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Effect of Seasonal Variation on Leaf Cuticular Waxes' Composition in the Mediterranean Cork Oak (*Quercus suber* L.)

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Abstract: *Quercus suber* L. (cork oak) leaves were analyzed along one annual cycle for cuticular wax content and chemical composition. This species, well adapted to the long dry summer conditions prevailing in the Mediterranean, has a leaf life span of about one year. The cuticular wax revealed a seasonal variation with a coverage increase from the newly expanded leaves (115.7 μg/cm^2 in spring) to a maximum value in fully expanded leaves (235.6 μg/cm^2 after summer). Triterpenoids dominated the wax composition throughout the leaf life cycle, corresponding in young leaves to 26 μg/cm^2 (22.6% of the total wax) and 116.0 μg/cm^2 (49% of the total wax) in mature leaves, with lupeol constituting about 70% of this fraction. The total aliphatic compounds increased from 39 μg/cm^2 (young leaves) to 71 μg/cm^2 (mature leaves) and then decreased to 22 μg/cm^2 and slightly increased during the remaining period. The major aliphatic compounds were fatty acids, mostly with C_{16} (hexadecanoic acid) and C_{28} (octacosanoic acid) chain lengths. Since pentacyclic triterpenoids are located almost exclusively within the cutin matrix (intracuticular wax), the increase in the cyclic-to-acyclic component ratio after summer shows an extensive deposition of intracuticular waxes in association with the establishment of mechanical and thermal stability and of water barrier properties in the mature leaf cuticle.

Keywords: *Quercus suber* L.; seasonal variation; cuticular waxes; leaves

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1. Introduction

The plant cuticle is a continuous extracellular hydrophobic membrane that forms a primary barrier between the plant's air-exposed surfaces and the external environment and also plays important functions in organ growth and development. Particularly in leaves, the cuticle plays a key role in limiting uncontrolled water loss and provides protection from biotic or abiotic stresses such as drought and high temperatures.

The cuticle is a heterogeneous membrane consisting of a matrix of polymeric cutin and cuticular waxes [1–3]. Cutin is a polyester formed by C_{16} and C_{18} hydroxy fatty acids and their derivatives and glycerol. Cuticular waxes predominantly comprise very long-chain fatty acids and their derivatives (including aldehydes, primary alcohols, and alkanes), as well as, in some species, cyclic molecules especially pentacyclic triterpenoids, sterols, and aromatics (e.g., *Ficus elastica* Roxb.) [1–3]. Cuticular waxes are distinguished according to their location in an intracuticular layer within the cutin matrix and an epicuticular layer deposited on the outer surface of the cutin [2].

The functional properties of the cuticle are largely related to the structural arrangement of the cuticular waxes' layers and their chemical compositions [1–3]. The limitation of the non-stomatal water loss is one of the most important features and has therefore been analyzed in terms of both the role of intracuticular and epicuticular waxes and the role of the different cuticular wax components. The functional barrier against water diffusion through the cuticle is preferentially established by the intracuticular wax, while the epicuticular wax does not contribute to the transpiration barrier [4]. Regarding the contribution of the

chemical wax components, it was shown that it is the very long-chain aliphatic fraction of the wax that establishes the transpiration barrier [1,2,5], whereas the triterpenoids provide mechanical and thermal stability to the plant cuticle [6,7]. For instance, the triterpenoids deposited within the cutin matrix restrict the polymer's thermal expansion and thus prevent thermal damage to the highly ordered aliphatic wax barrier, even at high temperatures [7].

The cork oak (*Quercus suber* L.) is one of the most important evergreen species in the western Mediterranean Basin, with important economic value because of the production of cork that feeds a dedicated industrial chain [8]. This species is well-adapted to the adverse conditions of the Mediterranean, that is, to the long, dry summer conditions with high solar irradiances, air temperatures and vapor pressure deficit, and little or no precipitation, as well as moderately cold winters. The leaves are sclerophilic, oval in shape with a dark green color on the adaxial face and without epidermal hairs (trichomes), and the abaxial face is lighter with numerous stomata and densely covered with trichomes in the form of starry multicellular hairs [8,9]. The leaf's cuticular membrane contains substantial amounts of cuticular wax (154.3–235.1 μg/cm^2) composed largely of pentacyclic triterpenoid compounds (61%–72% of the identified compounds, with lupeol as the main component), while long-chain aliphatic components are mainly fatty acids (mainly in C_{30}, C_{28}, and C_{16}) (17%–23% of the identified compounds) that contribute to building a nearly impermeable membrane [8]. Cutin is present in high amounts (518 μg/cm^2 of leaf area) and has as major monomeric constituents 10,16-dihydroxyhexadecanoic and 9,10,18-trihydroxyoctadecanoic acids [10].

Cork oak has a specific foliage phenology, characterized by short-lived leaves that usually fall within one year concurrently with spring growth, with a cycle that is much shorter than that of other evergreen oaks, such as the Iberian holm oak (*Quercus rotundifolia* Lam.= *Q. ilex* L. subsp. *ballota*), whose leaves last 1–3 years, or the kermes oak (*Q. coccifera* L.), whose leaves can last 5–6 years [11–13]. In *Q. suber*, physiological activity starts in February/March with bud development and shoot growth, and the development of new foliage begins in April and is terminated by June. Most leaves emerge and expand within 1 month. The leaf life duration is approximately 14 months, with a range of 11–18 months [12,14,15]. Most of the older leaves (1-year-old) fall in spring during the early part of shoot growth [11].

The present work addresses the study of the seasonal variation of the chemical composition of cuticular waxes in cork oak (*Quercus suber* L.) leaves in the Mediterranean climate. It is hypothesized that the content and composition of the leaf cuticular waxes depend on the seasonally related leaf development stage and on the prevailing climatic variables. The results aim at understanding the role of cuticular wax components of sclerophilic leaves and their protective role regarding biotic and abiotic stresses in association with leaf development, thereby contributing to climate change adaptation measures.

2. Material and Methods

2.1. Sampling

The study was carried out on a provenance trial of 21-year-old *Quercus suber* L. trees at Herdade Monte Fava, Santiago do Cacém, in central Portugal (38°00′ N, 08°70′ W, altitude 79 m). This trial was established with seedlings of cork oak trees raised from seeds of different provenances: Portugal (PT35), Spain (ES11), Italy (IT13), France (FR3), Morocco (MA27), and Tunisia (TU32). A more detailed trial and site description is given in Sampaio et al. [16] and Varela [17]. The sampling included the collection of leaves from two trees from each of the six provenances. The first sampling was in May 2019 on fully expanded leaves from the current year's spring flushing followed by samplings, in the same trees, in September 2019, December 2019, January 2020, and March 2020. The leaves were collected randomly from different branches on the south-exposed crown side, in the lower part of the canopy up to a height of approximately 2 m, making up a total sample per tree of about 100 leaves. A composite leaf sample per provenance was prepared with the leaves of the two trees.

2.2. Morphological Variables

The morphological variables leaf area and specific leaf area were measured in 40 leaves that were randomly selected from the leaves sampled for each provenance. Leaf area was measured by digitalizing and calculated with Leica Qwin vs. 3.0 Image Analysis Software. The leaves were oven-dried at 70 °C until no change in mass was detected, and the total dry mass per leaf was determined. Specific leaf area (SLA, cm^2/g) was calculated as the ratio between the measured leaf area and the dry weight and used to describe the sclerophyllous nature of leaves.

2.3. Extraction of Cuticular Waxes

Cuticular wax was extracted from whole fresh leaves with dichloromethane over 6 h in a Soxhlet apparatus [9]. This extraction method yields a solution containing both the epicuticular and intracuticular waxes from both leaf sides. The amount of the soluble cuticular compounds was determined from the mass difference of the extracted leaves after drying at 105 °C and was expressed on a leaf surface area and dry weight basis (the ratio between wax in μg and the two-sided leaf surface area in cm^2, obtained by digitalization) [9].

2.4. Cuticular Wax Composition

The cuticular wax, obtained as dichloromethane extracts, was analyzed using gas chromatography–mass spectrometry (GC-MS). Two milligrams of each leaf extract was taken and derivatized in 120 μL of pyridine; the compounds with hydroxyl and carboxyl groups were trimethylsilylated into trimethylsilyl (TMS) ethers and esters, respectively, by adding 80 μL of bis(trimethylsily)-trifluoroacetamide (BSTFA) and reacting at 60 °C for 30 min. The derivatized extracts (1 μL) were immediately analyzed by GC-MS (EMIS, Agilent 5973 MSD, Palo Alto, CA, USA). The detailed experimental conditions are given elsewhere [9]. The compounds were identified as TMS derivatives by matching with GC-MS spectral libraries (Wiley, NIST) and published fragmentation profiles [18,19]. Two replicates were made per extract.

2.5. Statistical Analysis

Data are presented as the means ± standard deviation of the six independent provenances' samples analyzed in duplicate. To compare data along the leaf cycle, one-way analysis of variance (ANOVA) was performed. Duncan's post hoc tests were used to analyze pairwise differences between provenances. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the Sigmaplot® (Version 11.0, Systat Software, Inc., Chicago, IL, USA).

3. Results

The seasonal changes in *Q. suber* leaves regarding leaf size, specific leaf area, and cuticular wax content and composition were followed throughout the first year, beginning with the newly expanded leaves in late spring (May) and ending with one-year-old leaves in the early spring (March) of the following year.

3.1. Leaf Area and SLA

In May, the newly developed leaves had a leaf size of 5.8 ± 1.7 cm^2, which increased through summer to a maximum size of 6.5 ± 1.4 cm^2 in September (corresponding to the fully expanded mature leaves) that remained unchanged throughout the winter months (6.5 ± 2.3 cm^2 in December and 6.5 ± 1.4 cm^2 in January). In March, when the 1-year-old leaves began to fall, the average leaf size decreased to 5.7 ± 1.7 cm^2 (Figure 1). Although differences in leaf size occurred during the study period, they were not statistically significant ($p = 0.449$).

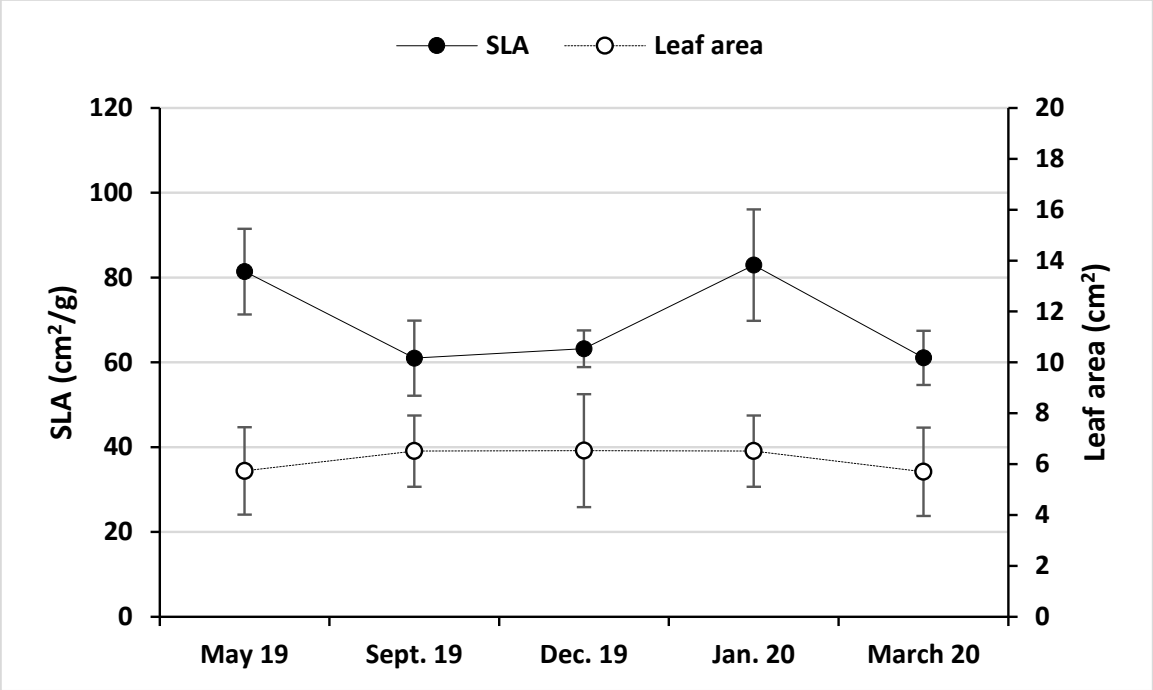

Figure 1. Seasonal variation in specific leaf area (SLA) and leaf area in *Quercus suber* L. (mean and standard deviation).

Specific leaf area (SLA), i.e., leaf surface per unit leaf dry mass, varied significantly throughout the study period ($p < 0.001$), with the values of May and January significantly different from those of September, December, and March (Figure 1). In May, the SLA of the young leaves was high (81.4 cm^2/g) and declined with time to a mean value of 61.0 cm^2/g, after the summer. SLA remained unchanged from September to December at a mean value of 63.2 cm^2/g and increased in January (82.3 cm^2/g). In March, the remaining one-year-old leaves were on average smaller, and the SLA was 61.1 cm^2/g.

3.2. Cuticular Wax Content

Our results revealed a seasonal variation in total wax quantity deposited in the cuticle of the cork oak leaves ($p < 0.001$), with the May value significantly different from the other ones (Figure 2). In May, the newly developed leaves exhibited the lowest value of total wax coverage over the annual cycle (115.7 $\mu g/cm^2$ on average, ranging between 68.9 and 172.5 $\mu g/cm^2$). In the next three months—within June, July and August —and until September, the biosynthesis of leaf wax lipids was very dynamic, doubling up to 235.6 $\mu g/cm^2$ (ranging from 173.7 to 267.4 $\mu g/cm^2$). Wax content decreased afterwards to 182.1 $\mu g/cm^2$ in December (ranging 151.0 and 208.0 $\mu g/cm^2$). The cuticular wax content in January and March (193.6 $\mu g/cm^2$ and 220.3 $\mu g/cm^2$, respectively) increased, although not significantly, due to sample variation.

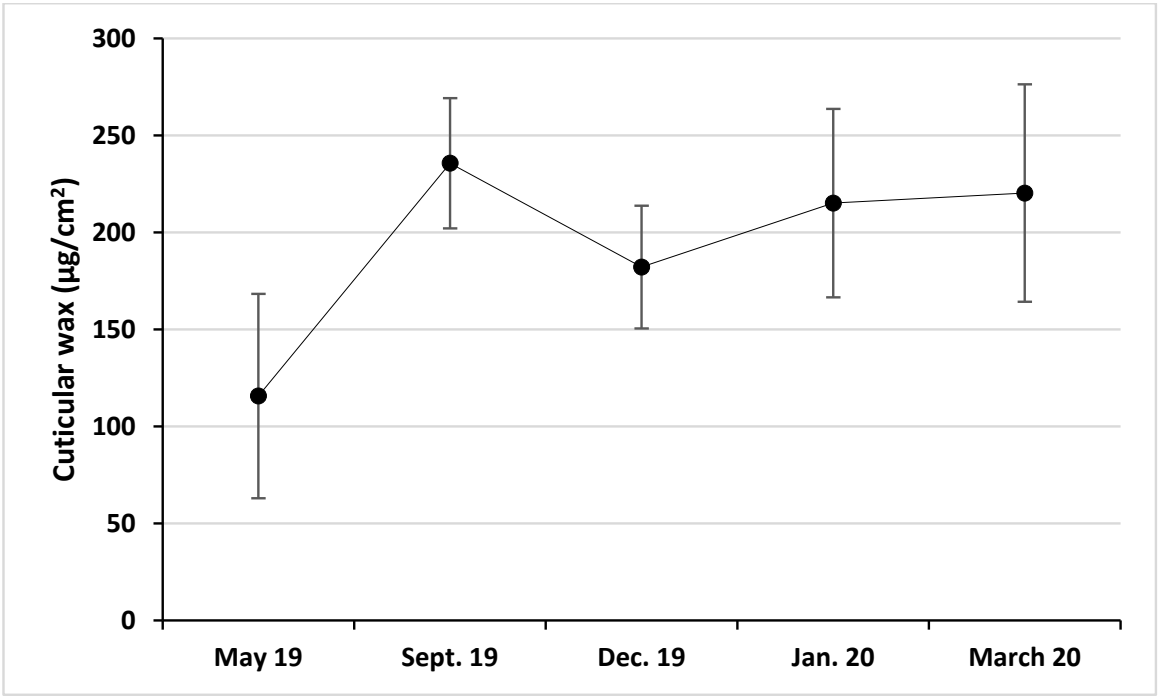

Figure 2. Seasonal variation of cuticular wax (µg/cm^2) in *Quercus suber* leaves (mean and standard deviation).

3.3. Cuticular Wax Composition

The seasonal changes in the composition of the leaf cuticular waxes grouped by major chemical families (µg/cm^2) are presented in Figure 3. The composition and the relative abundances of individual components are shown in Table 1.

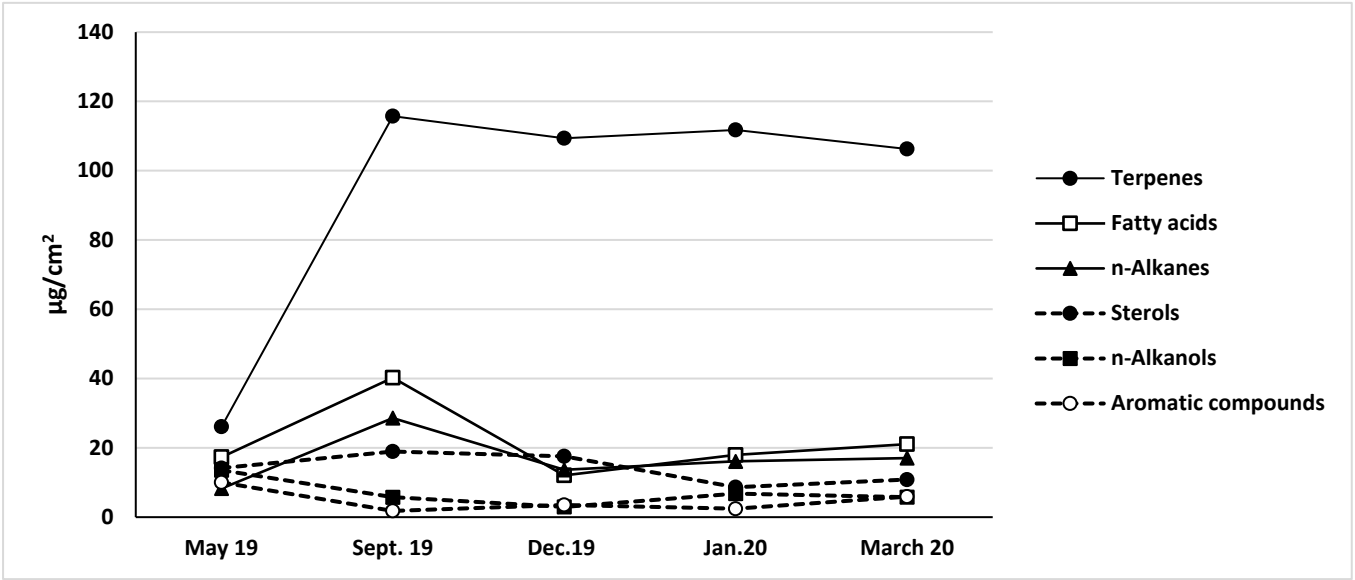

Figure 3. Seasonal variation in the cuticular wax chemical families (µg/cm^2) of *Quercus suber* leaves.

Table 1. Seasonal variation in the leaf cuticular waxes composition in cork oak (*Quercus suber*) (mean of six provenances), as determined by GC-MS, as a % of total peak area (only compounds with over 0.10% are shown).

	May 2019	**September 2019**	**December 2019**	**January 2020**	**March 2020**
Wax Content ($\mu g/cm^2$)	115.7 ± 52.6	235.6 ± 33.6	182.1 ± 31.6	215.1 ± 48.5	220.3 ± 56.0
n-Alkanes					
Hexacosane (C_{26})	-	0.16 ± 0.04	0.13 ± 0.15	0.13 ± 0.05	0.15 ± 0.17
Heptacosane (C_{27})	0.63 ± 0.24	1.06 ± 0.45	0.55 ± 0.45	0.68 ± 0.30	0.70 ± 0.17
Octacosane (C_{28})	0.21 ± 0.07	0.72 ± 0.20	0.46 ± 0.12	0.60 ± 0.19	0.46 ± 0.14
Nonacosane (C_{29})	4.75 ± 0.94	8.69 ± 3.38	7.23 ± 3.45	5.07 ± 2.61	5.24 ± 2.21
Triacontane (C_{30})	1.43 ± 0.54	1.38 ± 0.32	0.84 ± 0.40	0.93 ± 0.40	1.13 ± 0.29
n-Alkanols					
Hexadecan-1-ol ($C_{16}OH$)	0.10 ± 0.03	0.15 ± 0.05	0.14 ± 0.10	0.11 ± 0.09	0.12 ± 0.06
Docosan-1-ol ($C_{22}OH$)	0.89 ± 0.29	0.21 ± 0.10	0.12 ± 0.02	0.25 ± 0.10	0.18 ± 0.14
Tretracosan-1-ol ($C_{24}OH$)	9.92 ± 6.1	1.80 ± 0.89	1.14 ± 0.68	2.23 ± 1.05	1.84 ± 0.46
Pentacosan-1-ol ($C_{25}OH$	0.11 ± 0.07	-	-	-	-
Hexacosan-1-ol ($C_{26}OH$)	0.35 ± 0.30	-	-	-	-
Octacosn-1-ol ($C_{28}OH$)	-	-	-	0.32 ± 0.22	0.25 ± 0.26
Dotriacontan-1-ol ($C_{32}OH$)	-	0.11 ± 0.10	-	-	-
Fatty acids					
Saturated					
Decanoic acid ($C_{10:0}$)	-	-	-	-	0.21 ± 0.07
Dodecanoic acid ($C_{12:0}$)	0.10 ± 0.03	-	-	0.10 ± 0.07	0.22 ± 0.04
Tetradecanoic acid ($C_{14:0}$)	0.30 ± 0.16	0.12 ± 0.07	0.22 ± 0.11	0.15 ± 0.14	0.19 ± 0.23
Hexadecanoic acid ($C_{16:0}$)	4.97 ± 2.76	1.32 ± 0.65	4.31 ± 2.62	2.07 ± 0.60	2.02 ± 0.42
Octadecanoic acid ($C_{18:0}$)	0.51 ± 0.14	0.23 ± 0.09	0.17 ± 0.05	0.26 ± 0.14	0.20 ± 0.08
Eicosanoic acid ($C_{20:0}$)	0.20 ± 0.05	0.21 ± 0.11	-	0.18 ± 0.04	0.17 ± 0.08
Docosanoic acid ($C_{22:0}$)	0.32 ± 0.10	0.21 ± 0.17	-	0.26 ± 0.07	0.30 ± 0.17
Tetracosanoic acid ($C_{24:0}$)	0.80 ± 0.24	0.24 ± 0.15	-	0.24 ± 0.08	0.20 ± 0.17
Hexacosanoic acid ($C_{26:0}$)	0.71 ± 0.34	1.01 ± 0.43	-	0.56 ± 0.19	0.45 ± 0.16
Octacosanoic acid ($C_{28:0}$)	1.49 ± 1.09	4.65 ± 2.89	0.18 ± 0.09	1.46 ± 0.44	1.69 ± 0.36
Triacontanoic acid ($C_{30:0}$)	0.95 ± 1.01	8.15 ± 6.18	0.96 ± 0.74	2.28 ± 1.23	2.37 ± 0.61
Dotriacontanoic acid ($C_{32:0}$)	0.13 ± 0.09	0.41 ± 0.15	0.10 ± 0.7	0.32 ± 0.07	0.31 ± 0.12
Unsaturated					
9,12-Octadecadienoic acid ($C_{18:2}$)	1.42 ± 1.14	0.10 ± 0.05	-	-	0.15 ± 0.08
9,12,15-Octadecatrienoic acid ($C_{18:3}$)	2.54 ± 2.19	0.26 ± 0.16	0.24 ± 0.12	0.17 ± 0.12	0.51 ± 0.14
Glycerides					
Glycerol	3.52 ± 1.32	0.12 ± 0.05	0.16 ± 0.07	0.33 ± 0.38	1.03 ± 0.39
4-Hydroxyphenylglycolic acid	0.19 ± 0.21	0.35 ± 0.40	1.01 ± 0.45	1.18 ± 0.71	-
2-Palmitoglycerol	0.63 ± 0.09	0.21 ± 0.10	-	0.21 ± 0.26	-.
Glycerol monostearate	0.17 ± 0.07	-	-	-	-
1-Monolinoleate Glycerol	0.33 ±0.15	-	-	-	-
Linolenoylglycerol	0.13 ± 0.07	-	-	-	0.18 ± 0.12
Sterols					
β-Systosterol	11.94 ± 5.28	6.29 ± 1.86	9.64 ± 4.26	4.01 ± 1.11	4.91 ± 1.03

Table 1. *Cont.*

	May 2019	September 2019	December 2019	January 2020	March 2020
Terpenes					
Diterpenes					
Phytol	4.00 ± 2.40	0.61 ± 0.28	0.35 ± 0.39	0.49 ± 0.36	1.01 ±0.23
Pentacylic triperpenes					
α-Amyrin	1.61 ± 0.53	1.34 ± 0.40	0.72 ± 0.47	0.76 ± 0.29	0.64 ± 0.21
β-Amyrin	2.33 ± 0.95	3.98 ± 1.38	4.90 ± 2.74	3.50 ± 1.32	3.97 ± 1.25
Germanicol	0.27 ± 0.42	8.16 ± 1.69	8.10 ± 6.50	8.40 ± 2.47	6.38 ± 1.49
Lupeol	7.38 ± 5.71	28.20 ± 10.41	36.59 ± 11.13	27.75 ± 7.06	24.11 ± 6.81
Epifriedelanol	0.43 ± 0.32	2.38 ± 2.26	3.75 ± 3.25	1.92 ± 2.22	3.12 ± 2.38
Erythrodiol	0.21 ± 0.17	-	0.14 ± 0.12	1.62 ± 1.27	0.39 ± 0.30
Friedelin	1.14 ±0.96	2.47 ± 2.73	2.82 ± 2.82	2.16 ± 2.00	2.38 ±1.85
Betulin	0.40 ± 1.09	0.36 ± 0.10	0.80 ± 0.63	1.09 ± 0.81	1.46 ± 0.71
Betulinic acid	0.46 ± 0.25	0.42 ± 0.15	0.75 ± 0.63-	0.98 ± 0.38	1.21 ± 0.53
Ursolic acid	0.44 ± 0.16	0.27 ± 0.10	0.24 ± 0.19	0.27 ± 0.16	0.28 ± 0.15
Aromatic compounds					
Benzoic acid	0.33 ± 0.29	0.16 ± 0.07	0.33 ± 0.09	0.27 ± 0.05	0.36 ± 0.06
Hexadecy-(E)-p-coumarate	4.93 ± 2.87	-	0.20 ± 0.09	-	-
Vanillin acid	0.47 ± 0.17	0.11 ± 0.06	0.11 ± 0.06	0.40 ± 0.49	0.16 ± 0.15
4-(Hydroxymethyl)phenol	0.66 ± 0.31	0.10 ± 0.08	0.51 ± 0.54	-	1.36 ± 0.24
Other compounds					
Myo-inositol	1.35 ± 0.99	0.34 ± 0.18	0.10 ± 0.09	1.35 ± 2.31	1.18 ±1.27
D-Fructose	0.67 ± 0.20	0.32 ± 0.09	-	3.31 ± 2.80	1.04 ±1.36
α-Tocopherol	1.62 ±1.11	-	-	-	1.04 ± 1.36
β-Tocopherol	0.33 ± 0.13	0.95 ± 0.50	-	-	-
γ-Tocopherol	-	-	0.55 ± 0.62	-	-
α-Tocopherolquinone	0.19 ± 0.17	0.13 ± 0.10	0.10 ± 0.16	0.40 ± 0.46	2.05 ± 2.1
Erythrono-1,4-lactone	0.33 ± 0.14	-	-	0.37 ± 0.22	-
Ribonic acid, 1,4-lactone	0.33 ± 0.13	0.10 ± 0.06	-	0.51 ± 0.34	0.31 ± 0.22
Quinic acid	-	-	-	1.93 ± 0.76	-

In the new expanding spring leaves in May, the cuticular wax was mainly composed of cyclic terpene compounds and linear long-chain aliphatic molecules. Pentacyclic triterpenoids were abundant, accounting for 26 $\mu g/cm^2$ of the leaf wax coverage (22.6% of the total wax). The major component was lupeol (7.4% of all compounds, 32% of the triterpenic fraction), with β-amyrin (2.3% of all compounds) and betulin (2% of all compounds) as other important constituents. Sterols amounted to 14 $\mu g/cm^2$ (12.2% of the wax), mainly β-systosterol (12.0% of the compounds). Aromatics comprised 8.7% of all compounds (10 $\mu g/cm^2$), with hexadecy-(E)-p-coumarate as the major compound. The linear long aliphatic molecules of acids, alcohols, and alkanes together represented 39.1 $\mu g/cm^2$ (33.9% of the total wax) and were the most abundant wax components. Fatty acids were the main compounds (17.3 $\mu g/cm^2$, 15% of total wax), mostly C_{16} (hexadecanoic acid) and C_{28} (octacosanoic acid) fatty acids, which together accounted for 59% of the total fatty acids. The aliphatic alcohols contributed to 11.4 $\mu g/cm^2$ of leaf surface coverage (12% of total wax), mostly C_{24} (tretracosan-1-ol), with 85% of total aliphatic alcohols. Alkanes accounted for 7.2% of the wax, with C_{29} (nonacosane, 4.8% of all compounds) and C_{30} (triacontane, 1.4%) as predominant molecular species.

After the summer, the cuticular wax on the mature leaves increased significantly, mainly by pentacylic triperpenes, which reached 116.0 $\mu g/cm^2$ (49% of the total wax) (five times more than in spring leaves). Lupeol was the major constituent, followed by smaller amounts of epifriedelanol and friedelin. Pentacylic triterpenes were always the main wax components during the following months until the end of the annual cycle (March, with 12-month-old leaves), with a constant amount and composition (109.4 $\mu g/cm^2$, 111.8 $\mu g/cm^2$, and 106.3 $\mu g/cm^2$, respectively, in December, January, and March). The statistical analysis showed a significant difference ($p < 0.001$) in the time-related chemical

composition in the amount of terpenes (Figure 3), with the May value different from the other ones.

Sterols were relatively constant over time (8% of the wax compounds in September and 5% in March) and composed exclusively of β-systosterol. Aromatic compounds were present in minor amounts.

Fatty acids showed the highest variability in concentration, with a maximum in September (75.6 μg/cm^2, 17.1% of all compounds), decreasing to 28.7 μg/cm^2 in December and slightly increasing during the remaining period. The statistical analysis showed a significant difference ($p < 0.001$) in the amount of fatty acids (Figure 3), with the September value different from the other ones. Fatty acids were composed of a homologous series of even and saturated components from C_{16} to C_{30}, mostly including octacosanoic (C_{28}) and triacontanoic (C_{30}) acids, followed by hexadecanoic (C_{16}) acid.

The aliphatic alcohols, including mainly tretracosan-1-ol, were highest in spring but remained rather constant throughout the period.

n-Alkanes, including only nonacosane and triacontane, were highest in September (28.5 μg/cm^2) and then decreased and remained relatively constant (between 13.6 and 17.0 μg/cm^2).

4. Discussion

4.1. Leaf Area and SLA

In *Q. suber*, the shoot growth and the development of new foliage occur during the late spring and early summer, most likely reaching a maximum enlargement rate in summer (June/August) [11,20,21]. This was confirmed in the present study. The leaf size increased through summer to a maximum in September corresponding to the fully expanded mature leaves, which remained unchanged throughout the winter. The smaller leaf size measured in March is probably related to a canopy change related to leaf fall. In fact, the longevity of cork oak leaves is about one year, much shorter than other evergreen oaks; e.g., leaves last 1–3 years in *Quercus rotundifolia* and 5–6 years in *Q. coccifera* [13,14,22]. The leaf size measured in the present study matches the few available reports for mature *Q. suber* leaves. Mediavilla et al. [23] reported values between 5.5 ± 0.3 and 7.4 ± 0.5 cm^2 in leaves taken from different orientations in the canopy, and Prats et al. [21] observed 7.1 ± 1.5 cm^2 for full expanded leaves.

Specific leaf area (SLA) was the highest for the young leaves in May (Figure 1), when leaves were expanding and growing quickly, with the tree investing less in dry matter per leaf [24]. SLA declined after the summer as a result of the increase in leaf mass by the retention of the photosynthetic compounds produced during the late spring and early summer period, when the mature leaves were totally expanded. SLA changes resulting from leaf structure modification are an underlying mechanism facilitating acclimatization to a drought that increases with declining rainfall [25,26]. SLA increased in January when low temperatures limited photosynthesis and reflect the remobilization or loss of resources, namely, before leaf abcission [22]. In March, the remaining one-year-old leaves were on average smaller and maintained their loss of photosynthetic capacity. Photosynthetic activity has been shown to decrease with leaf age in other sclerophylls, and this strategy fits expectations for drought-tolerant species such as evergreen Mediterranean oaks [27].

The seasonal leaf variation in *Q. suber* was previously reported by Passarinho et al. [28] for three periods, spring to early summer, summer, and autumn–winter; the dry matter content of new leaves was low, increased with age to the maximum level in August/September, with SLA values of 85.0 ± 39 cm^2/g (April–July), 74.4 ± 41 cm^2/g (July–September), and 89.9 ± 37 cm^2/g (September–February). The findings of the present study accord with these reported results.

4.2. Cuticular Wax Content

The occurrence of cuticular waxes in the leaves revealed a significant seasonal variation in total wax quantity, as expressed by the unit area of the leaf surface, as shown in Figure 2.

The lowest value was found in May, in the newly developed cork oak leaves, suggesting that leaf growth was more efficient than the wax lipids biosynthesis during this spring period, when the environmental conditions are mild in terms of temperature and water availability, and therefore, the leaves did not require special protection. This changed drastically during the summer months, when biosynthesis of leaf wax lipids was very dynamic, doubling from May to September. This is in line with the need to provide efficient protection to minimize water loss through the cuticular layer when all stomata are closed due to the summer abiotic stress [24,29–32]. In fact, one of the leaf-related strategies that plants may adopt to cope with drought conditions is increasing wax accumulation on the leaf surface [33,34]. In winter, wax content decreased, likely because of natural wax erosion and evaporation due to environmental factors such as light, temperature, rain, and wind [29,31,35,36] and then increased slightly during the remaining period.There are no previous studies on the variation in the cuticular waxes along the development of *Q. suber* leaves, and the results reported here are the first for this species. They accord with the few available studies on leaves of several species that showed that the cuticular wax content in the early development stage is lower than that observed in mature leaves, reaching maximum values after full leaf expansion, e.g., on the leaves of *Quercus robur* L. [30], *Fagus sylvatica* L. [29], three *Hosta* genotypes (*Hosta plantaginea* (Lam) Asch., *H. lancifolia* Engl. and *H.* 'Krossa Regal') [31], *Hedera helix* L. [35], and *Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson [36].

The importance of a steadily present continuous outer leaf coverage as a protective strategy is shown by the self-healing of voids in the epicuticular wax layer on living plants, which is a dynamic process, at least in the early stages of leaf development [37–39]. Wax regeneration after removing the original epicuticular wax layer by peeling with a water-based glue applied to the leaf surface was observed on young leaves of *Prunus laurocerasus* L. [40] and on leaves with different ages of diverse species [37,39,41].

4.3. Cuticular Wax Composition

To our knowledge, no previous studies have been made on the seasonal changes in the composition of the cuticular waxes of *Q. suber* leaves. In this work, we have found a clear compositional variation, namely between the young and the mature leaves, i.e., between May and September (Figure 3), regarding the proportion of each chemical family. The cuticular wax composition of the young leaves (Table 1) included terpenes, sterols, linear long aliphatics (fatty acids, alcohols, and alkanes), and aromatics representing a proportion in the wax of 22.6%, 12.2%, 33.9%, and 8.7%, respectively. After the summer, the cuticular wax on the mature leaves increased significantly, mainly by pentacylic triperpenes, which represented nearly half of the total wax and about five times more than in spring leaves. The proportion of the four wax chemical classes of terpenes, sterols, linear long aliphatics, and aromatics in the mature leaves was, respectively, 60.5%, 9.6%, 15.7%, and 1.9%. Within each chemical family, the composition did not vary over time (Table 1).

In general, the *Q. suber* cuticular wax compositions found in this study were similar to that previously identified in 1-year-old *Q. suber* mature leaves sampled in March showing predominantly pentacyclic triterpenoids (61%–72% of the identified compounds, mainly lupeol) and aliphatic compounds (17%–23% of the identified compounds, mainly fatty acids (C_{30}, C_{28}, and C_{16})) [9].

A similar wax composition is found in other oaks. For instance, *Quercus coccifera* leaf wax contains pentacyclic triterpenoids as the most abundant class (49% and 61%), with germanicol and lupeol as major constituents, and very-long-chain aliphatic compounds from C_{20} to C_{51} (25%–34%) with *n*-alkanes as the main class comprising a homologous series from C_{25} to C_{32} [42]. The cuticular wax composition from the adaxial surface of fully expanded olive tree leaves (*Olea europaea* L.) was also mainly composed of pentacyclic triterpenoids (83% of total wax, with oleanolic acid as the major triterpenoid) with a ratio between the very long chain acyclic and the cyclic wax of 0.04 with *n*-alkanes as the main compound class (3% of the total wax) of the long-chain aliphatic fraction [43]. A high

proportion of pentacyclic triterpenoids is found in the leaf cuticles of desert plants, for instance, in *Rhazya stricta* Decne., for which pentacyclic triterpenoids make up 85.2% of the total cuticle wax, while long-chain aliphatics with chain lengths ranging from C_{20} to C_{33} represent only 3.4% with alkanes as the main class (2.5% of the total wax, 73.6% of the aliphatics) [7].

In the present study, the solubilized cuticular waxes were not differentiated into the epicuticular waxes, deposited on cutin, and the intracuticular waxes found within the cutin matrix, since the extraction method was aimed at the total removal of the lipophilic soluble components [9,10].

Recent studies revealed a substantial compositional gradient between the epicuticular wax and the intracuticular wax: while epicuticular wax is only composed of very-long-chain fatty acid derivatives (e.g., alkanes, primary alcohols, fatty acids), intracuticular wax is composed of alicyclic compounds (e.g., triterpenoids and steroids) and long-chain aliphatic molecules [4,44]. The wax compositional differences between the cork oak leaves throughout their development cycle may be linked to the proportional occurrence of epicuticular and intracuticular waxes over time, which can be estimated by analyzing the ratio between cyclic compounds and linear-chain compounds. In the young leaves from May, the pentacyclic triterpenoids (26 $\mu g/cm^2$) in the wax mixture were less than aliphatic compounds (39 $\mu g/cm^2$), with a cyclic-to-acyclic component ratio of 0.7, while in the mature leaves, this ratio was 1.6 in September, 3.8 in December, and 2.4 in March. Since the pentacyclic triterpenoids are located almost exclusively in the intracuticular wax compartment, the increase in the ratio after summer shows an extensive deposition of intracuticular waxes. This is in association with the establishment of mechanical and thermal stability given by the triterpenoids [6] and the water loss barrier in the mature leaf cuticle as given by the intracuticular waxes [7]; however, a natural effect of wax weathering with leaf age and with environmental factors was superimposed on the seasonal pattern of variation. This was detected by comparing the lowest ratio value from September with the highest value from December.

5. Conclusions

The seasonal dynamics of cuticular waxes in the cuticle of cork oak leaves were characterized for the first time, and the initial investigation hypothesis of an effect of seasonality on leaf wax coverage and composition was confirmed. There was an increase in wax coverage on the newly expanded leaves to a maximum value in the fully expanded leaves, and a decline after the summer as a result of the natural weathering of the cuticular waxes with leaf age and with environmental factors. A subsequent renewal of the external layer followed, thereby reflecting the importance of a steadily present continuous outer leaf coverage in such harsh climatic conditions. The leaf cuticular wax layer was mainly composed of cyclic terpene compounds and linear long-chain aliphatic molecules. Triterpenoids dominated the wax mixture throughout the leaf life cycle, with a high proportion of lupeol. In the very long-chain aliphatic wax fraction, fatty acids showed the highest concentration variability along the leaf-development cycle, with a decrease in autumn but an increasing trend in the next spring. The results on the chemical dynamics of the cuticular waxes along the leaf cycle support the role of the intracuticular layer and the long-chain lipids as a transpiration barrier during the summer drought in the Mediterranean climate.

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