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ARTICLE

Phenol Metabolism in the Leaves of the Olive Tree (*Olea europaea* L.) cv. Picual, Verdial, Arbequina, and Frantoio during Ripening

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The kinetic behavior and protein-expression level of phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) have been determined in the leaves of the olive tree (*Olea europaea* L.) of cv. Picual, Verdial, Arbequina, and Frantoio during fruit ripening. Moreover, the concentration of total phenolic compounds, oleuropein, hydroxytyrosol, and tyrosol has been also determined. This study was carried out in 20-year-old olive trees grown in Jaén (Spain). The concentration of total and specific phenols showed a specific pattern in each cultivar. Frantoio showed the highest phenol concentration followed by Arbequina, Picual, and Verdial. A coordinated response between PAL, PPO, and the concentration of total phenols in the four cultivars was found. Also, specific changes were shown over the course of ripening, indicating a regulation of PAL, PPO, and phenol concentration in the olive-tree leaves during fruit ripening.

KEYWORDS: *Olea europaea*; phenolic compounds; phenylalanine ammonia-lyase; polyphenol oxidase; ripening

INTRODUCTION

The study of the metabolism of phenolic compounds in the leaf of the olive tree (*Olea europaea* L.) is the current objective of several studies and the prime aim of the present work. Among all phenolic compounds, oleuropein (Ole) and hydroxytyrosol (Htyr, 3,4-dihydroxyphenylethanol) are two of the most abundant in the olive leaf that have important beneficial properties (*1*). Ole is the heterosidic ester of Htyr with β-glucosylated elenolic acid. This compound is typical of the Oleaceae family, and it has been proposed that it is biosynthesized from mevalonic acid in a complex metabolic pathway (*2*). In studies performed mainly in vitro, it has been demonstrated that Ole has important biological and health properties (*3*), including antiatherogenic, cardioprotective, hypoglycaemic, antihypertensive, antiviral, anti-inflammatory, cytostatic, molluscicidal, endocrinal, and enzymatic modulation activity (*4*). Htyr, in addition to conferring most of the biological properties of Ole, protects the human erythrocytes against oxidative damage, reduces superoxide anion production in the human promonocyte cells, inhibits peroxynitrite-dependent damage, induces cytochrome *C*-dependent apoptosis, inhibits tumor-cell proliferation, and inhibits leukocyte leukotriene B_4 (*5*,*6*). Recently, it has been reported that pure Htyr transiently associates with LDL lipoproteins in humans in vivo (*7*). Other phenolic compounds identified in olive leaves were verbascoside, ligstroside, and flavonoid glycosides including luteolin-7-rutinoside, luteolin 4′-glucoside, apigenin-7-glucoside, and apigenin-7-rutinoside (*4*).

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Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is a regulatory enzyme involved in the metabolism of phenolic compounds in the olive tree. PAL catalyzed the first reaction in the pathway of phenylpropanoid biosynthesis in which L-phenylalanine (L-Phe) is deaminated to form *trans*-cinnamic acid and free ammonium. This is the first reaction in the biosynthesis of a large group of phenylpropanoid-derived secondary products such as flavonoids, isoflavonoids, coumarins, lignins, wound-protective hydroxycinnamic acid esters, and other phenolic compounds such as tyrosol (*8*). PAL has been characterized by us in the olive tree cv. Picual during ripening (*9*). Polyphenol oxidase (PPO, EC 1.10.3.1) has a major function in the oxidative degradation of phenolic compounds. PPO catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-diquinones. These compounds provoke a browning color and are accumulated during ripening and during biotic as well as abiotic stress (*10*). We have reported the characterization of PPO of olive cv. Picual during ripening (*11*).

Currently, the olive leaf is being investigated at different levels and in different fields. Because of their richness in antioxidant phenols, olive leaves have recently been successfully mixed with overripe olives (2−3%) before processing to produce oils with a more marked flavor and higher resistance to oxidation (*12*, *13*). Tablets of olive leaf extracts have been marketed as a dietetic product or food integrator (*14*). Triterpenic acids, specifically oleanolic and maslinic acid, also are found in high concentration in olive leaves and have important beneficial properties related with health and nutrition (*15*−*17*). However, in practice, most of the leaves resulting from the pruning of olive trees are at present considered as a low-value byproduct/waste, used only for biomass production or farming.

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Regarding the ripening process, the metabolism of phenolic compounds in the olive tree is determined by the cultivar considered (*9*, *18*). We have previously reported that in fruits of the olive cultivars Picual, Verdial, Arbequina, and Frantoio, PAL and PPO showed a coordinated response that can explain the phenol concentration shown in the different cultivars over ripening. Also, we previously demonstrated that the olive leaf has active phenol metabolism (*9*, *10*, *19*).

The aim of the present work is to study whether the olive cultivar influences phenol metabolism in the leaves of olive trees. For this, we determined the concentration of total phenols, Ole, Htyr, and tyrosol (Tyr), in the leaves of olive cv. Picual, Verdial, Arbequina, and Frantoio, four major olive cultivars used for oil production. We also determined the kinetic and protein-expression level of PAL and PPO. Finally, this study has been made over the ripening period of olive fruits. This is a periodical annual situation involving olive trees that determines the metabolism of phenols in all the organs of the tree. The data compiled in this work will serve to re-evaluate the olive leaf in order to ascertain the best time to use it commercially and to evaluate the qualities of the different cultivars.

MATERIALS AND METHODS

Chemicals. Substrates, coenzymes, and other reagents such as β-mercaptoethanol, polyvinylpolypyrrolidone, soybean trypsin inhibitor Type II, tropolone, and 1,4-aminobenzotriazole were purchased from SIGMA Chemical Co. (St Louis, USA) and Fluka Chemie GmbH (Buchs, Switzerland). The chemicals used for SDS−PAGE and immunoblot were from BioRad Laboratories (Hercules, USA) and Pierce Biotechnology Inc. (Rockford, USA). Ole, Htyr, and Tyr were purchased from Extrasynthèse (Z.I. Lyon-Nord, Genay, France). The reagents used in HPLC were of HPLC grade.

Plant Material and Experimental Design. Olive trees (*Olea europaea*) of cv. Picual, Verdial, Arbequina, and Frantoio, 20 years old, located in the Agricultural Research and Training Centre "Estación de Olivicultura y Elaiotecnia Venta del Llano" (Mengíbar, Jaén, Junta de Andalucía, Spain) were used for this study. The single-trunked trees were grown at 7×7 m apart in the experimental farm located at 280 m above sea level under traditional rain-fed cultivation. The trees of each variety belonged to experimental orchards located near one of them. All orchards have similar soil conditions and the same climatic and cultivation (irrigation and fertilizers) conditions. Four different samples (called samples 1, 2, 3, and 4) were collected throughout the ripening period of olive fruits, from July to December. Samples 1, 2, 3, and 4 were picked on 31 July, 10 October, 1 November, and 3 December of 2002, respectively. In each orchard, five trees periodically distributed were chosen, and leaves were sampled from all orientations of the tree. From each orientation in each tree, five 25-cm segments of branches with fruits near the apical end were collected. The leaves and fruits were separated from the branches, and all the leaves and fruits from all five trees were pooled. The pool of leaves was divided into five replicates and frozen in liquid nitrogen (−70 °C) until analyzed. With the pool of fruits, the ripeness index (RI) was determined using a color evaluation of the skin and flesh (*20*). The procedure consists of distributing approximately 100 olive fruits into eight groups, according to the following characteristics: group 0, skin bright green; group 1, skin green-yellowish; group 2, skin green with reddish spots; group 3, skin reddish brown; group 4, skin black with white flesh; group 5, skin black with less than 50% of purple flesh; group 6, skin black with more than 50% purple flesh; and group 7, skin black with 100% purple flesh. Afterward, the number of fruits was counted in each group, and the RI was determined by the equation $RI = \sum(in_i)/100$, where i is the number of the group, and ni is the number of olives in it.

The water content of the leaves was determined by weighing 5 g of leaves and then drying at 55 °C in an oven to constant weight. The samples were cooled for 30 min in a dryer and reweighed.

Extraction and Assay of PAL of Leaves of Olive Trees. The procedure used to extract and assay PAL followed Ortega-García et al. (*9*). This procedure was carried out with leaves samples of the four olive-tree cultivars studied picked at the four ripening stages. Five replicates were made of each sample corresponding to each ripening state of each cultivar. After being defrosted, olive leaves were washed in NaCl 9 g L^{-1}, cut into fragments of 1 or 2 mm that were pulverized in a mortar with liquid nitrogen. A homogenate was made at a proportion of 1:5 w/v with an extraction buffer that contained 0.1 mol L^{-1} sodium phosphate buffer at pH 8.0, 50 g L^{-1} polyvinylpolypyrrolidone, 3 g L^{-1} soybean trypsin inhibitor Type II, and 2 mmol L^{-1} β-mercaptoethanol. The homogenate was filtered through glass wool, and the filtrate was centrifuged at 20000*g* for 20 min at 4 °C. The proteins of the supernatant were precipitated by adding ammonium sulfate to 80% saturation. The pH in the samples was maintained between 7 and 7.5. The precipitated proteins were collected by centrifugation at 20000*g* at 4 °C for 20 min. The pellet was dissolved and dialyzed overnight at 4 °C. The dialyzed samples were used to assay the PAL activity, protein determination, and immunoblot analysis.

PAL was assayed in a medium containing 37.5 mmol L^{-1} borate buffer at pH 8.8, 2 mmol L^{-1} mercaptoethanol, 6.67 μmol L^{-1} tropolone (an inhibitor of polyphenol oxidase), 66.67 μmol L^{-1} 1,4-aminobenzotriazole (an inhibitor of cinnamate-4-hydroxylase), L-Phe at variable concentration, from 0.05 to 10 mmol L^{-1}, and 0.12 mL of the enzyme extract. These media were incubated at 40 °C for 0, 10, 20, 30, 40, and 60 min. The reaction was stopped by adding 0.05 mL of 4.5 mol L^{-1} H_2SO_4. In the samples at time 0, first the acid was added and then the enzyme extract. The acidified samples were incubated in a boiling water bath for 10 min and afterward centrifuged at 1500*g* for 10 min. An aliquot of 2.5 mL of diethyl ether was added to each supernatant and vigorously shaken in a vortex for 2 min to extract the cinnamate. One milliliter of the ether phase was collected and evaporated. The resulting residue was dissolved in 0.05 mol L^{-1} NaOH, and the absorbance at 268 nm was recorded. The initial velocity of the reaction was determined considering the reaction-progress curve drawn on plotting the results found at the different assay times. One unit of PAL activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of cinnamic acid min^{-1} in a cuvette of 1 cm under optimum assay conditions. The protein concentration was determined by the Bradford method (*21*) using bovine serum albumin as the standard.

Determination of PAL Protein Levels by Immunoblot. PAL specific protein levels were determined by Western blot as described Ortega-García et al. (*9*). Samples prepared as described above were mixed with 62.5 mmol L^{-1} Tris-HCl at pH 6.8 buffer containing 20 g L^{-1} SDS, 100 mL L^{-1} glycerol, 25 g L^{-1} β-mercaptoethanol, and 0.045 mmol L^{-1} bromophenol blue and then heated for 3 min at 95 °C. Polypeptides were separated on 7.5% SDS−PAGE and afterward transferred to polyvinylidene fluoride membranes with a semidry electroblotting system at 1.5 mA cm^{-2} for 45 min in a medium containing 25 mmol L^{-1} Tris-HCl, 192 mmol L^{-1} glycine, 200 mL L^{-1} methanol, and 1 g L^{-1} SDS. Blots were blocked for 4.5 h at room temperature with a buffer containing 25 mmol L^{-1} Tris-HCl, 100 mmol L^{-1} NaCl, 2.5 mmol L^{-1} KCl, pH 7.6 (TBS), 1 mL L^{-1} Tween20, and 15 g L^{-1} bovine serum albumin (BSA) at pH 7.6. Membranes were washed with TBS containing 1 mL L^{-1} Tween20 (TBS-T) for 15 min and afterward incubated with a rabbit antiserum antiparsley PAL-1 (1:5000). The antiserum anti-PAL-1 was generously donated by Dr. N. Amrheim, Institut für Pflanzenwissenschafte, Eidgenössische Technische Hochschule of Zurich, Switzerland. After three washes with TBS-T containing 10 g L^{-1} BSA (TBS-T-BSA) for 10 min, membranes were incubated with a biotinylated goat antibody antirabbit-IgG. After three washes with TBS-T-BSA, immunodetection was performed using a chemiluminescent intensification system. Membranes were scanned with a Hewlett-Packard scanner and densitometer with a Bio-Rad Fluor-S Multimager and quantifier using Bio-Rad Quantity One software.

Extraction and Assay of PPO of Olive Leaves. The procedure used to extract and assay the PPO followed Ortega-García et al. (*11*). Acetone powders of the pulverized leaf samples were prepared using cold acetone and polyethylenglycol. Immediately before each PPO assay, acetone powder was resuspended in 0.1 mol L^{-1} sodium phosphate buffer, pH 6.2, with 0.3 g L^{-1} of type-II trypsin inhibitor. After filtration with glass wool, the filtrate was centrifuged at 10000*g* for 20 min at 4 °C. The supernatants were used for PPO assays, protein quantification, and PAGE.

Polyphenol oxidase activity was determined by spectrophotometry at 420 nm using catechol as the substrate. Assays were performed at 30 °C in

Table 1. Time Course of the Concentration of Water, Protein, and Total Phenols in Leaves of *Olea europaea* cv. Picual, Verdial, Arbequina, and Frantoio during Fruit Ripening[a]

	sample 1 (July)	sample 2 (October)	sample 3 (November)	sample 4 (December)
time (days)	1	42	63	96
		Fruit Ripeness Index		
Picual	0_a^x	$1.12 \pm 0.06_b^x$	$3.10 \pm 0.16_c^x$	$3.25 \pm 0.16_c^x$
Verdial	0_a^x	$1.22 \pm 0.07_b^x$	$2.88 \pm 0.14_c^x$	$3.91 \pm 0.20_d^y$
Arbequina	0_a^x	$1.18 \pm 0.06_b^x$	$1.78 \pm 0.09_c^y$	$2.55 \pm 0.13_d^z$
Frantoio	0_a^x	$2.97 \pm 0.15_b^y$	$4.34 \pm 0.22_c^z$	$3.72 \pm 0.19_c^{xy}$
		Water (g (kg weight)$^{-1}$)		
Picual	$500.1 \pm 1.5_a^x$	$500.0 \pm 6.7_a^x$	$482.3 \pm 11.0_{ab}^x$	$449.0 \pm 26.7_b^x$
Verdial	$530.5 \pm 4.5_a^y$	$520.0 \pm 10.8_a^{xy}$	$521.0 \pm 9.5_a^y$	$480.0 \pm 13.0_b^x$
Arbequina	$508.5 \pm 13.2_a^{xy}$	$507.0 \pm 10.0_a^x$	$551.0 \pm 22.5_a^z$	$492.4 \pm 9.6_a^x$
Frantoio	$526.8 \pm 10.7_a^y$	$540.2 \pm 9.8_a^y$	$541.2 \pm 6.0_a^{zy}$	$485.7 \pm 14.6_b^x$
		Protein (g (kg dry weight)$^{-1}$)		
Picual	$2.21 \pm 0.11_a^x$	$2.82 \pm 0.17_b^x$	$1.67 \pm 0.07_c^x$	$0.95 \pm 0.07_d^x$
Verdial	$1.49 \pm 0.07_a^y$	$1.43 \pm 0.09_a^y$	$3.33 \pm 0.07_b^y$	$1.65 \pm 0.11_a^y$
Arbequina	$0.39 \pm 0.02_a^z$	$0.98 \pm 0.06_b^z$	$0.67 \pm 0.09_c^z$	$2.03 \pm 0.15_d^z$
Frantoio	$0.42 \pm 0.01_a^z$	$2.35 \pm 0.23_b^x$	$3.44 \pm 0.18_c^y$	$2.24 \pm 0.19_b^z$
		Total Phenols (g (kg dry weight)$^{-1}$)		
Picual	$28.30 \pm 0.34_a^x$	$31.48 \pm 0.29_b^x$	$32.84 \pm 0.31_c^x$	$22.62 \pm 0.30_d^x$
Verdial	$37.81 \pm 0.48_a^y$	$23.73 \pm 0.11_b^y$	$21.44 \pm 0.60_c^y$	$27.55 \pm 0.17_d^y$
Arbequina	$49.52 \pm 0.14_a^z$	$26.30 \pm 0.17_b^z$	$34.23 \pm 0.20_c^z$	$26.96 \pm 0.21_b^z$
Frantoio	$40.53 \pm 0.15_a^w$	$41.95 \pm 0.19_a^w$	$54.33 \pm 0.20_b^w$	$41.63 \pm 0.15_a^w$

[a] Samples were harvested on four dates corresponding to different ripening status. Values are the means ± SEM of 10 data. The results were analysed by a two-way ANOVA and Student's *t*- test. For each parameter, the data in each row followed by different subindices (a−d) are statistically different ($p < 0.05$). For a comparison between cultivars, the data in each column followed by different superscripted indices (x, y, z, and w) are statistically different ($p < 0.05$).

a medium containing 0.1 mol L^{-1} sodium phosphate buffer, pH 6.2, 1.25−500 mmol L^{-1} catechol, and 50 μL of leaf enzyme extract in a total volume of 1 mL. The unspecific photooxidation of catechol was evaluated and corrected for. Specific activity was expressed as units (mg protein)$^{-1}$. One unit is defined as the amount of enzyme needed to prompt an increase of 0.1 optical density unit min^{-1} in a 1-cm cuvette at 30 °C under our standard assay conditions.

The PAL and PPO kinetic parameters (maximum velocity, V_{max}, and Michaelis constant, K_m) and kinetic behavior were determined by the nonlinear-regression analysis program GraFit (Erithacus Software Ltd., Surrey, UK).

SDS−PAGE and PPO Assay in-Gel. Partially denaturing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed as described by Ortega-García et al. (*11*). The samples used for SDS−PAGE were made without the addition of 2-mercaptoethanol and without heating to preserve PPO activity. Samples of supernatants of leaf acetone powders were mixed with 0.5 mol L^{-1} Tris-HCl, pH 6.8, containing 100 mL L^{-1} glycerol and 0.02 g L^{-1} bromophenol blue and subjected to 10% SDS−PAGE in a minigel Bio-Rad system (Bio-Rad Laboratories, Hercules USA), following Laemmli (*22*). Electrophoresis was performed for 1.5 h at constant voltage (100 V) in a buffer containing 15 g L^{-1} (w/v) Tris base, 72 g L^{-1} glycine, and 5 g L^{-1} (w/v) SDS. All operations were performed at 4 °C.

After electrophoresis, gels were incubated for 45 min with 5 mmol L^{-1} of DL-DOPA in the presence of 100000 U L^{-1} catalase dissolved in 0.1 mol L^{-1} sodium phosphate buffer pH 7.3. The reaction was stopped with 0.2 mmol L^{-1} of diethyldithiocarbamic acid. A second gel was stained specifically for peroxidase activity with 50 mmol L^{-1} guaiacol plus 0.2 mL of 10 mL L^{-1} H_2O_2.

Extraction, Analysis, and Quantification of Phenolic Compounds. The phenolic compounds in the leaves were extracted as in Ortega-García et al. (*11*). Leaf samples were pulverized in a mortar with liquid nitrogen and afterward homogenized in 800 mL L^{-1} methanol (1:4, w/v). The homogenates were vigorously shaken in a vortex for 2 min. After sedimentation, the methanol phase was decanted and the residue re-extracted with the same volume of 800 mL L^{-1} methanol. All of the methanol phases were pooled, and an equal volume of hexane was added. The mixture was again vigorously shaken in a vortex for 2 min, and after phase separation, the hexane was removed. This extraction was repeated twice more. Finally, for enhanced separation and removal of contaminating residues, the mixture was centrifuged at 1500*g* for 5 min. The resulting methanolic phase was used to analyze the phenolic compounds by high-performance liquid chromatography (HPLC) and for the measurement of total phenol content by spectrophotometry.

The HPLC analyses of the methanol extracts from the leaves were made using a reverse-phase Spherisorb ODS-2 column (5 μm, 25 cm by 4.6 mm; Waters Corporation, Milford, Massachusetts, USA) and a Shimadzu HPLC system consisting of two pumps, a column-heater module and a UV−visible detector that operates with LC-Solutions software (Shimadzu Corporation, Kyoto, Japan). The volume of sample injected into the column was 20 μL. Separation was achieved by an elution gradient using an initial composition of 900 mL L^{-1} water (pH adjusted to 3.1 with 2 mL L^{-1} acetic acid) and 100 mL L^{-1} methanol. The concentration of the latter solvent was increased to 300 mL L^{-1} in 10 min and maintained for 20 min. Subsequently, the methanol concentration was increased to 400 mL L^{-1} in 10 min, maintained for 5 min, increased to 500 mL L^{-1} in 5 min, and maintained for another 5 min. Finally, the methanol percentage was increased to 600 mL L^{-1}, 700 mL L^{-1}, and 1000 mL L^{-1} in 5-min periods. Initial conditions were reached in 15 min. A flux of 1 mL min^{-1} and a temperature of 35 °C were also used. Ole, Htyr, and Tyr were identified and quantified in leaf methanol extracts at 280 nm by using the external standard method. The concentration of total phenols was determined colorimetrically at 725 nm using the Folin−Ciocalteau reagent and caffeic acid as the standard (*23*).

Statistical Analysis. The results are expressed as the mean ± standard error of the mean (SEM). Initially, the data were analyzed by a one-way analysis of variance. The differences between means were analyzed using a Student's *t*-test. The criterion of significance was taken as $p < 0.05$.

RESULTS

The present work was conducted on four olive cultivars, Picual, Verdial, Arbequina, and Frantoio, under similar environmental and soil conditions at Jaén (southern Spain). Nevertheless, the

Table 2. Changes in Kinetic Parameters of PAL in the Leaves of Olive Trees (*Olea europaea*) cv. Picual, Verdial, Arbequina, and Frantoio during Fruit Ripening[a]

	sample 1 (July)	sample 2 (October)	sample 3 (November)	sample 4 (December)
		V_{max}[b]		
Picual	$0.63 \pm 0.05_a^x$	$4.16 \pm 0.40_b^x$	$2.49 \pm 0.20_c^x$	$1.22 \pm 0.10_d^x$
Verdial	$0.59 \pm 0.05_a^x$	$2.83 \pm 0.25_b^{xz}$	$1.45 \pm 0.08_c^y$	$1.17 \pm 0.08_d^x$
Arbequina	$0.92 \pm 0.06_a^y$	$0.90 \pm 0.07_a^y$	$1.18 \pm 0.10_a^y$	$1.40 \pm 0.10_b^x$
Frantoio	$1.32 \pm 0.12_a^z$	$2.63 \pm 0.21_b^z$	$1.49 \pm 0.12_{ac}^y$	$1.75 \pm 0.10_c^y$
		K_m[c]		
Picual	$0.21 \pm 0.02_a^x$	$0.27 \pm 0.02_a^x$	$0.22 \pm 0.02_a^x$	$0.23 \pm 0.02_a^x$
Verdial	$0.12 \pm 0.01_a^y$	$0.13 \pm 0.01_a^y$	$0.09 \pm 0.01_a^y$	$0.13 \pm 0.01_a^y$
Arbequina	$0.02 \pm 0.01_a^z$	$0.02 \pm 0.01_a^z$	$0.03 \pm 0.01_a^z$	$0.03 \pm 0.01_a^z$
Frantoio	$0.01 \pm 0.00_a^z$	$0.02 \pm 0.01_a^z$	$0.04 \pm 0.01_{ab}^z$	$0.07 \pm 0.01_b^w$
		Activity Ratio[d]		
Picual	$0.27 \pm 0.03_a^x$	$0.32 \pm 0.03_a^x$	$0.30 \pm 0.03_a^x$	$0.30 \pm 0.03_a^x$
Verdial	$0.47 \pm 0.04_a^y$	$0.40 \pm 0.05_a^x$	$0.51 \pm 0.05_a^y$	$0.41 \pm 0.01_a^y$
Arbequina	$0.69 \pm 0.07_a^z$	$0.68 \pm 0.04_a^y$	$0.63 \pm 0.04_a^z$	$0.67 \pm 0.05_a^z$
Frantoio	$0.81 \pm 0.08_a^z$	$0.63 \pm 0.05_a^y$	$0.48 \pm 0.04_b^y$	$0.47 \pm 0.04_b^y$
		Catalytic Efficiency[e]		
Picual	$3.00 \pm 0.28_a^x$	$15.40 \pm 1.10_b^x$	$11.32 \pm 1.00_c^x$	$5.30 \pm 0.45_d^x$
Verdial	$4.94 \pm 0.45_a^y$	$21.77 \pm 2.00_b^y$	$16.11 \pm 1.20_c^y$	$8.98 \pm 0.06_d^y$
Arbequina	$46.00 \pm 3.75_a^z$	$44.95 \pm 3.20_a^z$	$39.27 \pm 3.00_a^z$	$46.68 \pm 3.80_a^z$
Frantoio	$132.00 \pm 12.00_a^w$	$131.67 \pm 12.20_a^w$	$37.38 \pm 3.12_b^z$	$25.06 \pm 2.04_c^w$

[a] Samples were harvested on four dates corresponding to the different ripening status. Results are expressed as the mean ± SEM of 5 values. For each parameter, the data in each row followed by different subindices (a−d) are statistically different ($p < 0.05$). For the comparison between cultivars, the data in each column followed by different superscripted indices (x, y, z, and w) are statistically different ($p < 0.05$). [b] V_{max} is expressed as nmol min^{-1}. [c] K_m is expressed in mM. [d] Activity ratio is determined as the ratio between $V_{subsaturating}/V_{max}$. The subsaturating concentration considered was 0.05 mM. [e] Catalytic efficiency is determined as the V_{max}:K_m ratio and is expressed as nmol·min^{-1}·mM^{-1}.

olive-ripening pattern shown by each cultivar was different. **Table 1** presents the time course of the ripeness index of the fruit of each cultivar during the experiment. Frantoio was the cultivar that reached the highest RI, 4.34, in November, while Arbequina reached a value of only 2.55 in December. In Picual and Verdial, the highest RI values were 3.25 and 3.91, respectively. The water content in the leaf was, in all cases, around 500 g (kg weight)$^{-1}$. The total phenol concentration was different between cultivars and changed differentially during ripening. Thus, the mean value of the total phenols considering the entire experimental period was 44.61 ± 3.25 g (kg dry weight)$^{-1}$ in Frantoio; 34.25 ± 5.40 in Arbequina; 28.81 ± 2.27 in Picual; and 27.63 ± 3.62 in Verdial. The time course of total phenols is shown in **Table 1**. In Verdial and Arbequina, the total phenols concentration declined as ripening progressed; in Picual and Frantoio, the concentration increased until the last stage and then decreased.

Kinetic Behavior and Protein Expression of PAL. PAL specific activity was assayed in all leaf samples. The effect of L-Phe concentration on PAL specific activity was studied, and the kinetic parameters were calculated (**Table 2**). In the leaves of the four cultivars, hyperbolic kinetics was noted. At each ripening stage, the PAL specific activity differed between the four cultivars (**Table 2**, **Figure 1**). In Arbequina and Frantoio, the highest PAL activity values were recorded in July, the first stage of ripening studied. In Picual and Verdial, the lowest PAL activity was reached in July, while the highest was reached in October, the second ripening stage. The value reached in Verdial in October was the highest in the entire experiment. After the stage with the highest activity, all cultivars registered a significant decrease with ripening (**Table 2**, **Figure 1**). In the last two stages of ripening, the highest PAL-specific activity was found in Picual.

The K_m values of PAL for L-Phe differed among cultivars. In Picual, Verdial, and Arbequina, the K_m values were close to 0.22 mM, 0.12 mM, and 0.02 mM, in all ripening stages,

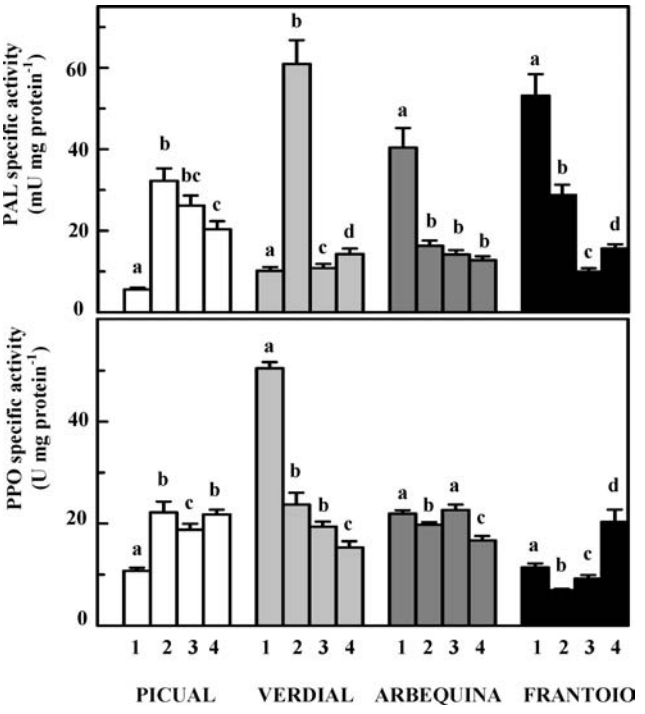

Figure 1. Changes in leaf phenylalanine ammonia-lyase (PAL, upper panel) and polyphenol oxidase (PPO, lower panel) specific activity at saturated substrate concentration of Picual, Verdial, Arbequina, and Frantoio cultivars during ripening. Data are the mean ± SEM of five values. For each cultivar, four samples, denoted 1−4, were harvested during the ripening process. Sample 1 was picked on July 31, 2002, sample 2 on October 10, 2002, sample 3 on November 1, 2002, and sample 4 on December 3, 2002. For each cultivar, columns with different letters are statistically different ($p < 0.05$).

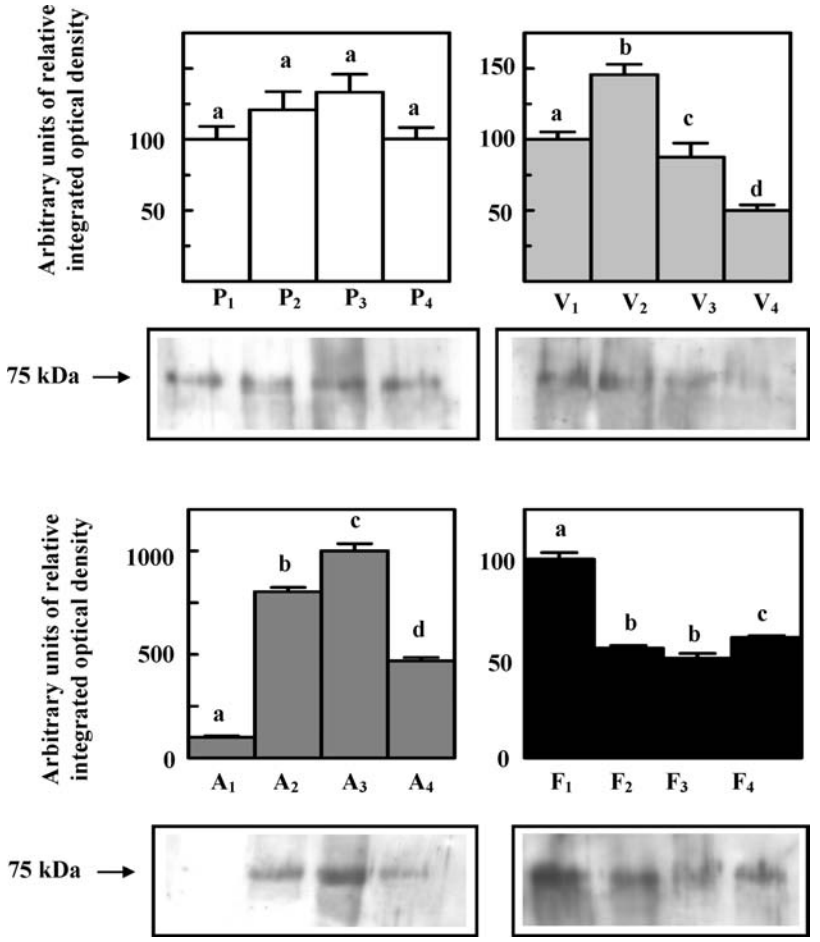

Figure 2. Western-blot analysis of phenylalanine ammonia-lyase (PAL) in leaves of *Olea europaea* cv. Picual (P), Verdial (V), Arbequina (A), and Frantoio (F) during fruit ripening. P_1, V_1, A_1, and F_1 give leaf samples from July; P_2, V_2, A_2, and F_2 give leaf samples from October; P_3, V_3, A_3, and F_3 give leaf samples from November; and P_4, V_4, A_4, and F_4 give leaf samples from December. Proteins from extracts of leaves of different indices of ripeness were separated by 7.5% SDS−PAGE (9.5 μg per lane) and then blotted onto a polyvinylidene difluoride (PVDF) membrane. The 75 kDa polypeptide corresponding to a monomeric form of the enzyme was detected with specific polyclonal anti-PAL rabbit serum. The quantification of PAL levels by densitometric analysis is shown in bar graphics. The results are the means ± SEM of five values and are expressed as arbitrary units of integrated optical density compared to sample number 1. Bars with different letters are significantly different ($p < 0.05$).

respectively. In Frantonio, the K_m changed from 0.01 to 0.07 mM during ripening. The activity ratio showed a behavior similar to that described for the K_m during ripening.

Catalytic efficiency significantly differed between cultivars, with Picual and Verdial showing the lowest values, Frantoio the highest, and Arbequina the intermediate (**Table 2**). In Picual and Verdial, a significant increment occurred between July and October followed by a decrease from October to December. In Arbequina, the values did not significantly change during ripening. In Frantoio, catalytic efficiency was maintained from July to October and afterward significantly fell.

The PAL protein content was determined by Western-blot using a specific antiserum anti-PAL-1 of parsley with leaf samples of the different cultivars at different ripening stages (**Figure 2**). Only one 75-kDa immunoreactive polypeptide was detected. In Verdial and Frantoio, coinciding with the specific activity, the intensity of the immunoreactive band declined as the ripeness progressed. In Picual, no significant changes were detected over the ripening period. In Arbequina, from July to November, a significant increment in the intensity of the immunoreactive band was found. From November to December, the immunoreactivity significantly fell.

Kinetic Behavior and Protein Expression of PPO. PPO-specific activity was assayed using catechol as the substrate in all leaf samples. Hyperbolic kinetics appeared in all cases (**Table 3**). The PPO specific activity and its changes during ripening differed between cultivars. In general, Verdial showed the highest PPO specific activity and Frantoio the lowest, and Picual and Arbequina showed intermediate levels. In Verdial, a significant and potential decrease was registered. In Arbequina and Frantoio, the PPO-specific activity also decreased over ripening, although in Frantoio the activity significantly intensified from November to December. In Picual, PPO activity increased from July to October, reaching a level that remained practically stable till December.

Significant differences were found between the K_m values for catechol in the four cultivars (**Table 3**). Picual registered the lowest K_m values in all the ripening stages, changing from 3.96 mM in July to 6.94 mM in December. Frantoio was the cultivar in which the highest K_m values were reached, changing from 45.80 mM in July to 84.88 mM in December. Verdial registered values that ranged from 7.78 mM in July to 21.22 mM in December, with 41.62 mM in October. Arbequina values ranged from 41.00 mM in July to 34.19 mM in December, with 51.70 mM in November.

The catalytic efficiency of PPO against catechol were expressed as min^{-1} M^{-1}. The low values of V_{max} and the high values of K_m resulted in the low values of catalytic efficiency reported. Frantoio was the cultivar that registered the lowest values of PPO

Table 3. Changes in Kinetic Parameters of PPO in Leaves of *Olea europaea* cv. Picual, Verdial, Arbequina, and Frantoio during Fruit Ripening[a]

	sample 1 (July)	sample 2 (October)	sample 3 (November)	sample 4 (December)
		V_{max}[b]		
Picual	$0.10 \pm 0.01_a^x$	$0.20 \pm 0.02_b^x$	$0.24 \pm 0.02_b^x$	$0.26 \pm 0.02_b^x$
Verdial	$1.18 \pm 0.05_a^y$	$0.38 \pm 0.03_b^y$	$0.34 \pm 0.02_b^y$	$0.30 \pm 0.02_b^x$
Arbequina	$0.34 \pm 0.02_a^z$	$0.24 \pm 0.01_b^z$	$0.31 \pm 0.02_a^y$	$0.26 \pm 0.01_{ab}^x$
Frantoio	$0.19 \pm 0.01_a^w$	$0.09 \pm 0.01_b^w$	$0.12 \pm 0.01_b^z$	$0.37 \pm 0.02_c^y$
		K_m[c]		
Picual	$3.96 \pm 0.20_a^x$	$7.49 \pm 0.70_b^x$	$6.61 \pm 0.65_b^x$	$6.94 \pm 0.30_b^x$
Verdial	$7.78 \pm 0.80_a^y$	$41.62 \pm 3.16_b^y$	$17.90 \pm 1.40_c^y$	$21.22 \pm 2.10_c^y$
Arbequina	$41.00 \pm 1.20_a^z$	$34.00 \pm 0.50_b^z$	$51.79 \pm 2.54_c^z$	$34.19 \pm 1.75_b^z$
Frantoio	$45.80 \pm 1.22_a^w$	$54.49 \pm 3.10_b^w$	$56.56 \pm 3.80_b^z$	$84.88 \pm 8.50_c^w$
		Activity Ratio[d]		
Picual	$0.56 \pm 0.06_a^x$	$0.47 \pm 0.03_{ab}^x$	$0.53 \pm 0.05_a^x$	$0.42 \pm 0.02_b^x$
Verdial	$0.38 \pm 0.01_a^y$	$0.12 \pm 0.01_b^y$	$0.29 \pm 0.02_c^y$	$0.27 \pm 0.02_c^y$
Arbequina	$0.29 \pm 0.03_a^z$	$0.11 \pm 0.01_b^y$	$0.17 \pm 0.01_c^z$	$0.27 \pm 0.02_a^y$
Frantoio	$0.17 \pm 0.01_a^w$	$0.09 \pm 0.01_b^y$	$0.10 \pm 0.01_b^w$	$0.12 \pm 0.01_b^z$
		Catalytic Efficiency[e]		
Picual	$25.25 \pm 1.55_a^x$	$38.72 \pm 3.10_b^x$	$36.31 \pm 2.80_b^x$	$37.46 \pm 1.80_b^x$
Verdial	$151.67 \pm 10.78_a^y$	$9.13 \pm 0.70_b^y$	$18.99 \pm 1.62_c^y$	$14.14 \pm 1.25_d^y$
Arbequina	$8.29 \pm 0.37_a^z$	$7.06 \pm 0.25_b^z$	$5.98 \pm 0.28_c^z$	$7.60 \pm 0.50_{ab}^z$
Frantoio	$4.07 \pm 0.15_a^w$	$1.69 \pm 0.10_b^w$	$2.17 \pm 0.08_c^w$	$4.36 \pm 0.30_a^w$

[a] Samples were harvested on four dates corresponding to the different ripening status. Results are expressed as the mean ± SEM of 5 values. For each parameter, the data in each row followed by different subindices (a−d) are statistically different ($p < 0.05$). For the comparison between cultivars, the data in each column followed by different superscripted indices (x, y, z, and w) are statistically different ($p < 0.05$). [b] V_{max} is expressed as $10 \cdot \Delta DO\ min^{-1}$. [c] K_m is expressed in mM. [d] Activity ratio is determined as the ratio between $V_{subsaturating}/V_{max}$. The subsaturated concentration considered was 2.5 mM. [e] Catalytic efficiency was determined as the V_{max}/K_m ratio and is expressed as $min^{-1} \cdot M^{-1}$.

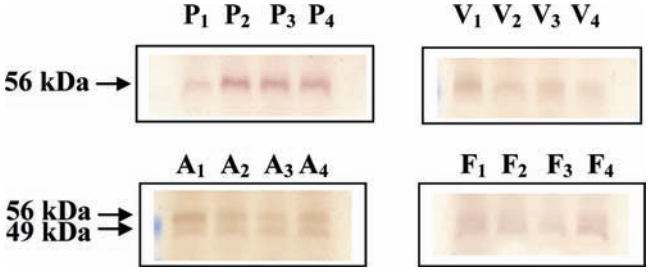

Figure 3. Partially denaturing SDS−PAGE followed by in-gel staining for polyphenol oxidase (PPO) activity of native extracts of leaves of *Olea europaea* cv. Picual (P), Verdial (V), Arbequina (A), and Frantoio (F) during ripening. From each cultivar, four samples, denoted 1−4, were harvested during the ripening process. Leaf extracts were dissolved in native buffer and separated by 10% SDS−PAGE at 4 °C. In each lane, 4.5 μg of protein was loaded. Gels were stained with DL-DOPA and catalase. The molecular mass of each band with PPO activity is indicated.

catalytic efficiency at all ripening stages studied, followed by Arbequina and Picual. Verdial showed the highest values in July with a significant decline in October, November, and December to intermediate values with respect to Arbequina and Picual.

The PPO activity, following partially denaturing SDS−PAGE, was assayed in all the leaf samples (**Figure 3**). After electrophoretic separation of polypeptides and in-gel staining with DL-DOPA, one band (Picual and Verdial) or two bands (Arbequina and Frantoio) with PPO activity appeared. The apparent molecular masses of these proteins were 56 kDa and 49 kDa, respectively. The intensity of these bands changed during ripening according to the changes described by the PPO-specific activity assayed in a cuvette.

Concentrations of Ole, Htyr, and Tyr. Samples of phenolic extracts of the leaves of Picual, Verdial, Arbequina, and Frantoio cultivars were analyzed by HPLC to determine the concentration of Ole, Htyr, and Tyr during ripening (**Figure 4**). Ole, Htyr, and Tyr were identified with retention times of 47.9 ± 0.3, 9.9 ± 0.1, and 13.9 ± 0.1 min, respectively. The Ole concentration in Picual ranged from 4.77 g (kg dry weight)$^{-1}$ in July to 6.34 in December with values of 9.37 and 8.35 in October and November (**Table 4**). Frantoio and Verdial showed the highest values in July and October, falling afterward in November and December. Arbequina was the cultivar with the highest Ole concentration in November and December.

With respect to the Htyr concentration, significant differences appeared between cultivars (**Table 4**). Picual was the cultivar that, in general, presented the lowest Htyr values, from 0.21 to 0.39 g (kg dry weight)$^{-1}$. Verdial, Arbequina, and Frantoio had values of around 1 g (kg dry weight)$^{-1}$ in July. From July to November, these values significantly decreased, and afterward increased in December.

With respect to the Tyr concentration, Picual showed the lowest values followed by Arbequina, Frantoio, and Verdial. Only in Arbequina were changes over the ripening period detected, values ranging from 0.51 g (kg dry weight)$^{-1}$ in July to 0.13 g (kg dry weight)$^{-1}$ in December.

DISCUSSION

The aim of our work was to study the metabolism of the phenolic compounds in the leaves of olive trees of four important cultivars over the ripening period. We made this study at the same time in which we investigated what occurred in the fruits of the same trees. The results found in the fruits have been previously published (*9*), and here, we present the results found in the leaves. Considering all of the values found in our study, the concentration of total phenols in leaves differed between cultivars. Frantoio was the cultivar that showed the highest concentration,

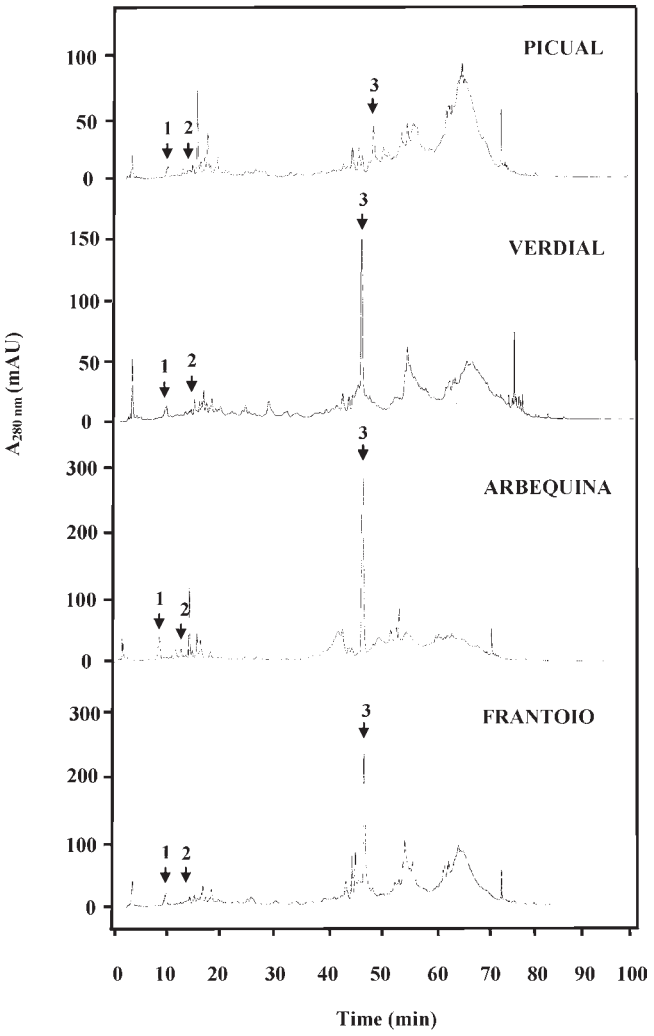

Figure 4. HPLC chromatograms at 280 nm of methanol extracts of olive leaves of *Olea europaea* cv. Picual, Verdial, Arbequina, and Frantoio. Samples of 20 μL methanol extracts of olive leaves were HPLC chromatographed in a Spherisorb ODS-2 column and eluted with a methanol−water gradient for 100 min. The peaks corresponding to hydroxytyrosol (1), tyrosol (2), and oleuropein (3) are marked on the chromatogram made at 280 nm. The chromatograms shown in this figure are representative of five independent determinations. The chromatograms for Picual, Verdial, and Arbequina correspond to the sample picked on 31 July, 2002. The chromatogram for Frantoio corresponds to the sample picked on 10 October, 2002.

Arbequina the intermediate, and Verdial and Picual the lowest. A rather similar pattern has been described for the total phenol concentration in the fruits of these cultivars (*9*) with the exception of the behavior of Picual cultivar, which showed a higher relative concentration of total phenols in fruits than in leaves. Nevertheless, our results found in Picual and Frantoio are not in accordance with that described by Papoti and Tsimidou (*18*) in experiments performed in Greece. With the same type of assay, these authors reported a higher total phenol content in the leaves of the Picual cultivar than in Frantoio. With a fluorometric assay, no differences were found in their study. The differences in the values between cultivars found in the two studies may be due to the different agricultural and climatic conditions, which can also influence the behavior of different cultivars.

In absolute value, the concentration of total phenols, expressed as g (kg dry weight)$^{-1}$ found in our study in the leaves of the four cultivars of olive trees, is higher than those found in the fruits of these same cultivars (*9*). This indicates that these compounds must be important in the metabolism and function of the olive leaf. The differences in the concentration between cultivars constitute a specific characteristic of each cultivar that must be related with specific properties, for example, adaptation to ambient conditions or the production of fruits with a specific phenol concentration. This implies different cosmetic or dietetic applications. Specifically, the phenol concentration of the leaves of the four cultivars may be related to resistance to biotic or abiotic stress such as cold or even infection by pathogens such as *Verticillium dahliae*. In this sense, some works have described how phenols can act as a natural defense of the olive tree against fungi (*24*, *25*) or cold stress (*19*).

Also in this work, we reported that the differences found in the concentration of total phenols between cultivars can be correlated with the properties of PAL and PPO, which also differed specifically between cultivars. With respect to PAL, on the basis of the different K_m values against L-Phe, it seems that different isoenzymes are expressed in the different cultivars. K_m is a kinetic parameter that makes reference to the substrate concentration at which the half of V_{max} is obtained. Therefore, $1/K_m$ indicated the strength of binding of substrate to the enzyme, and in some specific cases, $1/K_m$ is a measure of the affinity of the substrate for its binding site on the enzyme. In Frantoio and Arbequina, the low K_m values found indicate that the enzyme had a high affinity for L-Phe. The K_m of PAL in Verdial was intermediate and in Picual was highest, indicating that this latter cultivar had the lowest affinity of PAL for L-Phe. These differences may be related to the total phenol concentration in the leaves of the four cultivars. Although these differences existed, the average specific activity found in the four cultivars did not significantly differ. Nevertheless, major changes in the specific activity have been found in the cultivars during ripening. In Frantoio and Arbequina, the highest level of PAL activity was found in the first sampling (July), followed by a significant decrease with the progress of ripening. In Picual, the PAL activity increased over ripening, reaching the highest level in the October sample, as in Verdial. Nevertheless, in this last cultivar, no differences were found among the samples of July, November, or December. With respect to the Frantoio cultivar, it is interesting to note that a parallelism exists among the highest values of RI, total phenols content, and the highest values of the catalytic efficiency of PAL in November, July, and October. These are warming signs in the biosynthesis of phenols in July and October in the Frantoio variety.

With respect to the oxidative metabolism of phenols catalyzed by PPO, a different behavior was observed between the olive cultivars. PPO-specific activity was detected in the four cultivars, although at a level significantly lower than that detected in the fruit (*9*). This indicates that PPO is present also in the leaf and has a specific role in the function of this organ. When comparing between cultivars, a different kinetic behavior is observed. Picual was the cultivar in which PPO showed the low values of K_m against catechol, indicating that this cultivar showed the highest affinity for it and therefore the existence of a high phenolic oxidative metabolism. Verdial showed medium values and Arbequina and Frantoio the highest. Thus, Arbequina and Frantoio were the cultivars with the lowest affinity for phenol oxidation and, consequently, a lower oxidative metabolism of these compounds by PPO. This behavior is consistent with the highest concentration of these compounds found in these cultivars. Also, Frantoio and Arbequina were the two cultivars in which two bands of PPO activity appeared in the partially denaturing SDS−PAGE.

The PPO kinetic behavior in the olive leaf changed during ripening, depending on the cultivar. In Picual, the specific activity significantly increased as ripening progress. Nevertheless, in

Table 4. Time Course of the Concentration of Hydroxytyrosol, Tyrosol, and Oleuropein in the Leaves of *Olea europaea* cv. Picual, Verdial, Arbequina, and Frantoio during Fruit Ripening[a]

	sample 1 (July)	sample 2 (October)	sample 3 (November)	sample 4 (December)
		Hydroxytyrosol (g (kg dry mass)$^{-1}$)		
Picual	$0.21 \pm 0.01_a^x$	$0.19 \pm 0.01_a^x$	$0.31 \pm 0.02_b^x$	$0.39 \pm 0.02_c^x$
Verdial	$0.97 \pm 0.01_a^y$	$0.46 \pm 0.02_b^y$	$0.48 \pm 0.05_b^y$	$0.54 \pm 0.04_b^y$
Arbequina	$1.12 \pm 0.10_a^y$	$0.61 \pm 0.02_b^z$	$0.28 \pm 0.01_c^x$	$0.60 \pm 0.03_b^y$
Frantoio	$0.89 \pm 0.05_a^y$	$0.80 \pm 0.02_a^w$	$0.30 \pm 0.01_b^x$	$0.77 \pm 0.05_a^z$
		Tyrosol (g (kg dry mass)$^{-1}$)		
Picual	$0.09 \pm 0.02_a^x$	$0.10 \pm 0.03_a^x$	$0.12 \pm 0.02_a^x$	$0.17 \pm 0.04_a^x$
Verdial	$0.66 \pm 0.11_a^y$	$0.55 \pm 0.02_a^y$	$0.41 \pm 0.09_a^y$	$0.18 \pm 0.02_b^x$
Arbequina	$0.51 \pm 0.05_a^y$	$0.18 \pm 0.02_b^z$	$0.16 \pm 0.01_b^x$	$0.13 \pm 0.01_b^x$
Frantoio	$0.37 \pm 0.03_a^z$	$0.32 \pm 0.02_a^w$	$0.30 \pm 0.02_a^y$	$0.29 \pm 0.03_a^y$
		Oleuropein (g (kg dry mass)$^{-1}$)		
Picual	$4.77 \pm 0.37_a^x$	$9.37 \pm 1.00_b^x$	$8.35 \pm 0.79_b^x$	$6.34 \pm 0.04_c^x$
Verdial	$25.05 \pm 1.58_a^y$	$13.03 \pm 0.24_b^y$	$7.18 \pm 0.77_c^x$	$2.90 \pm 1.13_d^y$
Arbequina	$10.56 \pm 0.69_a^z$	$8.37 \pm 0.90_b^x$	$26.70 \pm 2.70_c^y$	$20.54 \pm 0.50_c^z$
Frantoio	$30.17 \pm 0.51_a^w$	$23.61 \pm 1.55_b^z$	$6.35 \pm 0.09_c^z$	$12.90 \pm 0.33_d^w$

[a] Samples were harvested on four dates corresponding to the different ripening states. Results are expressed as the means ± SEM of five values. The results were analysed by a two-way ANOVA and Student's *t*-test. For each compound, the data in each row followed by different subindices (a−d) are statistically different ($p < 0.05$). For the comparison between cultivars, the data in each column followed by different superscripted indices (x, y, z, and w) are statistically different ($p < 0.05$).

Table 5. Resume of the Phenol Metabolism Parameters Found in Our Study

	Picual	Verdial	Arbequina	Frantoio
total phenols	low	low	medium	high
Ole concentration	low	medium	high	high
Htyr concentration	low	high	high	high
Tyr concentration	low	high	medium	medium
PAL activity	medium	medium	medium	medium
PAL efficiency	low	medium	high	highest
PPO activity	medium	high	medium	low
PPO efficiency	high	high	low	lowest

Verdial, Arbequina, and Frantoio a significant decrease was observed.

Table 5 summarizes the behavior of the different parameters analyzed in the four cultivars. Frantoio and Arbequina showed high or medium content of total phenols, high concentration of Ole and Htyr, high PAL efficiency, and low PPO activity and efficiency. Picual showed a low concentration of total phenols, Ole, and Htyr; low PAL efficiency; and medium and high PPO activity and efficiency. Verdial showed low total phenol concentrations, medium or high Htyr and Tyr concentrations, and medium and high PAL and PPO activity. Overall, these results reflect a relationship between the level and efficiency of PAL and PPO with the phenol concentration in the leaves of each cultivar. Thus, the cultivars that showed high PAL efficiency and low or medium PPO activity (Frantoio and Arbequina) registered high phenol concentrations; cultivars that showed medium PAL efficiency and high PPO activity (Verdial) have a medium level of phenols; meanwhile, cultivars with low PAL efficiency and high PPO efficiency (Picual) had lower levels of phenols. In short, these results indicate a corelated pattern between PAL and PPO that could determine the phenol levels in olive leaves in a manner similar to what we described previously in the fruits of these four cultivars (*9*).

In conclusion, the results reported in our work showed that olive leaves have a high concentration of total and specific phenols and that there exists an important variety component, even influenced by ripening, that influences phenol metabolism in leaves. These results can serve as a basis to re-evaluate the olive leaf as a phenol source for different proposes.

ABBREVIATIONS USED

Ole, oleuropein; Htyr, hydroxytyrosol; Tyr, tyrosol; PAL, phenylalanine ammonia-lyase; PPO, polyphenol oxidase; L-Phe, L-phenylalanine; TBS, Tris buffer solution; TBS-T, Tris buffer solution−Tween20; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide-gel electrophoresis; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; K_m, Michaelis constant; V_{max}, maximum velocity.

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