chemical composition, in parallel with the associated cuticular wax deposition under the specific leaf morphological characteristics, thereby allowing a full insight into the cuticle development along the leaf cycle in association with leaf development and season climatic features. It is hypothesized that cutin coverage increases in the developing leaves and shows a compositional variation along the cycle that, in conjunction with the cuticular waxes, will favor the strengthening of the leaf and the cuticle development for establishing a transpiration barrier. It should be noted for the sake of experimental accuracy that when referring to cutin it is the material solubilized by transesterification of the solvent-extracted leaves that is meant while cuticular waxes refer to the Soxhlet solvent solubilized compounds.

2. Materials and Methods

2.1. Sampling

Leaves were collected from 21-year-old *Quercus suber* L. trees grown in a provenance trial at Herdade Monte Fava, Santiago do Cacém, in central Portugal (38,000′ N, 08070′ W, altitude 79 m) from seedlings obtained with seeds from different geographical provenances. The site has a Mediterranean-type climate with dry and hot summers, mildly cold and wet winters, with most rainfall between October and May, and a long term mean annual temperature of 15.8 °C and mean annual precipitation of 587 mm. A detailed trial and site description is given elsewhere [26,27]. The leaves were collected from several branches on the south exposed lower part of the canopy below a height of approximately 2 m, from two trees from six provenances: Portugal (PT35), Spain (ES11), Italy (IT13), France (FR3), Morocco (MA27), and Tunisia (TU32). The sampling was carried out along one annual leaf cycle with five leaf collections: in May 2019, when the new leaves from the current year spring flushing are almost fully expanded, in September 2019, December 2019, January 2020, and March 2020. The same trees were sampled in the different periods. Each sampling amounted to about 100 leaves per tree, therefore, making up a total of approximately 1200 leaves at each sampling date.

2.2. Morphological Variables

Leaf area and specific leaf area (SLA, cm^2/g of leaf dry mass) were measured in 40 leaves for each provenance and sampling time. The detailed methodology based on image analysis of the leaves was reported in Simões et al. [25].

2.3. Determination of Cuticular Waxes

Whole fresh leaves corresponding to approximately 1.5 g dry mass were extracted with dichloromethane during 6 h in a Soxhlet apparatus for removal of the epicuticular and intracuticular waxes from both leaf sides. The amount of the solubilized cuticular material was determined and expressed on a dry weight basis (g/1000 g dry leaf mass) and on a leaf surface area (as $\mu g/cm^2$), corresponding to a leaf surface coverage, and the area being the two-sided leaf surface area. The detailed methodology was already reported showing that little amounts of internal lipids or polysaccharides were solubilized under these conditions [25].

2.4. Determination of Cutin Content

The depolymerization of cutin was carried out by transesterification with a sodium methoxide (NaOMe)-catalyzed methanolysis applied to the whole leaves after removal of the cuticular waxes. The protocol followed the methodology applied to cork suberin depolymerization [28] and previously adapted to cutin determination in cork oak leaves [24]. A sample of approximately 1.5 g dewaxed leaves was refluxed during 3 h with 100 mL of a methanolic 3% $NaOCH_3$ solution, filtrated, washed with CH_3OH, refluxed again with 100 mL CH_3OH during 15 min, and filtrated. The combined filtrates were acidified to pH 6 with 2 M H_2SO_4, and evaporated to dryness. The residue was suspended in 50 mL of water and extracted successively three times with dichloromethane (50 mL each). The combined dichloromethane extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. Cutin was quantified by determining the mass loss by methanolysis after drying and weighing the leaves residue, and was expressed in percent of the initial dry weight basis (g/1000 g dry leaf mass) and on a leaf surface area (as $\mu g/cm^2$), corresponding to a leaf surface coverage, the area being the two-sided leaf surface area.

2.5. Chemical Composition of Cutin

The cutin monomers obtained by transesterification of the dewaxed leaves were solubilized in dichloromethane and derivatized into silylated derivatives for GC–MS analysis. Aliquots (5 mL) of the dichloromethane extracts were evaporated under N_2 flow and were dried overnight at ambient conditions under a vacuum. The samples were derivatized in 100 µL of pyridine with 100 µL of bis(trimethylsilyl) trifluoroacetamide at 60 °C for 30 min, in order to trimethylsilylate (TMS) the hydroxyl and carboxyl groups into ethers and esters, respectively. The derivatized samples were immediately injected in a GC–MS Agilent 5973 MSD with the following GC conditions: Zebron 7HG-G015-02 column (30 m, 0.25 mm; ID, 0.1 µm film thickness), flow 1 mL/min, injector 280 °C, oven temperature program, 100 °C (1 min), rate of 8 °C/min up to 250 °C, rate of 5 °C/min up to 300 °C (5 min), rate of 5 °C/min up to 350 °C (5 min), rate of 10 °C/min up to 380 °C (5 min). The MS source was kept at 220 °C and the electron impact mass spectra (EIMS) taken at 70 eV. The compounds were identified as TMS derivatives by comparing with a GC–MS spectral library (Wiley, NIST) and with published data, as reported in Simões et al. [24]. The peak area in the GC–MS total ion chromatograms was integrated, and each peak was quantified in area proportion of the total chromatogram area. Duplicate analyses were made (with standard deviation below 5%).

2.6. Statistical Analysis

The results are given for each sampling time as the means and their standard deviation of the six independent provenances' samples which were analyzed in duplicate, i.e., with two trees. One-way repeated measures analysis of variance (ANOVA) was performed to compare the different sampling times. Pairwise differences were evaluated with a Holm–Sidak post hoc test. Statistical significance was set at $p < 0.05$. Sigmaplot® (Version 11.0, Systat Software, Inc., Chicago, IL, USA) was used for all statistical analyses.

3. Results

The seasonal changes in cuticle composition of *Quercus suber* leaves were followed along their first year, starting with the young leaves in late spring (May), and ending with the one-year-old leaves in early spring (March) of the following year before leaf fall. Leaf size and specific leaf area were measured, and the cuticle development was analyzed in relation to content and leaf coverage of cuticular waxes and of cutin, as well as with the study of cutin compositional variation. Microscopic observations of *Q. suber* leaf anatomy were already published by our group and are not repeated here [23].

3.1. Leaf Area and Specific Leaf Area

The average leaf area was constant along the studied period at 6.1 ± 2.0 cm^2 (Figure 1). Leaf size variability was large, as shown by the standard deviation of the mean at each sampling period, and the differences that occurred along the period were statistically significant ($p = 0.002$) with the March value different from all the others. Specific leaf area (SLA) was high in the young leaves (83.7 cm^2/g) and declined subsequently to a mean value of 62.8 cm^2/g. The differences were highly significant ($p < 0.001$), with the May value being different from all the others (Figure 1).

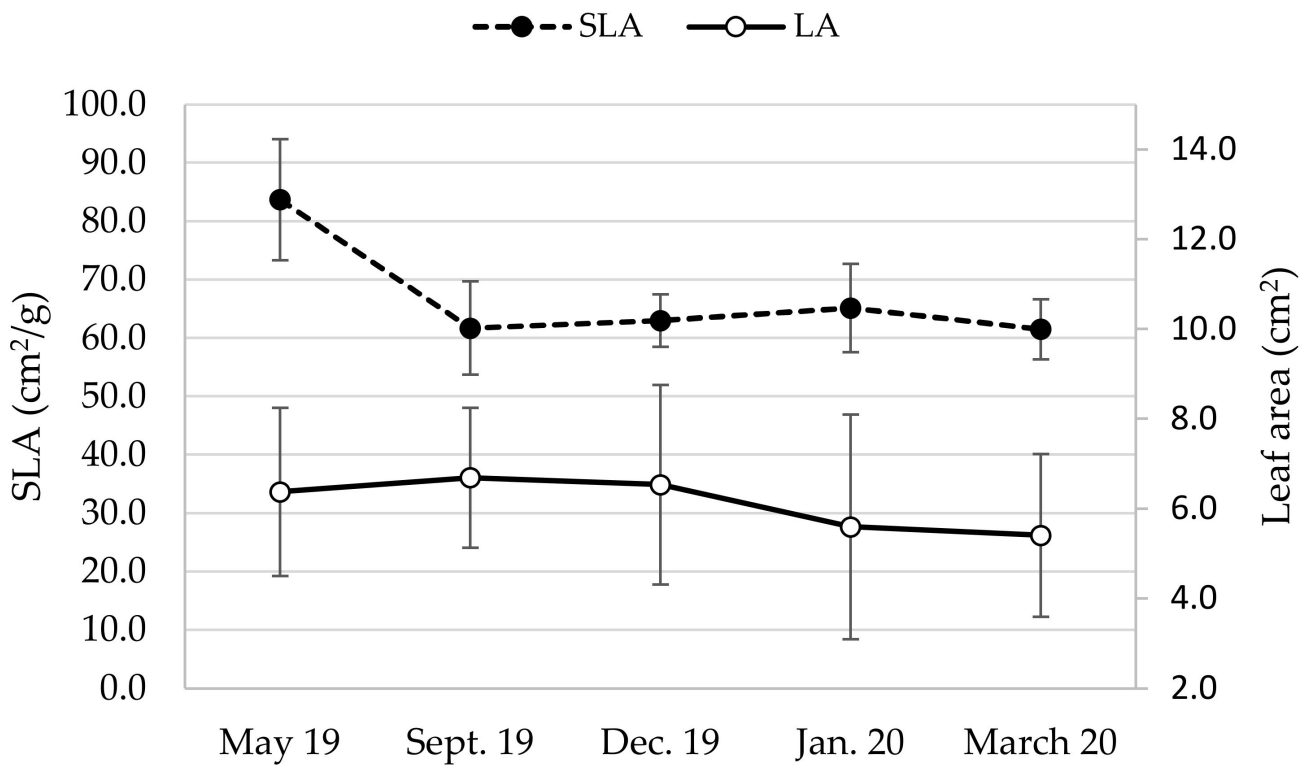

Figure 1. Variation in leaf area (LA, empty circles) and specific leaf area (SLA, filled circles) in *Quercus suber* along one year (mean of six provenances with two trees per provenance and standard, deviation as bars).

3.2. Cutin and Cuticular Wax Contents

The variation in cutin and cuticular waxes along the year is summarized in Table 1 as proportion of the leaf dry mass, in g/1000 g, and shown in Figure 2 as leaf coverage, in μg/cm^2.

Table 1. Seasonal variation in cuticular waxes (as solubles by dichloromethane), cutin (as monomers solubilized by transesterification), and cuticle (as the sum of cutin and cuticular waxes), in mass proportion of dry leaf mass (g/1000 g) in leaves of *Quercus suber* (mean of six provenances with two trees per provenance and standard deviation).

	May 2019	**September 2019**	**December 2019**	**January 2020**	**March 2020**
Cutin (g/1000 g dry leaves)	71.0 ± 16.2	66.2 ± 6.2	71.2 ± 8.7	63.5 ± 6.8	65.5 ± 1.6
Cutin (% of cuticle)	79.3 ± 5.5	70.1 ± 5.0	75.7 ± 4.9	66.3 ± 4.7	70.4 ± 3.4
Cuticular waxes (g/1000 g dry leaves)	18.4 ± 5.8	28.5 ± 6.0	22.8 ± 4.7	32.8 ± 8.6 b	27.6 ± 4.0
Cuticular waxes (% of cuticle)	20.7 ± 5.5	29.9 ± 5.0	24.3 ± 4.9	33.7 ± 4.7	29.6 ± 3.4
Cuticle (cutin + cuticular waxes) (g/1000 g dry leaves)	89.3 ± 18.3	94.6 ± 9.0	94.0 ± 8.4	96.3 ± 13.8	93.1 ± 3.3

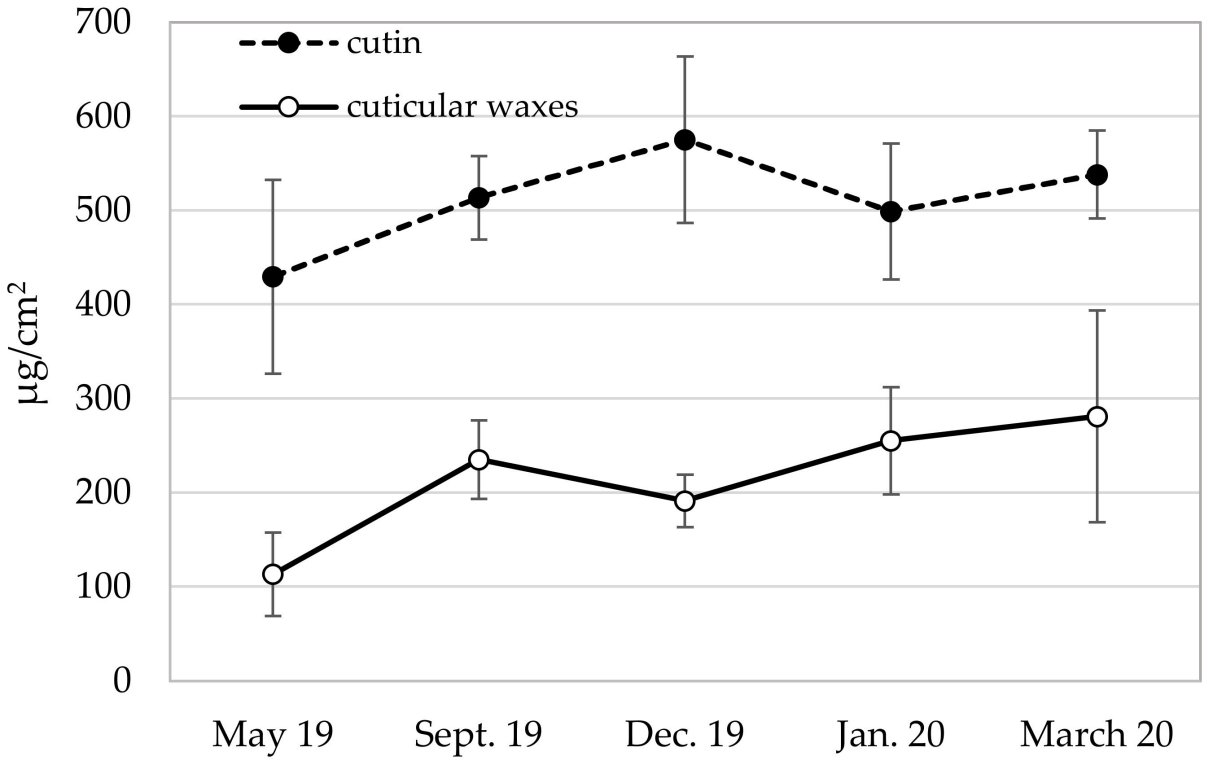

Figure 2. Variation in cuticular waxes and cutin contents in *Quercus suber* L. leaves along one year (mean of six provenances with two trees per provenance and standard deviation as bars).

In the young leaves in May, cuticular waxes amounted to an average of 18.4 g/1000 g, and cutin to 71.0 g/1000 g, corresponding to a summed mass of 88.4 g/1000 g. Cuticular waxes increased substantially during the following summer months to 28.5 g/1000 g in September, and maintained that proportion with some variations, for instance 22.8 g/1000 g in December and 32.8 g/1000 g in January (Table 1). The seasonal variation in the cuticular wax mass proportion in the cork oak leaves was statistically significant ($p = 0.004$), with the May value being significantly different from the September, January, and March values. As regards cutin mass proportion variation along the leaf cycle (Table 1), overall constant values were found with some small differences (e.g., from 71.2 g/1000 g to 63.5 g/1000 g, respectively, in December and January) that were not statistically significant ($p = 0.510$). Cutin is the major component of the cuticle in cork oak leaves, representing on average 72.4% of the cuticle across the leaf cycle (Table 1).

The cuticular wax coverage was lowest in the young leaves in May (113.5 µg/cm^2) and increased sharply to 235.2 µg/cm^2 in September, decreased to 191.3 µg/cm^2 in December, and increased subsequently with the highest value in March (281.2 µg/cm^2). The seasonal variation in the cuticular wax quantity deposited in the cuticle of the cork oak leaves was statistically significant ($p = 0.005$), with the May value being significantly different from the September and March values (Figure 2).

Cutin also showed the lowest coverage in the young leaves in May (429.7 µg/cm^2) and increased steadily in September and December when the highest value was attained (575.4 µg/cm^2), after which a small decrease was observed (Figure 2). The seasonal variation in the cutin deposited in the cuticle of the cork oak leaves was statistically significant ($p = 0.032$), with the January value being significantly different from the December and March values.

3.3. Cutin Chemical Compostion

The leaf cutin chemical composition grouped by major chemical families is shown in Figure 3 in proportion of the total solubilized monomers as determined by GC–MS. Overall, the major components of cutin are aliphatic long-chain compounds, mainly ω-hydroxyacids followed by fatty acids and a lower amount of α, ω-diacids and alcohols, accompanied by a substantial proportion of aromatics. Small amounts of cuticular waxes such as alkanes, terpenes, and sterols, representing on average 0.3%, 1.1%, and 1.4%, respectively, were found in the cutin extracts.

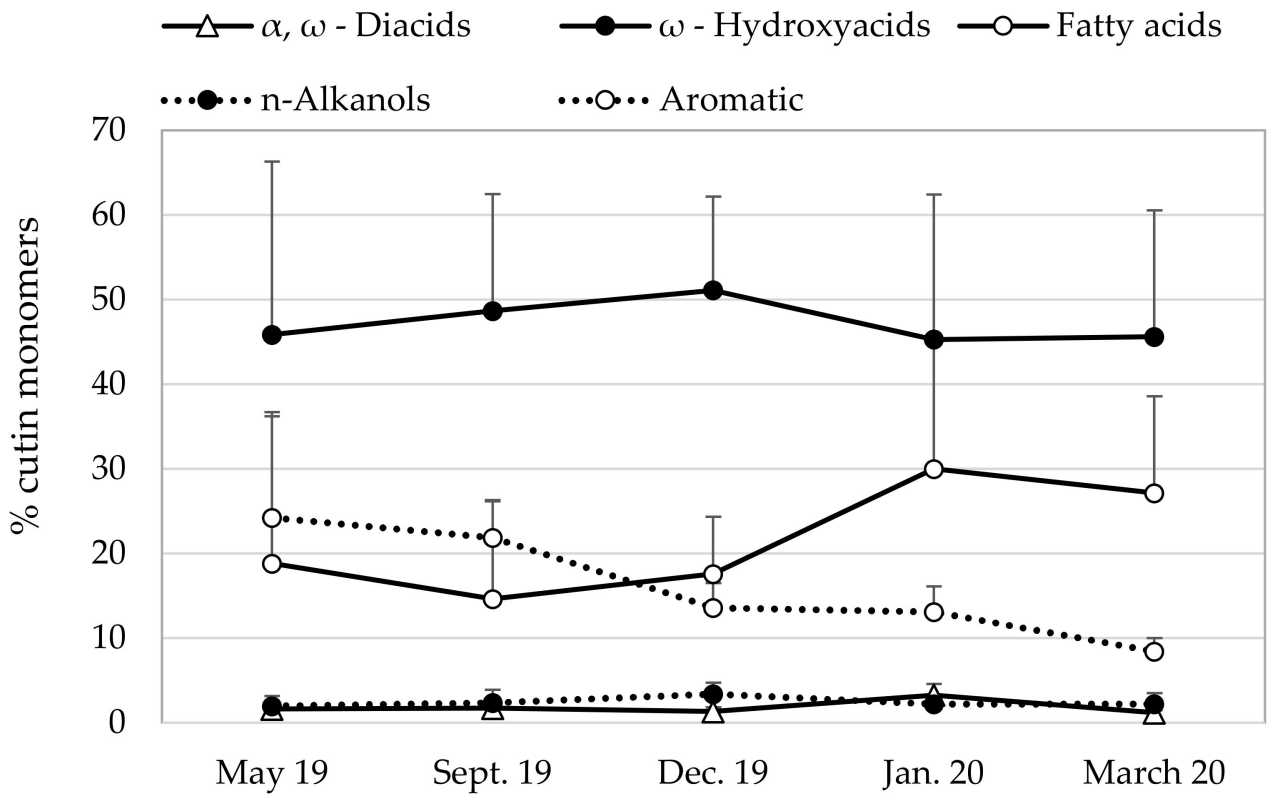

Figure 3. Seasonal variation in the cutin chemical families of *Quercus suber* leaves, in % of the total monomers released by transesterification of the extracted leaves (mean of six provenances with two trees per provenance and half standard deviation as bars).

Cutin showed a compositional variation along the leaf development cycle. In the new spring leaves in May, cutin was composed mainly of ω-hydroxyacids (45.8% of the total compounds), aromatics (24.2%), and carboxylic acids (16.5%), while α,ω-diacids represented only 1.6% and alkanols 2.0%. After the summer, the proportion of ω-hydroxyacids increased (48.4% in September and 50.8% in December) while the proportion of aromatics decreased significantly from September on (e.g., 21.0% in September, 13.6% in December, and 8.5% in March).

The complete chemical composition of cutin is summarized in Table 2, showing that the main monomers are 10,16-dihydroxyhexadecanoic and 9,10,18-trihydroxyoctadecanoic acid for the ω-hydroxyacids, hexadecanoic acid and 2-hydroxytetradecanoic acid for the fatty acids, and methyl *p*-coumarate for the aromatics. The proportion of 10,16-dihydroxyhexadecanoic (29.3% in May and 18.4% in March) and of the methyl-*p*-coumarate (23.0% and 6.3%, respectively) decreased along the leaf cycle.

Table 2. Seasonal variation in cutin composition in cork oak (*Quercus suber*) leaves (mean of six provenances with two trees per provenance), as determined by GC–MS, as a % of total peak area (only compounds with a proportion over 0.10% are included).

	May 2019	**September 2019**	**December 2019**	**January 2020**	**March 2020**
n-Alkanols					
Hexadecan-1-ol	0.24 ± 0.12	0.65 ± 0.48	1.00 ± 0.54	0.77 ± 0.45	0.84 ± 0.51
Octadecan-1-ol	0.21 ± 0.08	0.26 ± 0.13	0.15 ± 0.08	0.10 ± 0.03	0.16 ± 0.06
Eicosan-1-ol	0.17 ± 0.09	0.14 ± 0.09	0.14 ± 0.08	0.13 ± 0.05	0.22 ± 0.33
Docosan-1-ol	0.22 ± 0.10	0.16 ± 0.06	0.18 ± 0.04	0.15 ± 0.02	0.21 ± 0.06
Tetracosan-1-ol	0.81 ± 0.42	0.44 ± 0.23	1.17 ± 0.30	0.65 ± 0.36	0.39 ± 0.15
Hexacosan-1-ol	0.16 ± 0.21	0.40 ± 0.26	0.34 ± 0.12	0.17 ± 0.03	-
1,2-Dodecanediol	0.04 ± 0.07	0.25 ± 0.23	0.27 ± 0.09	0.12 ± 0.09	0.27 ± 0.11
Alkanes					
Heptacosane	0.22 ± 0.01	0.06 ± 0.05	0.06 ± 0.03	0.06 ± 0.01	0.07 ± 0.04
Nonacosane	0.08 ± 0.01	0.21 ± 0.16	0.08 ± 0.04	0.17 ± 0.17	0.39 ± 0.34
Triacontane	–	0.11 ± 0.06	0.06 ± 0.01	0.06 ± 0.02	0.08 ± 0.03
α, ω–Diacids					
Butanedioic acid	0.03 ± 0.02	0.24 ± 0.22	0.02 ± 0.01	0.67 ± 0.20	0.04 ± 0.01
Nonanedioic acid	0.58 ± 0.39	–	0.61 ± 0.25	–	0.74 ± 0.34
Decanedioic acid	1.12 ± 0.66	0.45 ± 0.33	0.13 ± 0.02	9.99 ± 5.40	6.50 ± 3.78
Hexadecanedioic acid	0.40 ± 0.16	0.55 ± 0.21	0.50 ± 0.12	0.31 ± 0.19	0.47 ± 0.11
Eicosanedioic acid	0.54 ± 0.45	0.37 ± 0.28	–	–	–
Octadecanedioic acid	0.08 ± 0.03	0.08 ± 0.02	0.13 ± 0.02	0.13 ± 0.06	0.15 ± 0.02
Tetracosanedioic acid	0.07 ± 0.03	0.11 ± 0.05	0.05 ± 0.02	0.07 ± 0.04	0.08 ± 0.05
Cis-4-Decene-1,10-dioic acid	0.02 ± 0.01	–	0.10 ± 0.05	–	0.04 ± 0.01
9-Octadecenedioic acid 1,18-dimethyl ester	0.14 ± 0.13	0.24 ± 0.12	0.15 ± 0.09	0.13 ± 0.06	0.10 ± 0.03
10,12-Docosadiynedioic acid	0.09 ± 0.04	–	0.04 ± 0.01	2.10 ± 1.77	–
9,10-Dihydroxyoctadecanedioic acid	0.31 ± 0.17	0.38 ± 0.16	0.43 ± 0.26	0.54 ± 0.61	0.39 ± 0.09
2-Hydroxydecanedioic acid	0.48 ± 0.43	–	0.17 ± 0.11	0.34 ± 0.34	0.30 ± 0.15
2-Hydroxy-3-isopropylsuccinic acid	0.13 ± 0.05	0.17 ± 0.11	0.07 ± 0.03	0.07 ± 0.03	0.11 ± 0.05
ω–Hydroxyacids					
16-Hydroxyhexadecanoic acid	1.24 ± 1.02	1.82 ± 1.22	2.66 ± 0.83	1.41 ± 0.92	2.20 ± 0.27
22-Hydroxydocosanoic acid	0.37 ± 0.29	0.45 ± 0.24	1.43 ± 0.79	2.87 ± 2.15	2.02 ± 1.05
9-Octadecenoic acid 18-hydroxy-methyl ester	1.15 ± 1.78	2.69 ± 1.82	2.08 ± 1.17	1.09 ± 0.69	0.50 ± 0.40
10,16-Dihydroxyhexadecanoic acid	29.28 ± 8.32	25.93 ± 4.15	26.47 ± 3.92	20.22 ± 3.22	18.39 ± 3.46
9,10-Epoxy-18-hydroxyoctadecanoic acid	2.30 ± 2.09	2.14 ± 1.91	6.51 ± 2.63	0.49 ± 0.25	8.96 ± 3.82
9,16-Dihydroxyhexadecanoic acid	0.49 ± 0.25	0.30 ± 0.16	0.43 ± 0.13	9.59 ± 5.63	1.41 ± 3.41
9,12-Octadecadienoic acid 18-hydroxy- methyl ester	0.60 ± 0.61	0.30 ± 0.26	0.24 ± 0.10	0.18 ± 0.11	0.50 ± 0.32
9,10,18 Trihydroxyoctadecanoic acid	10.37 ± 6.08	14.97 ± 4.07	11.23 ± 1.49	9.41 ± 4.15	11.57 ± 2.22
Carboxylic acids					
Ethanoic acid	0.55 ± 0.42	–	0.13 ± 0.11	0.37 ± 0.22	0.23 ± 0.12
Octanoic acid	2.34 ± 3.17	0.51 ± 0.69	0.52 ± 0.72	0.47 ± 0.90	0.84 ± 0.78
Nonanoic acid	0.18 ± 0.09	0.06 ± 0.11	0.17 ± 0.05	0.10 ± 0.11	0.26 ± 0.09
Dodecanoic acid	0.20 ± 0.27	0.07 ± 0.02	0.08 ± 0.06	0.12 ± 0.10	0.08 ± 0.05
Tetradecanoic acid	0.16 ± 0.19	0.13 ± 0.21	0.30 ± 0.10	0.23 ± 0.21	0.42 ± 0.10
Hexadecanoic acid	3.24 ± 2.52	2.68 ± 1.38	5.49 ± 0.98	5.86 ± 1.74	7.25 ± 1.32
Octadecanoic acid	0.31 ± 0.20	0.43 ± 0.30	0.34 ± 0.10	0.26 ± 0.19	0.41 ± 0.20
Eicosanoic acid	0.21 ± 0.10	0.32 ± 0.20	0.29 ± 0.08	0.22 ± 0.14	0.24 ± 0.10
Docosanoic acid	0.49 ± 0.29	0.31 ± 0.26	0.23 ± 0.15	0.29 ± 0.11	–
Tetracosanoic acid	1.22 ± 1.01	0.38 ± 0.10	0.26 ± 0.07	0.50 ± 0.15	0.69 ± 0.36
Octacosanoic acid	0.07 ± 0.04	0.17 ± 0.17	0.10 ± 0.04	0.13 ± 0.14	0.11 ± 0.04
Triacontanoic acid	0.16 ± 0.13	0.29 ± 0.28	0.23 ± 0.07	0.20 ± 0.19	0.31 ± 0.12
7-Hexadecenoic acid (Z)-	–	0.03 ± 0.01	0.06 ± 0.02	–	0.12 ± 0.15
9-Hexadecenoic acid (Z)-	–	0.14 ± 0.19	0.03 ± 0.01	0.07 ± 0.01	0.17 ± 0.44
9,12,15-Octadecatrienoic acid (Z,Z,Z)-	0.42 ± 0.85	0.85 ± 0.71	0.39 ± 0.28	1.36 ± 1.33	0.30 ± 0.27
9,12-Octadecadienoic acid (Z,Z)-	0.38 ± 0.62	2.09 ± 2.28	–	0.40 ± 0.20	0.10 ± 0.08
9-Octadecenoic acid (Z)-	0.20 ± 0.21	1.35 ± 1.81	1.39 ± 1.01	1.35 ± 0.73	0.09 ± 0.08
3,6,9-Octadecatrienoic acid	0.30 ± 0.38	0.23 ± 0.12	–	0.15 ± 0.04	0.11 ± 0.02
13-Octadecenoic acid	–	–	0.15 ± 0.07	0.12 ± 0.06	0.17 ± 0.06
5,8,11-Eicosatriynoic acid	1.96 ± 2.66	0.20 ± 0.18	0.83 ± 0.47	1.34 ± 0.95	1.51 ± 0.46

Table 2. *Cont.*

	May 2019	September 2019	December 2019	January 2020	March 2020
2-Oxobutanoic acid	0.11 ± 0.04	0.13 ± 0.11	0.05 ± 0.03	0.07 ± 0.03	0.04 ± 0.02
9-Oxononanoic acid	0.13 ± 0.07	0.14 ± 0.02	0.10 ± 0.04	0.13 ± 0.07	0.24 ± 0.08
Methyl 10-oxo-8-decenoate	0.07 ± 0.04	0.30 ± 0.12	0.06 ± 0.04	0.15 ± 0.08	0.36 ± 0.15
12-Methyl tetradecanoic acid	0.08 ± 0.04	0.07 ± 0.02	0.08 ± 0.08	0.06 ± 0.01	0.13 ± 0.05
14-Methyl hexadecanoic acid	0.08 ± 0.02	0.09 ± 0.03	0.15 ± 0.06	0.11 ± 0.04	0.14 ± 0.04
7-Hydroxyoctanoic acid	0.22 ± 0.15	0.19 ± 0.11	0.27 ± 0.12	0.20 ± 0.09	0.28 ± 0.09
21-Methyl docosanoic acid	0.11 ± 0.04	0.07 ± 0.07	0.14 ± 0.01	0.16 ± 0.04	0.19 ± 0.07
2-Hydroxytetradecanoic acid	2.87 ± 2.07	2.50 ± 1.23	4.15 ± 1.03	4.16 ± 1.48	4.07 ± 1.24
2-Hydroxypentanoic acid	–	–	0.30 ± 0.41	0.18 ± 0.06	0.35 ± 0.40
Aromatic compounds					
Methyl *p*-coumarate	22.99 ± 12.0	19.58 ± 4.07	11.35 ± 2.01	11.79 ± 2.61	6.29 ± 1.23
Other aromatic compounds	1.09 ± 0.42	2.14 ± 0.36	2.04 ± 0.84	1.24 ± 0.36	2.14 ± 0.36
Glycerol + derivatives					
Ethylene glycol	0.13 ± 0.10	0.21 ± 0.16	0.04 ± 0.05	0.12 ± 0.10	0.07 ± 0.05
Glycerol	0.02 ± 0.01	–	0.03 ± 0.02	0.05 ± 0.02	0.10 ± 0.06
Oleoylglycerol	0.20 ± 0.17	0.22 ± 0.18	0.44 ± 0.16	0.32 ± 0.18	0.18 ± 0.06
Monopalmitin	–	0.26 ± 0.34	–	–	–
9,12-Octadecadienoic acid (Z,Z)-2,3-dihydroxypropyl ester	0.22 ± 0.10	0.29 ± 0.21	0.30 ± 0.18	0.25 ± 0.10	0.37 ± 0.14
Terpernes					
Phytol	–	0.10 ± 0.10	0.11 ± 0.05	0.11 ± 0.04	–
β-Amyrin	0.24 ± 0.16	0.35 ± 0.20	0.26 ± 0.16	0.35 ± 0.27	0.26 ± 0.09
α-Amyrin	0.16 ± 0.12	0.10 ± 0.10	0.22 ± 0.14	0.10 ± 0.06	0.17 ± 0.15
Germanicol	0.15 ± 0.17	0.13 ± 0.07	0.14 ± 0.14	0.27 ± 0.26	0.16 ± 0.13
Lupeol	–	0.33 ± 0.10	0.25 ± 0.18	0.14 ± 0.10	0.24 ± 0.20
D:A-Friedooleanan-7-ol (7α)-	0.07 ± 0.02	0.36 ± 0.39	0.09 ± 0.03	–	0.08 ± 0.04
Friedelan-3-one	–	0.19 ± 0.10	0.05 ± 0.02	0.07 ± 0.04	0.09 ± 0.09
Betulin	0.11 ± 0.11	0.08 ± 0.01	–	–	0.04 ± 0.02
Steroids					
Epimethendiol	0.07 ± 0.03	0.11 ± 0.03	0.14 ± 0.14	0.12 ± 0.06	0.11 ± 0.13
12α-Hydroxy-5α-pregnane-dimethyl(isopropyl)silyl ether	0.74 ± 0.45	1.01 ± 0.43	1.29 ± 0.24	0.88 ± 0.21	1.08 ± 0.29
β-Sistosterol	–	0.46 ± 0.36	0.32 ± 0.24	0.14 ± 0.10	0.27 ± 0.13
Vitamin E					
α-Tocospiro A	0.14 ± 0.18	0.13 ± 0.10	0.10 ± 0.05	0.28 ± 0.28	0.39 ± 0.73
α-Tocospiro B	0.10 ± 0.02	0.11 ± 0.05	0.11 ± 0.05	0.11 ± 0.04	0.11 ± 0.04
Other compounds					
2-Pentadecanone 6,10,14-trimethyl-	–	0.18 ± 0.12	0.10 ± 0.04	0.12 ± 0.05	0.14 ± 0.09
D-Pinitol	0.04 ± 0.01	–	0.08 ± 0.04	0.05 ± 0.05	0.10 ± 0.11
Sitosteryl-3β-D-glucopiranoside	0.08 ± 0.01	0.17 ± 0.11	–	0.021 ± 0.002	0.04 ± 0.01

4. Discussion

The results reported here are the first to be published on the variation in cutin content and chemical composition in the cuticle along the development of *Quercus suber* leaves in association with cuticular wax deposition. Our group recently published data that revealed a significant seasonal variation in leaf area, specific leaf area, and content and composition of cuticular wax in cork oak leaves [25].

The leaf developmental pattern of increasing area during the late spring and early summer to a maximum in the fully expanded leaves in September, followed by a rather unchanged leaf area throughout the winter [22,29,30], was confirmed in the present study that showed an average constant leaf area from May to March. The young leaves in May were already substantially expanded, with only a small leaf area increase to the maximal area of the fully developed leaves in September (Figure 1). Leaf maturation in the context of leaf mass development as measured by the specific leaf area (SLA) was highest in the young leaves in May (Figure 1), showing that in this period leaves expanded quickly and the tree invested less in dry matter per leaf [31], while after the summer, leaf mass increased by retention of photosynthetized compounds, and therefore SLA declined since leaf area remained particularly stable in the fully expanded leaves. Such leaf structure modifications

facilitate an adaptation to droughts [32,33]. The present results of the seasonal variation in cork oak leaf morphological traits along the life cycle closely accord with previous reports [24,34]. Nevertheless, further studies should be carried out with a more extensive sampling that may elucidate aspects of leaf structural variability within the canopy, since the present sampling was carried out in the lower canopy, and in trees grown under different edapho-climatic conditions.

A significant seasonal variation in the cuticular wax quantity by unit area of the leaf surface has been previously reported [25]. This was confirmed here (Figure 2) with the lowest cuticular wax leaf coverage in May, in the newly developed leaves, but that significantly increased subsequently. This suggests that in the spring, with overall mild temperatures and water availability, priority was given to leaf growth in relation to wax lipids biosynthesis, since leaves did not yet require special protection. However, in the dry summer months, from May to September, biosynthesis of leaf wax lipids increased significantly by a factor of 1.6 (Table 1), allowing for transpiration protection. Reports on cuticular waxes variation along the development of *Q. suber* leaves are limited to the present results and to the previously reported data from our group [25]. Information is also scarce for other species, but the few available studies also confirm that cuticular wax content is lower in the initial development stage than in mature leaves after their full expansion, e.g., in *Quercus robur* [35], *Fagus sylvatica* [36], *Hedera helix* [37], and *Actinidia deliciosa* [38]. Increasing wax accumulation and building up of a continuous outer leaf coverage are one strategy to cope with drought conditions [39,40], as a protective mechanism to decease water loss through the cuticular layer [31,35,36,41]. The need to have a continuous outer leaf coverage is shown by the regeneration of a wax layer whenever the original epicuticular wax layer was removed from leaves of various species [42,43].

Cutin biosynthesis and accumulation on the outer surface of the leaf epidermis was always higher than that of the cuticular waxes (Table 1). While the mass proportion of cutin in the leaves was constant along the leaf cycle, which may indicate a similar photosynthate allocation to the cutin monomers, in terms of mass surface coverage, there was an increase from spring onwards until December (Figure 2), suggesting a structural evolution of the leaves with a higher increase in thickness than in surface expansion. Detailed anatomical studies on cork oak leaf development are needed to clarify these dynamics.

As regards cutin chemical composition, the present results clearly show a compositional variation between the young and the mature leaves regarding the proportion of each chemical family (Table 2). One striking aspect is the fact that methyl coumarates are important cutin components in the young leaves, with a proportion of 23.0% of the total monomers, but decreased substantially afterwards to 6.3% in March (Table 2). It is argued that methyl coumarates are bridging to the polysaccharide layer on the epidermal cell walls [44], thereby anchoring the cutin macromolecule on the epidermis and overall strengthening the leaf structure. This process is therefore established in the first leaf development stage and out-phased subsequently. The strong links to the polysaccharide layer on the epidermal cells through esterification may be one of the reasons that justify the difficulty in isolating the cuticle from cork oak leaves using the applied enzymatic treatments, as discussed in Simões et al. [24]. With an opposite trend, the proportion of alkanoic acids increased in the later stages of the leaf cycle (Figure 3).

Cork oak leaf cutin composition is reported only in one previous publication referring to one single sampling made in March before leaf fall, and its results match those obtained now for the same sampling time of March (Table 2), respectively, ω-hydroxyacids 44.4% and 45.5%, fatty acids 20.7% and 29.9%, α, ω-diacids 6.5% and 2.9%, and aromatics 12.8% and 8.5% [24]. The findings reported here are the first ones reported on the cutin compositional variation along the leaf cycle, showing that the cork oak leaf cuticle has seasonal dynamics in its cutin and cuticular waxes components with most differences occurring between the young leaves from May and the fully expanded and mature leaves after summer.

5. Conclusions

The dynamics of cutin proportion and composition in the cuticle of cork oak leaves were characterized for the first time along one annual development cycle. Cutin builds up over two thirds of the sum of the cuticle cutin and waxes, and is an important strengthening component that accompanies the sclerophyllous development of the leaf in the dry summer months after the major spring surface expansion. There was a seasonal variation in cutin monomeric moieties, and methyl coumarates were important components during the early leaf development, in accordance with their potential bridging role onto the epidermal polysaccharide cell wall layer. The cuticular waxes also accompanied the deposition pattern associated to maximize protection against dehydration in the summer months of the Mediterranean climate.

Author Contributions: Conceptualization, H.P. and I.M.; methodology, I.M. and R.S.; validation, H.P., I.M. and R.S.; experimental investigation, R.S.; data curation, R.S., H.P. and I.M.; writing—original draft preparation, I.M. and R.S.; writing—review and editing, H.P. and I.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fundação para a Ciência e a Tecnologia (FCT) through funding of the Forest Research Center (UIDB/00239/2020). Funding for this work was also provided by a doctoral scholarship from FCT SUSFOR Doctoral Program (PD/BD/128259/2016).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data are available upon request to the corresponding author.

Acknowledgments: Rita Simões acknowledges a doctoral scholarship from FCT with the SUSFOR Doctoral Program (PD/BD/128259/2016). The cork oak provenance field trials were funded by the European Commission (FAIR1-CT-95-0202) and national programs (PBIC/AGR/2282/95, PAMAF 4027, PRAXIS/3/3.2/Flor/2110/95). We thank Ana Rodrigues for her technical assistance in field sampling.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Kerstiens, G. Cuticular water permeability and its physiological significance. *J. Exp. Bot.* **1996**, *47*, 1813–1832. [CrossRef]
2. Krauss, P.; Markstadter, C.; Riederer, M. Attenuation of UV radiation by plant cuticles from woody species. *Plant Cell Environ.* **1997**, *20*, 1079–1085. [CrossRef]
3. Buschhaus, C.; Jetter, R. Composition differences between epicuticular and intracuticular wax substructures: How do plants seal their epidermal surfaces? *J. Exp. Bot.* **2011**, *62*, 841–853. [CrossRef] [PubMed]
4. Jeffree, C.E. The fine structure of the plant cuticle. In *Biology of the Plant Cuticle*; Riederer, M., Müller, C., Eds.; Blackwell: Oxford, UK, 2007; pp. 11–125. [CrossRef]
5. Samuels, L.; Kunst, L.; Jetter, R. Sealing plant surfaces: Cuticular wax formation by epidermal cells. *Annu. Rev. Plant Biol.* **2008**, *59*, 683–707. [CrossRef]
6. Yeats, T.H.; Rose, J.K. The formation and function of plant cuticles. *Plant Physiol.* **2013**, *163*, 5–20. [CrossRef]
7. Guzmán-Delgado, P.; Graça, J.; Cabral, V.; Gil, L.; Fernández, V. The presence of cutan limits the interpretation of cuticular chemistry and structure: *Ficus elastica* leaf as an example. *Physiol. Plant.* **2016**, *157*, 205–220. [CrossRef]
8. Leide, J.; Nierop, K.G.J.; Deininger, A.C.; Staiger, S.; Riederer, M.; de Leeuw, J.E. Leaf cuticle analyses: Implications for the existence of cutan/non-ester cutin and its biosynthetic origin. *Ann. Bot.* **2020**, *126*, 141–162. [CrossRef]
9. Zeisler-Diehl, V.; Müller, Y.; Schreiber, L. Epicuticular wax on leaf cuticles does not establish the transpiration barrier, which is essentially formed by intracuticular wax. *J. Plant Physiol.* **2018**, *227*, 66–74. [CrossRef]
10. Buschhaus, C.; Jetter, R. Composition and physiological function of the wax layers coating Arabidopsis leaves: β-amyrin negatively affects the intracuticular water barrier. *Plant Physiol.* **2012**, *160*, 1120–1129. [CrossRef]
11. Jetter, R.; Riederer, M. Localization of the transpiration barrier in the epi- and intracuticular waxes of eight plant species: Water transport tesistances are associated with fatty acyl rather than alicyclic componentes. *Plant Physiol.* **2016**, *170*, 921–934. [CrossRef]
12. Seufert, P.; Staiger, S.; Arand, K.; Bueno, A.; Burghardt, M.; Riederer, M. Building a barrier: The influence of different wax fractions on the water transpiration barrier of leaf cuticles. *Front. Plant Sci.* **2022**, *12*, 766602. [CrossRef]
13. Schuster, A.-C.; Burghardt, M.; Alfarhan, A.; Bueno, A.; Hedrich, R.; Leide, J.; Thomas, J.; Riederer, M. Effectiveness of cuticular transpiration barriers in a desert plant at controlling water loss at high temperatures. *AoB Plants* **2016**, *8*, plw027. [CrossRef]

14. Tsubaki, S.; Sugimura, K.; Teramoto, Y.; Yonemori, K.; and Azuma, J.I. Cuticular membrane of Fuyu persimmon fruit is strengthened by triterpenoid nano-fillers. *PLoS ONE* **2013**, *8*, 75275. [CrossRef]
15. Fich, E.A.; Segerson, N.A.; Rose, J.K. The plant polyester cutin: Biosynthesis, structure, and biological roles. *Annu. Rev. Plant Biol.* **2016**, *67*, 207–233. [CrossRef]
16. Li-Beisson, Y.; Verdier, G.; Xu, L.; Beisson, F. Cutin and suberin polyesters. In *Essential for Life Science (eLS)*; John Wiley & Sons, Ltd.: Chichester, UK, 2016; pp. 1–12. [CrossRef]
17. Pereira, H. *Cork: Biology, Production and Uses*; Elsevier: Amsterdam, The Netherlands, 2007.
18. IPCC. *Climate Change 2022: Impacts, Adaptation, and Vulnerability. Contribution of Working Group II to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*; Pörtner, H.-O., Roberts, D.C., Tignor, M., Poloczanska, E.S., Mintenbeck, K., Alegría, A., Craig, M., Langsdorf, S., Löschke, S., Möller, V., et al., Eds.; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2022; p. 3056. [CrossRef]
19. Escudero, A.; Del Arco, J.M.; Sanz, I.C.; Ayala, J. Effects of leaf longevity and retranslocation efficiency on the retention time of nutrients in the leaf biomass of different woody species. *Oecologia* **1992**, *90*, 80–87. [CrossRef]
20. Fialho, C.; Lopes, F.; Perreira, H. The effect of cork removal on the radial growth and phenology of young cork oak trees. *For. Ecol. Manag.* **2001**, *141*, 251–258. [CrossRef]
21. Oliveira, G.; Correia, O.; Martins-Loução, M.A.; Catarino, F. Phenological and growth patterns of the Mediterranean oak *Quercus suber* L. *Trees* **1994**, *9*, 41–46. [CrossRef]
22. Pereira, J.S.; Beyschlag, G.; Lange, O.L.; Beyschlag, W.; Tenhunen, J.D. Comparative phenology of four mediterranean shrub species growing in Portugal. In *Plant Response to Stress*; Tenhunen, J.D., Catarino, F.M., Lange, O.L., Oechel, W.C., Eds.; Springer: Berlin/Heidelberg, Germany, 1987; pp. 503–514.
23. Simões, R.; Rodrigues, A.; Ferreira-Dias, S.; Miranda, I.; Pereira, H. Chemical composition of cuticular waxes, pigments and morphology of leaves of *Quercus suber* trees from different provenances. *Plants* **2020**, *9*, 1165. [CrossRef]
24. Simões, R.; Miranda, I.; Pereira, H. Chemical composition of leaf cutin in six *Quercus suber* provenances. *Phytochemistry* **2021**, *181*, 112570. [CrossRef]
25. Simões, R.; Miranda, I.; Pereira, H. Effect of seasonal variation on leaf cuticular waxes' composition in the Mediterranean cork oak (*Quercus suber* L.). *Forests* **2022**, *13*, 1236. [CrossRef]
26. Sampaio, T.; Gonçalves, E.; Patrício, M.S.; Cota, T.M.; Almeida, M.H. Seed origin drives differences in survival and growth traits of cork oak (*Quercus suber* L.) populations. *For. Ecol. Manag.* **2019**, *448*, 267–277. [CrossRef]
27. Varela, M.C. *European Network for the Evaluation of Genetic Resources of Cork Oak for Appropriate Use in Breeding and Gene Conservation Strategies*; INIA: Lisbon, Portugal, 2020.
28. Pereira, H. Chemical composition and variability of cork from *Quercus suber* L. *Wood Sci. Technol.* **1988**, *22*, 211–218. [CrossRef]
29. Grant, O.M.; Tronina, L.; Ramalho, J.C.; Besson, C.K.; Lobo-Do-Vale, R.; Pereira, J.S.; Jones, H.G.; Chaves, M.M. The impact of drought on leaf physiology of *Quercus suber* L. trees: Comparison of an extreme drought event with chronic rainfall reduction. *J. Exp. Bot.* **2010**, *61*, 4361–4371. [CrossRef]
30. Prats, K.A.; Brodersen, C.R.; Ashton, M.S. Influence of dry season on *Quercus suber* L. leaf traits in the Iberian Peninsula. *Am. J. Bot.* **2019**, *106*, 656–666. [CrossRef]
31. Dwyer, J.M.; Hobbs, R.J.; Mayfield, M.M. Specific leaf area responses to environmental gradients through space and time. *Ecology* **2014**, *95*, 399–410. [CrossRef]
32. Aranda, I.; Pardos, M.; Puértolas, J.; Jiménez, M.D.; Pardos, J.A. Water-use efficiency in cork oak (*Quercus suber*) is modified by the interaction of water and light availabilities. *Tree Physiol.* **2007**, *27*, 671–677. [CrossRef]
33. Gouveia, A.C.; Freitas, H. Modulation of leaf attributes and water use efficiency in *Quercus suber* along a rainfall gradient. *Trees* **2009**, *23*, 267–275. [CrossRef]
34. Passarinho, J.A.P.; Lamosa, P.; Baeta, J.P.; Santos, H.; Ricardo, C.P.P. Annual changes in the concentration of minerals and organic compounds of Quercus suber leaves. *Physiol. Plant* **2006**, *127*, 100–110. [CrossRef]
35. Gülz, P.-G.; Müller, E. Seasonal variation in the composition of epicuticular waxes of *Quercus robur* leaves. *Z. Naturforsch. C.* **1992**, *47*, 800–806. [CrossRef]
36. Prasad, R.B.N.; Gülz, P.G. Surface structure and chemical composition of leaf waxes from *Quercus robur* L., *Acer pseudoplatanus* L. and *JugIans regia* L. *Z. Naturforsch. C.* **1990**, *45*, 813–817. [CrossRef]
37. Hauke, V.; Schreiber, L. Ontogenetic and seasonal development of wax composition and cuticular transpiration of ivy (*Hedera helix* L.) sun and shade leaves. *Planta* **1998**, *207*, 67–75. [CrossRef]
38. Celano, G.; D'Auria, M.; Xiloyannis, C.; Mauriello, G.; Baldassarre, M. Composition and seasonal variation of soluble cuticular waxes in *Actinidia deliciosa* leaves. *Nat. Prod. Res.* **2006**, *20*, 701–709. [CrossRef] [PubMed]
39. Zhang, J.Y.; Broeckling, C.D.; Blancaflor, E.B.; Sledge, M.K.; Sumner, L.W.; Wang, Z.Y. Overexpression of WXP1, a putative *Medicago truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (*Medicago sativa*). *Plant J.* **2005**, *42*, 689–707. [CrossRef] [PubMed]
40. Cameron, K.D.; Teece, M.A.; Smart, L.B. Increased accumu- lation of cuticular wax and expression of lipid transfer protein in response to periodic drying events in leaves of tree tobacco. *Plant Physiol.* **2006**, *140*, 176–183. [CrossRef]

41. Fang, Y.; Xiong, L. General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell. Mol. Life Sci.* **2015**, *72*, 673–689. [CrossRef]
42. Jetter, R.; Schäffer, S. Chemical composition of the Prunus laurocerasus leaf surface. dynamic changes of the epicuticular wax film during leaf development. *Plant Physiol.* **2001**, *126*, 1725–1737. [CrossRef]
43. Neinhuis, C.; Koch, K.; Barthlott, W. Movement and regeneration of epicuticlar waxes through plant cuticles. *Planta* **2001**, *213*, 427–434. [CrossRef]
44. Riley, R.G.; Kolattukudy, P.E. Evidence for covalently attached *p*-voumaric acid and ferulic acid in cutins and suberins. *Plant Physiol.* **1975**, *56*, 650–654. [CrossRef]