JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.7b03049 • Publication Date (Web): 07 Sep 2017

Just Accepted

ACS Publications

Journal of Agricultural and Food Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Title: Chemical Composition and Water Permeability of Fruit and Leaf Cuticles of *Olea europaea* L.

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Manuscript Type: Research article

ABSTRACT

The plant cuticle, protecting against uncontrolled water loss, covers olive (*Olea europaea*) fruits and leaves. The present study describes the organ-specific chemical composition of the cuticular waxes and the cutin and compares three developmental stages of fruits (green, turning and black) with the leaf surface. Numerous organ-specific differences, such as the total coverage of cutin monomeric components (1034.4 µg cm^{-2} and 630.5 µg cm^{-2}) and the cuticular waxes (201.6 µg cm^{-2} and 320.4 µg cm^{-2}) among all three fruit stages and leaves, respectively, were detected. Water permeability as the main cuticular function was five-fold lower in adaxial leaf cuticles (2.1 x 10^{-5} m s^{-1}) in comparison to all three fruit stages (9.5 x 10^{-5} m s^{-1}). The three fruit developmental stages have the same cuticular water permeability. It is hypothesized that a higher weighted average chain length of the acyclic cuticular components leads to a considerably lower permeability of the leaf as compared to the fruit cuticle.

Key Words: average chain-length; cuticular transpiration, cutin, fruit, leaf, plant cuticle, triterpenoids, very-long-chain acyclic components, wax

INTRODUCTION

Olea europaea L. (Oleaceae) is an evergreen tree with silvery green, elongated leaves. Producing small non-climacteric drupes, it is one of the predominant fruit tree crops in the Mediterranean area and, thus, is of high economic importance. The total world production of olive fruits has nearly doubled from 8.0 to 15.4 million tons during the past four decades (1974 to 2014; FAOSTAT 2017, http, //www.fao.org/faostat/en/#data/QC). About 58% of the crop is produced in Europe, mainly Spain, Italy, and Greece. Hundreds of olive cultivars are documented, such as the economically important `Arbequina´ cultivar originating from Spain. Plants growing in Mediterranean-type climates often suffer from various environmental stresses, such as elevated temperatures, high vapor pressure deficits, and limited water availability.[1] The importance of protecting olive leaves and fruits from uncontrolled water loss is given throughout the whole fruit developmental process, which includes fruit set, growth, maturation and oil accumulation in the fruit pulp.[2] During fruit growth, stomata lose their shape and are covered by cuticular waxes.[3,4] Trichomes and stomata are mainly occurring on the abaxial leaf surface, very few incompletely degraded trichomes with suberized stalks exist on the adaxial leaf surface.[5-7] Thus, the plant cuticle covering fruit and leaf surfaces is the main barrier limiting uncontrolled water loss.

The plant cuticle is considered an important structure affecting olive fruit and leaf transpiration, especially under drought conditions. Water deficit induced an increase of fruit cuticle thickness when compared to olive fruits grown under normal irrigation.[8,9] However, thicker plant cuticles do not provide more effective transpiration barriers.[10,11] Plant cuticles are lipoid in nature consisting of a cutin matrix, a polyester of C_{16} and C_{18} fatty acids and hydroxy fatty acids, often with additional hydroxy, carboxy, epoxy and oxo groups in secondary mostly mid-chain position with cuticular waxes embedded within or deposited onto its surface.[12,13] The cuticular waxes form the main transport-limiting barrier.[10,14] However, there is no simple relationship between cuticular wax quantity and transpiration. Also, it was

proposed that cutin and the corresponding ester-linkage might play a major role in establishing barrier properties by providing the framework in which the cuticular waxes are arranged.[15,16]

The cuticular waxes from different cultivars and different developmental stages of olive fruits and leaves are dominated by pentacyclic triterpenoids and additionally contain minor amounts of very-long-chain fatty acid derivatives, the very-long-chain acyclic wax compounds.[17-21] Up to now, most of the information available is about the cuticular wax composition of olive fruits while the leaf waxes have not been investigated to the same degree. Furthermore, there is no study on the composition of the cutin polymer neither of olive fruits nor leaves.

During maturation, olive fruits having attained their final size change color turning from green to black with an intermediate red-to-purple stage called the turning stage. The turning stage is considered to be important for the tolerance and/or resistance to biotic and abiotic environmental factors.[21] However, the contribution of the cuticular components to cuticle functions, particularly the transpiration barrier properties, have not been comprehensively studied.

The present study aims at elucidating differential chemical compositions of the plant cuticle, for the first time comparing fruit developmental stages (here green, turning and black stage) with the leaf surface within one plant species. The cuticular waxes and the cutin monomeric composition were used to determine the contribution of cuticular components to the transpiration barrier properties.

MATERIALS AND METHODS

Plant Material

Trees of *Olea europaea* L. cultivar `Arbequina´ (Oleaceae) were grown in El Soleràs, Lleida, Spain (41°24'48.71"N, 0°40'50.05"E). The trees were non-irrigated and only rain-fed. Fruit samples at different development stages (green, turning and black) were harvested during the ripening period in November 2014. Leaves and fruits were collected from the same trees during the same ripening period. For the chemical analysis (monomeric cutin and cuticular wax composition) five replicates were used. The permeability experiments were repeated with 12 fruits/leaves, respectively.

Chemicals

For cuticle isolation pectinase (Trenolin, Erbslöh, Geisenheim, Germany), cellulase (Celluclast, NCBE, University of Reading, UK), citric acid monohydrate (Applichem, Darmstadt, Germany) and sodium azide (Sigma-Aldrich, Steinheim, Germany) were used.

For extraction and analysis chloroform (≥ 99.8%), pyridine, sodium chloride and sodium sulfate were purchased from Roth (Karlsruhe, Germany). Boron trifluoride in methanol (1.3 M) was obtained from Fluka (Neu-Ulm, Germany). *N*-tetracosane and *n*-dotriacontane were provided by Sigma-Aldrich (Steinheim, Germany). *N,O*-bis(trimethylsilyl) trifluoroacetamide was from Macherey-Nagel (Düren, Germany).

Authentic standards of ß-amyrin, erythrodiol, ursolic acid and betulinic acid were purchased from Roth (Karlsruhe, Germany), oleanolic acid from Sigma-Aldrich (Steinheim, Germany).

Cutin Depolymerization for Chemical Analysis

Cuticular membranes from both fruits and adaxial leaf surfaces were isolated enzymatically in citric acid buffer containing pectinase (1%), cellulase (1%) and 1 mM sodium azide to avoid growth of microorganism.[22]

For the cutin analysis, the air-dried isolated cuticular membranes were immersed in chloroform to remove the cuticular waxes. The wax-free polymer matrix was depolymerized

with boron trifluoride in methanol (1.3 M) at 70°C for 16 h. *N*-dotriacontane as an internal standard was added. Subsequently, a saturated aqueous sodium chloride solution was added. The mixture was extracted three times with chloroform. The collected extracts were dried over sodium sulfate, and the organic solvent was gently evaporated under a continuous flow of nitrogen.

Cuticular Wax Extraction for Chemical Analysis

To extract the cuticular waxes from the fruit surface, whole fruits were dipped in chloroform. To avoid contact of the solvent with the pedicel, approximately 90% of the fruit was vertically dipped into chloroform for 60 s. The cuticular wax from the adaxial leaf surface was extracted by dipping the enzymatically isolated adaxial cuticle two subsequent times in chloroform. Each fruit sample was extracted three times consecutively. *N*-tetracosane was added to the extracts as an internal standard. The solvent was evaporated under a gentle stream of nitrogen gas until dryness.

Chemical Analysis of Cuticular Components

Prior to the gas chromatographic analysis, the cuticular wax and cutin samples were derivatized with *N,O*-bis(trimethylsilyl) trifluoroacetamide in pyridine for 30 min at 70°C. For the quantitation of the compounds, a capillary gas chromatograph with flame ionization detector (6850N, GC-System; Agilent Technologies, Santa Clara, California) and on-column injection with a capillary column (30 m × 0.32 mm, DB-1 ms, 0.1 µm film; J&W Scientific, Agilent Technologies) was used. For separation of the cuticular wax compounds, the samples were injected at 50°C followed by 2 min at 50°C, temperature raised by 40°C min^{-1} to 200°C, held for 2 min at 200°C, raised by 3°C min^{-1} to 320°C and held for 30 min at 320°C. For separation of the cutin monomers, samples were injected at 50°C, followed by 1 min at 50°C, temperature raised by 10°C min^{-1} to 150°C, held for 2 min at 150°C, raised by 3°C min^{-1} to 320°C and held for 30 min at 320°C.

Qualitative analysis was carried out with a gas chromatograph (6890N, Agilent Technologies) equipped with a mass spectrometric detector (*m/z* 50-750, MSD 5973; Agilent Technologies) under the same gas chromatographic conditions except that helium was used as carrier gas. Identification of the compounds was carried out by their mass spectra using authentic standards, the Wiley 10th/NIST 2014 mass spectral library (W10N14; John Wiley & Sons, Hoboken, New Jersey) or by interpretation of the spectra, by their retention times and/or by comparison with literature data. Based on the coverage of very-long-chain acyclic wax compounds and their carbon chain length distribution, the weighted average chain length (ACL) of very-long-chain acyclic wax components was calculated. ACL is the weight-averaged number of carbon atoms, defined as:

$$\mathrm{ACL} = \frac{\sum(C_n \times n)}{\sum C_n} \quad (1)$$

with C_n being the coverage of each very-long-chain acyclic wax compound with n carbon atoms.

Determination of Cuticular Transpiration

The transpiration from whole fruits and leaves was determined gravimetrically by measuring the water loss over time. Before measurement, the attachment site of the fruit pedicel and the leaf petioles were sealed with paraffin wax (melting point 65°C; Roth, Karlsruhe, Germany). The abaxial leaf surface was sealed with paraffin wax to ensure that the water transpiration occurs only via the stomata-free adaxial leaf surface. The fruit and leaf samples were placed in boxes over silica gel (Applichem, Darmstadt, Germany) to reduce the relative humidity to nearly zero. The boxes were placed in an incubator (IPP 110, Memmert, Schwabach, Germany) to control the surrounding temperature (25°C). The amount of water transpired from whole fruit and adaxial leaf surfaces versus time (five to six data points per individual sample) was measured using an analytic electronic balance with a precision of 0.1 mg (MC-1 AC210S, Sartorius, Göttingen, Germany). The temperature in the incubator was controlled

with a digital thermometer (Testoterm 6010, Lenzkirch, Germany), and the actual temperature of fruit and leaf surfaces was measured by an infrared laser thermometer (Harbor Freight Tools, Calabasas, California). The transpiration rate (flux of water vapor; J in g m^{-2} s^{-1}) was obtained from the change of the fresh weight of the samples (ΔW in g) over time (Δt in s) and surface area (A in m^2):

$$J = \frac{\Delta W}{\Delta t \cdot A} \quad (2)$$

The form of the fruit of the olive cultivar used in this study deviates only slightly from a perfect sphere. So the fruit surface area (A_{fruit}) was calculated for a sphere with a radius calculated as the mean of the polar and equatorial radii of the fruit. The leaf surface area (A_{leaf}) of the adaxial surface was obtained by scanning the leaf surface. The permeance (P in m s^{-1}) was calculated from the transpiration rate (J) divided by the driving force:

$$P = \frac{J}{c^*_{wv}\,(a_{fruit/leaf} - a_{air})} \quad (3)$$

The water vapor saturation concentration at the actual fruit or leaf temperature (water vapor content of air at saturation; c^*_{wv}) was obtained from tabulated values.[23] Air water activity (a_{air}) over silica gel was close to zero. The water activity in the fruit or leaf ($a_{fruit/leaf}$) was assumed to be unity.[24]

Statistical Analysis

Statistical analyses were performed using SigmaPlot 13 (Systat Software GmbH, Erkrath, Germany). The total amount of cutin monomers, total wax amount, surface area and cuticular permeance were checked for normal distribution by the Shapiro-Wilk normality test (p-value to reject 0.05). Normally distributed data are given as means with the corresponding standard deviations. Comparisons between leaf parameters and black-stage fruit parameters were tested for significance with Student's t-test (level of significance $p < 0.05$) and, when comparing the different fruit stages, Kruskal-Wallis one way analysis of variance (ANOVA).

RESULTS

Composition of the Cutin Polymer of *Olea europaea* Fruits and Leaves

During fruit development, the quantity, as well as the quality of the cutin matrix covering olive fruits, changed. The amount of cutin monomers was 969 μg cm^{-2}, 1005 μg cm^{-2} and 1129 μg cm^{-2} in green, turning and black fruits, respectively, with no significant differences among these values (Table 1; Figure 1A). This trend during fruit development resulted mainly from an accumulation of fatty acids, ω-hydroxy fatty acids and ω-hydroxy fatty acids with mid-chain hydroxy groups.

In comparison to fruits, the total amount of cutin monomers of the fully expanded, adaxial leaf surface was 35% to 44% lower (631 μg cm^{-2}). In the fruit cutin matrix, the predominant cutin monomers were ω-hydroxy fatty acids with mid-chain hydroxy groups (72% to 77% of the total cutin). The main cutin compounds were 9/10,16-dihydroxyhexadecanoic acid (33% to 35%), 9,10,18-trihydroxyoctadecenoic acid (14% to 26%) and 9,10,18-trihydroxyoctadecanoic acid (13% to 17%). Similarly, the major cutin monomers on the adaxial leaf surface were ω-hydroxy fatty acids with mid-chain hydroxy groups (43% of the total cutin) dominated by 9/10,16-dihydroxyhexadecanoic acid (26%) and 9,10,18-trihydroxyoctadecanoic acid (16%). The ω-hydroxy fatty acid with a mid-chain epoxy group (9,10-epoxy-18-hydroxyoctadecanoic acid, 39% of the total cutin) was only detected in the leaf cutin matrix. Small amounts of unsubstituted fatty acids with even-numbered carbon chain lengths from C_{16} to C_{28} (2% to 3% of the total cutin), ω-hydroxy fatty acids (2% to 9%), α,ω-dicarboxylic fatty acids with mid-chain epoxy group (1%) and phenolic compounds (< 1% to 5%) were also found in the cutin matrix of fruit and leaf surfaces. ω-Hydroxy fatty acids with a mid-chain oxo group (1%) and α,ω-dicarboxylic fatty acids (< 1%) were identified exclusively in the fruit cutin matrix.

The degree of unsaturation of the cutin polymer was 18% to 32% in green, turning, and black fruits, and 2% in the leaf but the degree of epoxidation was higher for the leaf (40%) as

compared to the fruit cutin (1%). Furthermore, the ratio of the main cutin monomers with a carbon chain length of C_{16} and C_{18} shifted from 1.0 to 0.8 in green, turning and black fruits, and amounted to 0.5 in the leaf cutin matrix (Table 3).

Composition of the Cuticular Wax of *Olea europaea* Fruits and Leaves

The cuticular wax compositions and amounts of olive fruits at different developmental stages and the adaxial leaf surface was determined. The total cuticular wax coverage did not change significantly from green to turning to black fruits (195, 202 and 209 μg cm^{-2}, respectively; Table 2; Figure 1B). Compared to the fruit waxes, the wax coverage of the adaxial leaf surface was significantly higher (320 μg cm^{-2}).

Significant amounts of pentacyclic triterpenoids were present in the cuticular waxes of fruits, which did not change significantly over the three developmental stages investigated (Table 3). At the same time, the very-long-chain acyclic coverage significantly increased from 39 (green) to 62 μg cm^{-2} (black; Table 3) leading to a decrease in the relative contribution of triterpenoids to the total wax from 74% (green), 67% (turning) to 62% black. The leaf cuticular waxes consisted to an even higher degree of triterpenoids (83%) with a coverage of 266 μg cm^{-2}; Table 3). The cyclic wax fraction consists of the triterpenoid acids oleanolic acid, maslinic acid, ursolic acid, betulinic acid, and the triterpenoid alcohols β-amyrin, erythrodiol, and uvaol. The major triterpenoid was oleanolic acid (35% to 37% in fruits, 60% in leaves). Additionally, an oleanolic acid derivative (11% to 20%) and maslinic acid (10% to 14%) were found to be highly abundant in the cuticular fruit waxes. In comparison to fruits, higher amounts of erythrodiol (6%), uvaol (7%) and ursolic acid (9%) were found on the adaxial leaf surface (Table 2). Ursolic acid was not identified in olive fruits and the oleanolic acid derivative and betulinic acid not in olive leaves.

Compared to the high amounts of pentacyclic triterpenoids, smaller proportions of very-long-chain acyclic wax compounds were detected (Table 3). The main component classes were fatty acids (8% to 9%) and primary alcohols (9% to 11%) in fruit cuticular waxes, most

prominently hexacosanoic acid (C_{26}) and hexacosanol (C_{26}). In leaf cuticular waxes, fatty acids (1%) and *n*-alkanes (3%) were dominated by octacosanoic acid (C_{28}), *n*-hentriacontane (C_{31}) and *n*-tritriacontane (C_{33}). Minor proportions of aldehydes, *n*-alkanes, alkyl esters, methyl esters, phenyl methyl esters, diacylglycerols, and sterols were also found in the fruit cuticular wax mixture (< 1% to 4%). Aldehydes, alkyl esters, methyl esters, phenyl methyl esters, diacylglycerols, and sterols were not identified in cuticular waxes of the adaxial leaf surface. Only small amounts of primary alcohols (< 1%) were found.

The homologous series of fatty acids, primary alcohols, aldehydes, alkyl esters, methyl esters and phenyl methyl esters showed a very pronounced even-numbered carbon chain length distribution, while odd-numbered homologs dominated the distributions of *n*-alkanes and diacylglycerols. The carbon chain length distribution of both fatty acids and primary alcohols ranged from C_{20} to C_{30} in all three fruit developmental stages. Aldehydes were identified from C_{22} to C_{30} in fruit cuticular waxes. These compound classes made up 92% of the total very-long-chain acyclic wax fraction in green, 83% in turning and 74% in black fruits. In contrast to fruit waxes, *n*-alkanes with odd-numbered carbon chain lengths primarily from C_{27} to C_{33} were the main very-long-chain acyclic wax compounds of the total very-long-chain acyclic wax fraction on the adaxial leaf surface (78%). Fatty acids in adaxial leaf waxes ranged from C_{20} to C_{28}, while the fraction of the primary alcohols contained only small amounts of hexacosanol (C_{26}) and octacosanol (C_{28}). The average chain length (ACL) of very-long-chain acyclic wax compounds was 25.8 for green, 26.6 for turning and 27.3 for black fruits and 30.1 for the adaxial leaf surface (Table 3). The ratios of cuticular wax and cutin coverages were in the range of 0.2 for all three developmental stages of fruits and 0.52 for leaves (Table 3).

Cuticular Water Permeability of *Olea europaea* Fruits and Leaves

The fruit surface area was determined for relating the water loss to the transpiring area. It increased significantly by 28% from 4.6 cm^2 in green to 6.1 cm^2 in turning and 6.4 cm^2 in black fruits (Figure 2A). The cuticular transpiration of astomatous fruit and adaxial leaf

surfaces was determined gravimetrically. For both fruits and leaf, the cuticular water loss followed a linear time course. The cuticular water loss of fruits at the three considered developmental stages (green, turning and black) showed no significant differences but it was considerably higher than the water loss rate across the adaxial leaf surface. Based on the driving force, the cuticular water permeance was calculated. The cuticular water permeances of green, turning and black fruits exhibited no significant differences and were on the average 9.5×10^{-5} m s^{-1} (Figure 2B). The cuticular water permeance of the adaxial leaf surface was 2.1 $\times 10^{-5}$ m s^{-1} and, thus, was significantly lower by a factor of five than that of the fruits.

DISCUSSION

The predominant cutin monomers of olive fruits in all three developmental stages are C_{16} and C_{18} type monomers, which were dominated by 9/10,ω-dihydroxyhexadecanoic acid similarly as in many other fruit crops such as *Prunus avium* L. (drupe)[25], *Rubus chamaemorus* L. (aggregate fruit with drupelets)[26], *Capsicum annuum* L. (berry)[27] and *Solanum lycopersicum* L. (berry).[28] Overall, the amounts of cutin monomers from the green to the turning and black stage did not change significantly. A shift to a higher amount of C_{18} type monomers e.g. octadecenoic acid, ω-hydroxyoctadecenoic acid and 9,10,ω-trihydroxyoctadecenoic acid, resulting in an alteration of the C_{16}/C_{18} ratio from 1.1 to 0.8 during fruit development, was detected. Kosma et al.[29] showed an increase in total cutin monomers for *Solanum lycopersicum* L. during fruit development. In contrast, a decrease of total cutin was detected during the final ripening of *Prunus avium* L. fruits.[25]

In comparison to the black-stage fruit, the adaxial leaf surface had a significantly smaller amount of cutin monomers. The prominent 9,10-epoxy-ω-hydroxyoctadecanoic acid found in the olive leaf cutin had also been detected in leaves of *Agave americana* L., *Clivia miniata* (Lindl.) Bosse, *Ilex aquifolium* L., *Nerium oleander* L. and *Sansevieria trifasciata* Prain (evergreen leaves)[30,31] and was not present in the olive fruits. Furthermore, the phenolic cutin

fraction exclusively consisting of coumaric acid and derivatives was distinctly reduced in the cutin matrix of olive leaves. The ratio between the long-chain acyclic and the phenolic cutin proportions was 23.7 for black fruits but 215.4 for fully expanded leaves.

The ratio of C_{16}/C_{18} cutin monomers was 0.5 for the leaf cutin and 0.8 for the black fruit cutin. The leaf and fruit cutin of olive displayed a mixed C_{16} and C_{18} type, which is very common in plants, e.g. in *Ilex aquifolium* L..[32] The fruit cutin matrix was also characterized by a substantial proportion of unsaturated cutin monomers. The compositional differences between fruit and leaf cutin of olive (carbon chain lengths, the degree of unsaturation, and the extent of epoxidation) may lead to unique polymeric structures in both organs.

The cuticular waxes from the olive fruit surface of three developmental stages were dominated by pentacyclic triterpenoids. Triterpenoid coverage did not significantly change during fruit development. A decrease of triterpenoids in developing fruits was reported for *Prunus avium* L..[25] The very-long-chain acyclic components, mainly fatty acids and primary alcohols, constituted the smaller portion of cuticular waxes. An increase of the very-long-chain acyclic fraction during fruit development was primarily due to a higher accumulation of alkyl esters and primary alcohols. Bianchi et al.[17] compared the green and black developmental stages of fruits of the `Coratina´ cultivar. Similarly as found for the cultivar `Arbequina,' the black-stage fruits exhibit a higher percentage of very-long-chain acyclic components compared to the green-stage fruit. Also in the case of the `Coratina´ cultivar, the fraction of pentacyclic triterpenoids decreased from the green to the black stage.

Overall, the total amount of cuticular waxes covering olive fruits was more or less constant at the green, turning and black stages, which comprise the late fruit expansion phase. For *Solanum lycopersicum* L. and *Prunus avium* L., a slight decrease of the cuticular wax quantity during the later fruit developmental stages was shown.[25,28] This reduction of cuticular waxes was described as not a mere dilution effect, which could be due to the surface area expansion, but resulted from specific changes of individual constituents.[25]

Oleanolic acid was the major cuticular wax component in olive fruits over all three developmental stages. As for olive fruits, this triterpene acid was also found as the main triterpenoid in other fruit crops, e.g., *Prunus domestica* L. (drupe)[33], *Vaccinium myrtillus* L. (berry)[34] and *Vitis vinifera* L. (berry).[35-37] Fatty acids and primary alcohols with carbon chain lengths between C_{20} and C_{30} were the most pronounced very-long-chain acyclic wax compound classes in olive fruits. The very-long-chain acyclic wax fraction showed a continuous increase in the average chain length (ACL), which describes the average number of carbon atoms of very-long-chain acyclic components[38], from 25.8 to 27.3 over the three developmental stages. The ACL value is widely accepted as a proxy indicator for the cuticular wax quality in plants.

Comparing the black-stage fruit with the adaxial leaf surface, the total cuticular wax amount was significantly higher for the leaf surface than for the fruit surface. The leaf cuticular waxes were mainly composed of pentacyclic triterpenoids and a smaller fraction of very-long-chain acyclic components. Like in fruits, oleanolic acid was the major cuticular wax compound in leaves, which is not common for leaf cuticular waxes.[39] In leaves of *Ligustrum vulgare* L., also a member of the Oleaceae plant family, ursolic acid dominated the cuticular wax mixture.[40]

Fruits and leaves of olive were characterized by an organ-specific pattern of triterpenoid composition, which might indicate differences in the triterpenoid biosynthesis in both organs. Cuticular waxes of olive contained mainly pentacyclic triterpenoids from the oleanane type: predominantly oleanolic acid and maslinic acid in fruits and oleanolic acid and erythrodiol in leaves. With the exception of the deposition on the olive fruit surface, maslinic acid has been very rarely detected in fruits, e.g. in *Ziziphus jujuba* Mill. (drupe)[41], *Rubus chingii* Hu (aggregate fruit with drupelets)[42] and *Malus pumila* Mill. (pome)[43]. Additionally, cuticular waxes of olive leaves exhibited a high amount of ursolic acid and uvaol that belong to the

ursane type of triterpenoids, whereas only fruit cuticular waxes had betulinic acid of the lupane type.[44]

The ratio between the very-long-chain acyclic and the cyclic wax proportions was 0.5 for black fruits and only 0.04 for fully expanded leaves. The very-long-chain acyclic wax fraction of olive leaves exhibited a considerably higher average chain length (ACL) value of 30.1. *N*-alkanes and fatty acids with carbon chain lengths between C_{26} and C_{33} as well as C_{20} and C_{30} were the prominent very-long-chain acyclic wax compound classes in the cuticular waxes of olive leaves. Bianchi et al.[18] analyzed the leaf cuticular waxes of the olive cultivars `Cipressino´ and `Coratina´. They also found that the main proportion were triterpenoids, and that the very-long-chain acyclic fraction was mainly composed of *n*-alkanes and fatty acids. Similar very-long-chain acyclic component classes were detected for the `Arbequina´ cultivar, but the percentage of individual component classes might be cultivar-specific.

Olive fruits and leaves were characterized by a high amount of cuticular waxes but significant organ-specific modifications in both the very-long-chain acyclic wax compound classes and triterpene types, which might have a major influence on the barrier properties of the olive leaf and fruit surface. The total amounts and composition of the cuticles of olive fruits and leaves were different, also showing a different ratio between cuticular waxes and cutin monomers (0.2 for black fruits and 0.5 for fully expanded leaves), which may have an impact on the cuticular functions.

The cuticular water permeability of the three developmental fruit stages was not significantly different and was on the average 9.5×10^{-5} m s^{-1}. The increasing coverage of very-long-chain acyclic compounds from 39 (green) to 62 µg cm^{-2} (black) did not lead to changes in cuticular permeability.

The adaxial leaf surface of the `Arbequina´ cultivar had a cuticular permeance of 2.1×10^{-5} m s^{-1} which is significantly lower by a factor of five than the water permeability of the black-stage fruits. Cuticular water permeances of leaves within the Oleaceae reported so far ranged

from 1.5×10^{-5} m s^{-1} to 1.9×10^{-5} m s^{-1} measured for *Ligustrum vulgare* L., *Forsythia suspensa* (Thunb.) Vahl and *Syringa vulgaris* L..[45,46] Schreiber and Riederer[45] determined a value of 5.5×10^{-6} m s^{-1} for isolated leaf cuticles from an unspecified olive cultivar, which was distinctly lower than that found for the `Arbequina´ cultivar in this study. A study comparing leaves and fruits of several species showed that fruits generally had higher cuticular permeabilities than leaves.[45] Up to now, there was no study directly comparing the cuticular permeability of fruits and leaves of the same species.

Alterations in cuticle composition are commonly proposed to affect the cuticular transport barrier. Within the plant cuticle, the cuticular waxes establish the main transport-limiting barrier. Differences in the cutin polymer composition and the corresponding primary and secondary ester linkages have also been proposed to influence the barrier properties.[15,16,47] The transpiration barrier properties of the cuticular waxes are associated with the very-long-chain acyclic wax components rather than with the cyclic wax compounds.[28,48,49] A model of the physical arrangement of the wax constituents suggests that very-long-chain acyclic components are arranged in impermeable crystalline domains and amorphous zones in between which may also contain cyclic components.[14,50] Water diffusion is assumed to occur only in the amorphous zone following a very tortuous pathway around the crystalline flakes. Therefore, it has been proposed that the physical properties derived from wax composition and not wax quantity determine the efficacy of the transpiration barrier.[10,14] In analogy to polyethylene, which is a semi-crystalline long-chain acyclic material comparable to cuticular waxes, the degree of crystallinity should determine the barrier properties of cuticular waxes. The higher the crystallinity is, the longer is the effective pathway, and consequently, the lower is the effective diffusion coefficient of water molecules across this barrier.[51] The average chain length (ACL) of the very-long-chain acyclic wax components is one of several parameters influencing crystallinity.

In olive, the coverage of very-long-chain acyclic components in the cuticular waxes is almost 6-fold lower in leaves than in black-stage fruits.At the same time leaves have a two-fold higher coverage of cyclic components. So, at first sight, one might expect that the fruits should have a lower cuticular permeability since it has been shown in the past that the very-long-chain acyclic components primarily make up the transpiration barrier while the contribution of the cyclic components to the barrier is small or absent. Therefore, the reason for the observed higher efficacy of the leaf transpiration barrier might be caused by differences in the molecular properties of the very-long-chain fraction. We hypothesize that the higher ACL in leaves as compared to fruits might be a prominent factor leading to the considerably lower permeability of the leaf cuticle. Additional factors like cutin polymer structure and cutin/wax interactions may also contribute to the organ-specific differences in cuticular permeability observed in this study.

Funding Sources

This project was supported by an Oversea Study Program of Guangzhou Elite Project (GEP, Approval No. Sui Jiaoke [2013]94) to HH and by a Chinese Academy of Sciences Visiting Professorship for Senior International Scientists grant no. 2011T2S31 to MR.

ASSOCIATED CONTENT

Supporting Information

Table S1. Detailed chemical composition of the cutin polymer of *Olea europaea* L. cultivar `Arbequina´ fruits in green, turning and black stages and the fully expanded, adaxial leaf surface. Data were given as means ± standard deviation in μg cm^{-2} (n = 5).

Table S2. Detailed chemical composition of the cuticular waxes of *Olea europaea* L. cultivar `Arbequina´ fruits in green, turning and black stages and the fully expanded adaxial leaf surface. Data were given as means ± standard deviation in μg cm^{-2} (n = 5).

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Figure Legends

Figure 1. The total amount of cutin monomers (a) and the total amount of cuticular waxes (b) for the three developmental stages of *Olea europaea* L. cultivar `Arbequina´ fruits (green, turning and black) and the adaxial leaf surface (n = 5).

There were no statistical differences between the three fruit stages ($p = 0.074$ and $p = 0.565$ for cutin and wax respectively). The leaf cutin monomer amount was significantly lower ($p < 0.001$) and the leaf total cuticular wax amount was significantly higher ($p < 0.001$) in comparison to the fruit (black stage).

Figure 2. Surface area (a) and the permeance for water (b) of *Olea europaea* L. cultivar `Arbequina´ fruits (green, turning and black) and the adaxial leaf surface of fully expanded leaves (n = 12).

The surface area of the three fruit stages differed significantly ($p < 0.001$, green and turning $p < 0.001$, green and black $p < 0.001$, turning and black $p = 0.051$). The water permeances of the fruit stages did not differ significantly ($p = 0.187$). The water permeance of the adaxial leaf surface was significantly lower than the water permeance of the black-stage fruits ($p < 0.01$).

Table 1. Composition of the cutin polymer of *Olea europaea* L. cultivar `Arbequina´ fruits in green, turning and black stages and the fully expanded, adaxial leaf surface. Data were given as means ± standard deviations (µg cm^{-2}; n = 5).

compound classes	**fruit**			**leaf**
	green stage	**turning stage**	**black stage**	**adaxial surface**
fatty acids	17.92 ± 2.47	19.21 ± 1.99	32.06 ± 11.34	11.68 ± 3.93
ω-hydroxy fatty acids	46.09 ± 11.91	49.56 ± 9.34	96.30 ± 31.42	13.56 ± 2.26
ω-hydroxy fatty acids with mid-chain hydroxy group	698.90 ± 43.17	742.11 ± 60.68	864.72 ± 143.71	273.03 ± 39.28
ω-hydroxy fatty acids with mid-chain epoxy group	-	-	-	247.36 ± 40.68
ω-hydroxy fatty acids with mid-chain oxo group	11.09 ± 9.74	5.60 ± 2.93	5.85 ± 2.53	-
α,ω-dicarboxylic fatty acids	1.38 ± 0.25	2.91 ± 2.10	1.70 ± 0.28	-
α,ω-dicarboxylic fatty acids with mid-chain epoxy group	9.61 ± 3.69	7.26 ± 4.53	5.81 ± 2.47	3.66 ± 0.60
phenolic compounds	46.61 ± 5.14	44.19 ± 5.54	42.39 ± 4.53	2.55 ± 0.58
not identified	137.68 ± 13.96	134.10 ± 16.08	106.29 ± 13.12	78.62 ± 11.12
total cutin	**969.29 ± 54.10**	**1004.94 ± 71.42**	**1129.05 ± 151.04**	**630.46 ± 88.57**

Table 2. Composition of the cuticular waxes of *Olea europaea* L. cultivar `Arbequina´ fruits in green, turning and black stages and the fully expanded adaxial leaf surface. Data were given as means ± standard deviations (μg cm^{-2}; n = 5).

compound classes	fruit			leaf
	green stage	turning stage	black stage	adaxial surface
fatty acids	15.23 ± 2.50	18.60 ± 2.48	18.62 ± 1.60	2.14 ± 0.64
primary alcohols	17.86 ± 2.16	21.43 ± 2.07	23.4 ± 2.27	0.25 ± 0.05
aldehydes	3.19 ± 0.79	4.67 ± 0.66	4.55 ± 0.94	-
n-alkanes	1.12 ± 0.22	2.27 ± 0.68	3.23 ± 0.84	8.47 ± 3.09
alkyl esters	0.53 ± 0.13	3.77 ± 1.32	8.60 ± 3.00	-
methyl esters	0.10 ± 0.02	0.20 ± 0.03	0.17 ± 0.09	-
phenyl methyl esters	0.55 ± 0.23	1.24 ± 0.28	1.41 ± 0.50	-
diacylglycerols	0.82 ± 0.26	1.71 ± 0.48	2.98 ± 0.62	-
triterpenoids	144.45 ± 19.43	135.82 ± 12.97	129.19 ± 12.18	266.36 ± 38.97
sterols	0.08 ± 0.04	0.06 ± 0.03	0.08 ± 0.03	-
not identified	10.69 ± 3.82	11.84 ± 2.97	16.41 ± 1.92	43.19 ± 13.71
total wax	**194.61 ± 22.84**	**201.61 ± 19.65**	**208.64 ± 17.94**	**320.41 ± 39.54**

Table 3. Amount of cyclic and very-long-chain acyclic wax fractions (µg cm^{-2}), weighted average chain length (ACL) of the very-long-chain acyclic wax fraction, ratio of cuticular waxes and cutin, ratio of main cutin monomers of C_{16} and C_{18} of *Olea europaea* L. cultivar `Arbequina´ fruits in green, turning and black stages and the fully expanded adaxial leaf surface. Data were given as means ± standard deviations (n = 5).

organ		cyclic fraction	very-long-chain acyclic fraction	ACL	wax/cutin	C_{16}/C_{18}
fruit	green stage	145.07 ± 19.31	38.85 ± 5.26	25.80 ± 0.11	0.20 ± 0.03	1.10 ± 0.11
	turning stage	137.12 ± 13.07	52.65 ± 6.18	26.55 ± 0.19	0.20 ± 0.03	1.06 ± 0.12
	black stage	130.68 ± 12.09	61.55 ± 5.94	27.27 ± 0.26	0.19 ± 0.03	0.78 ± 0.17
leaf	leaf adaxial	266.36 ± 38.97	10.86 ± 3.51	30.06 ± 0.27	0.52 ± 0.07	0.45 ± 0.02

Figure 1.

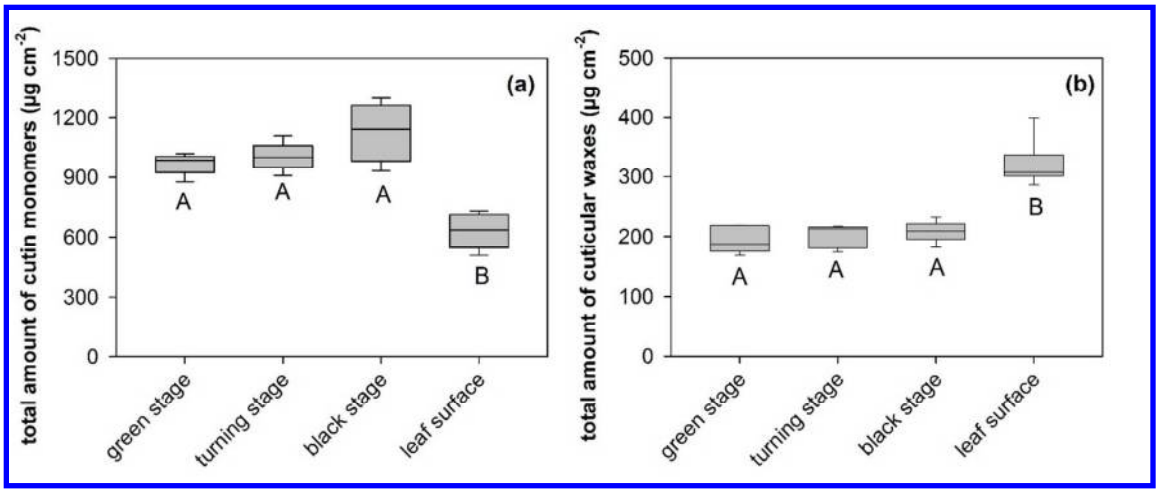
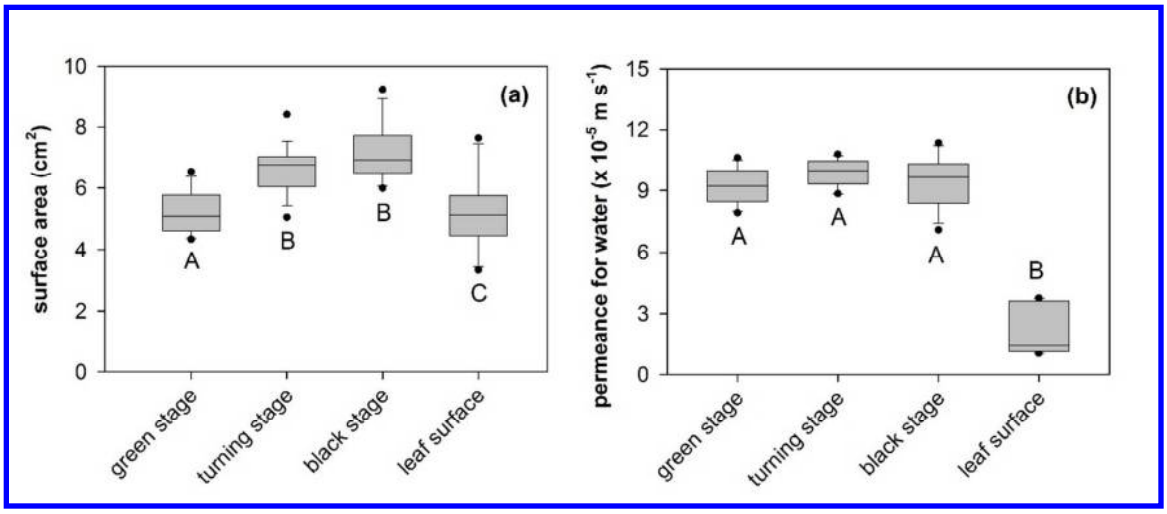
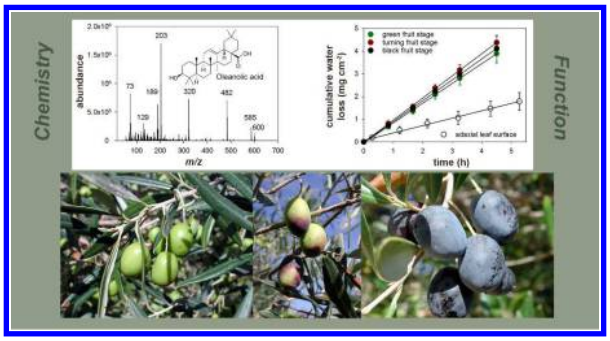

total amount of cutin monomers (μg cm-2)
1500
1200
900
600
300
0
(a)
A
A
A
B
green stage
turning stage
black stage
leaf surface
total amount of cuticular waxes (μg cm-2)
500
400
300
200
100
0
(b)
A
A
A
B
green stage
turning stage
black stage
leaf surface

Figure 2.


surface area (cm^2)
(a)
A
B
B
C
green stage
turning stage
black stage
leaf surface
permeance for water (x 10^{-5} m s^{-1})
(b)
A
A
A
B
green stage
turning stage
black stage
leaf surface

TOC graphic


Chemistry
Function
abundance
m/z
Oleanolic acid
cumulative water loss (mg cm⁻²)
time (h)
green fruit stage
turning fruit stage
black fruit stage
adaxial leaf surface