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β-Glucosidase Involvement in the Formation and Transformation of Oleuropein during the Growth and Development of Olive Fruits (*Olea europaea* L. cv. Arbequina) Grown under Different Farming Practices

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ABSTRACT: The present study investigates oleuropein metabolism, as well as the involvement of β-glucosidase during the growth, development, and ripening of olive fruit. The results show that in olive fruit the *in vivo* formation and transformation of oleuropein takes place in three different stages. The first one is characterized by a net accumulation of oleuropein and occurs in the immature fruit. In the second stage, associated with the green and light-green fruits, oleuropein content is maintained practically constant, and finally, a third stage that begins in the green-yellow fruit is characterized by a progressive decline of the oleuropein concentration. Our findings confirm that in the absence of β-glucosidase the Damtoft-proposed pathway is active and that net synthesis of oleuropein is unquestionable. β-Glucosidase activity plays a key role in the oleuropein metabolism catalyzing its *in vivo* hydrolysis.

KEYWORDS: *olive fruit, oleoside-11-methyl ester, ligstroside, tyrosol, oleuropein, aglycones, elenolic acid, metabolism, oleuropein precursors, β-glucosidase*

INTRODUCTION

Phenolic compounds are complex mixtures that display a rich structural variety and a wide range of biological activities as evidenced by their antioxidant and health-enhancing properties. Recently, an increasing interest in the olive biophenol pathway has been observed, which correlates well with related investigations into olive farming practices for the production of extra virgin olive oil and table olives. Multidisciplinary research has been conducted on the biophenol composition and histological distribution of olives. The histochemical enzymatic localization of phenolic compounds has also been investigated to determine the biomolecular function of these compounds, which are activated by native β-glucosidase on secoiridoid conjugates such as oleuropein, which releases phytoalexins against pathogens.[1]

The mechanism of oleuropein biosynthesis in *Olea europaea* is complex and not yet well understood. Oleuropein is typical of the Oleaceae family, and it is thought to be biosynthesized from mevalonic acid via a complex metabolic pathway.[2,3]These studies show that the pathway to deoxyloganic acid, 7-epi-loganic acid, 7-ketologanic acid, and 7-ketologanin as last-stage carbocyclic iridoid precursors (Figure 1) could exist, although this sequence may vary among species and depend on the season of the year.

As was mentioned in a previous work,[4] Damtoft et al.[2,3] have tested also possible secoiridoid intermediates, including 7-ketologanin and oleoside-11-methyl ester, the compound from which oleuropein[3] and similar iridoids in the Oleaceae family are derived. However, even in plant species selected by their high biosynthetic ability, intermediates of neither kingiside type nor secologanin type are incorporated to the expected degree for a true intermediate between 7-ketologanin and oleoside-11-methyl ester. These authors conclude that 7-ketologanin is the immediate precursor for oleoside-11-methyl ester, and this indicates that the conversion is most likely a single-step reaction[3] through a Baeyer−Villiger-type intermediate and that up to three different processes might be feasible (Figure 1). A reaction (R-1) initiated by rupture of the peroxide bond followed by cleavage of the C7−C8 bond and simultaneous abstraction of H9 would give rise to oleoside-11-methyl ester. A similar reaction (R-2) with abstraction of a proton from C10 would produce secoxyloganin. Finally, 8-epi-kingiside acid and related compounds (R-3) could be formed via an alkyl shift of C8. Such a mechanism could explain the presence of the other iridoid products found with oleosides.

The final steps in oleoside synthesis can be inferred as the direct conversion of 7-ketologanin to oleoside-11-methyl ester (R-1) followed by the conversion to 7-β-1-D-glucopyranosyl-11-methyl oleoside, which undergoes an esterification reaction with tyrosol to form ligstroside, and finally, oleuropein is formed via a hydroxylation reaction (Figure 1).

In the paper mentioned by the authors,[4] the evolution of the Damtoft oleuropein precursors[2,3] and the enzymatic degradation

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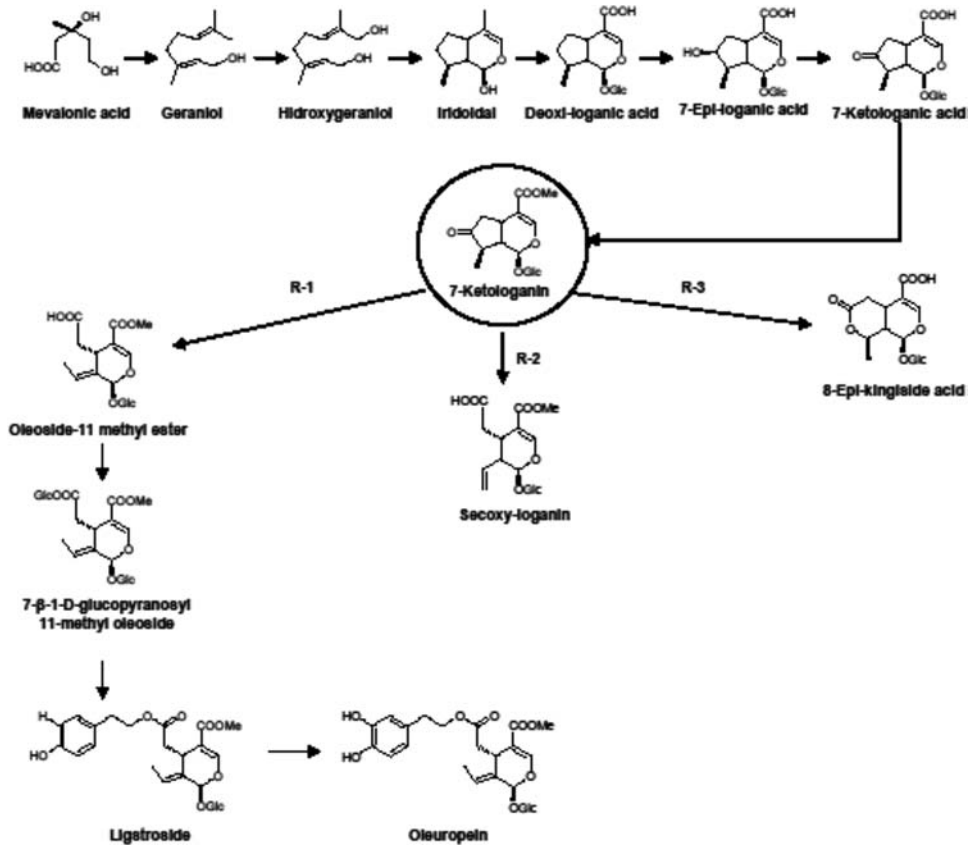

Figure 1. Biosynthesis route of oleuropein according to Damtoft et al.[2,3]

products (Figure 2), elenolic acid and the aglycone forms of oleuropein and ligstroside, during the vegetative cycle of *Olea europaea* fruits from the Arbequina and Hojiblanca varieties produced in Córdoba (Spain) were discussed. The results support the oleuropein biosynthetic pathway proposed previously[2,3] for the Hojiblanca and Arbequina olive varieties. In addition, the high activity of β-glucosidase, which acts directly on glucosylated phenols, that was observed in both varieties (6−8 μkat/g acetonic powder) modulates the *in vivo* synthesis of oleuropein and its precursors. This observation confirms the idea that this enzyme is responsible for the hydrolysis of oleuropein and ligstroside to their respective aglycones, leading to a decreased accumulation of oleuropein, which is a major compound in a wide range of olive varieties. Notably, this complex metabolic process of oleuropein differs from one variety to another, with the Arbequina variety exhibiting active metabolic routes over a sustained time and at a higher yield than the Hojiblanca variety. Considering that climate and agronomic conditions affect the polyphenolic content of olive fruits, further studies are required to confirm this working hypothesis. The type and quantity of polyphenol compounds in olive tissues vary greatly depending on the species, variety, stage of ripening and development, and growing conditions.

The present study is focused on the Arbequina variety grown using different farming practices (including organic and conventional farming). The study was conducted in two olive-producing regions with different climates and agronomical characteristics, Lleida (northeast Spain) and Seville (southwest Spain), and its aim is to confirm the route proposed by Damtoft et al.[2] (Figure 1) through the quantitation of oleuropein precursors, as well as of its catabolites by the activity of the endogenous β-glycosidase (Figure 2), reported in a previous work.[4] Furthermore, we investigate the impact that the cultivar's origin and cultivation method (organic and conventional) have on the net synthesis of oleuropein during the biological cycle of the olive fruit.

MATERIALS AND METHODS

Raw Material. This study was conducted using selected olive fruits of the Arbequina variety (*Olea europaea* L.) grown via organic and conventional farming methods. The fruits were collected in 2009 from two Spanish olive-producing regions with contrasting climatic conditions: Lleida (Catalonia), located in northeast Spain (altitude 167 m and latitude 41°36′54″ N), and Ecija (Seville, Andalucía), located in southwest Spain (altitude 106 m and latitude 37°32′26″ N).

Samples from Lleida were collected in two olive orchards situated in Els Torms, Lleida, Spain. The organic and conventional orchards were set up 200 m apart to minimize the variability due to climatic conditions. The organic orchard occupied a field of 13 Ha, and the spacing between trees was 6 × 5 m. The orchard was fertilized with natural compost obtained by mixing olive pomace and manure. Additional enrichment in potassium with NaturFruit olive (Daymso, Zaragoza, Spain), a slow-releasing nitrogen fertilizer, was applied. The conventional orchard occupied a field of 1.5 Ha, and the olive trees were spaced 6 × 5 m apart. The olive trees were also treated annually with copper to prevent infestation by the peacock spot (*Spilocaea oleaginea*) pest. The orchard was fertilized with an organic fertilizer rich in potassium (5−6%). It was also treated with the broad spectrum systemic herbicide Roundup at a concentration of 3 L/Ha [isopropylamine salt of *N*-(phosphonomethyl)glycine, Monsanto Europe, Belgium] and the systemic chloronicotinyl insecticide Condifor 20LS (20% w/v, Imidacloprid, Bayer Crop Sciences AG, Monheim am Rhein, Germany) at a concentration of 0.5 L/Ha. During the study, the presence of the olive fruit fly was not detected, and therefore, specific treatment for this pest was not required.

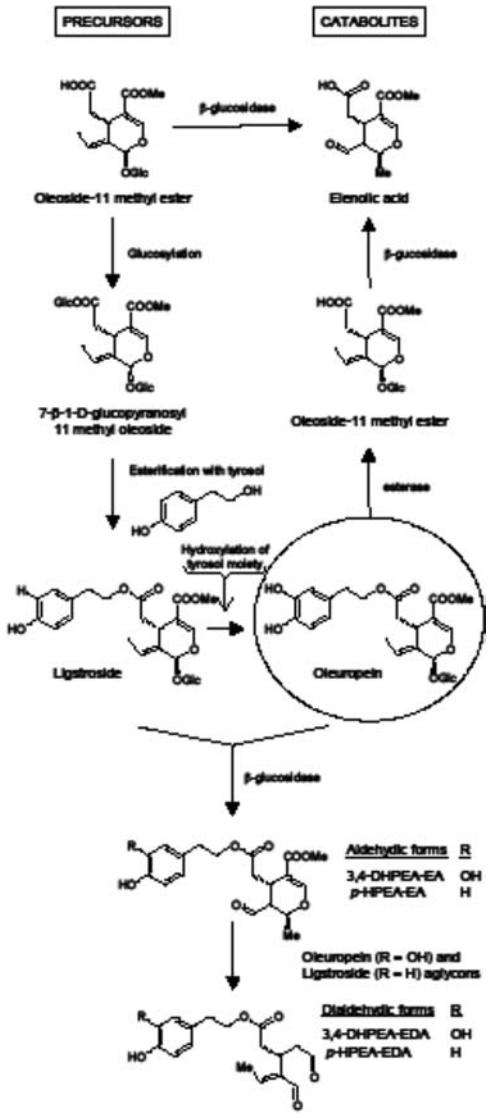

Figure 2. Proposed reactions for the effect of *β*-glucosidase on oleuropein metabolism in olive fruits (*Olea europaea*) cv. Arbequina.

Samples from Seville were collected in orchards situated in Ecija, Seville, Spain. The 65-Ha orchard was divided into two sections: one section used organic farming methods, and the other employed conventional farming. The trees were planted using a 10 × 10 m frame. The organic section was fertilized with liquid herbal manure (using lucerne, thyme, and rosemary as compost) applied at concentrations of 100−200 mL/Ha during spring, and animal manure, oil-foot, and leaves were used during autumn. The most common pest of these areas, "Prays" (*Prays oleae* Bern), was treated with *Bacillus thuringiensis*; the "Fly" (*Dacus oleae* Rossi) was controlled with natural pyrethrins. The most widespread disease, "Repilo" (*Spilocaea oleaginea*), was treated with a Bordeaux mixture. In the conventionally grown olive groves, urea and potassium nitrate were used as fertilizers. Pests were treated using malathion [*O*,*O*-dimethyl- and *S*-(1,2-diethoxycarbonylethyl)-dithiophosphate, Agrodan S.A., Madrid, Spain] at a concentration of 20−25 kg/Ha and Formothion [33% w/v, *O*,*O*-dimethyl- and *S*-(*N*-formyl-*N*-methylcarbamoyl)dithiophosphate, Sandoz, Barcelona, Spain] at a concentration of 1−1.5 L/Ha. The diseases were treated with glyphosate [12% w/v, *N*-(phosphonomethyl)glycine, Sipcam Inagra] at 2 L/Ha along with 4 L/Ha Oxyfluorfen 24% w/v, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-trifluoromethylbenzene, Rhône-Poulenc, Madrid, Spain] as a postemergent herbicide in the autumn.

Lleida has a Mediterranean climate with continental influences. The year 2009 was a dry year in Spain. During the harvesting seasons of 2009 (from mid-June to the first of November), the temperature ranged from a minimum of −2 °C to a maximum of 38 °C, and an accumulated precipitation of 142 mm and a mean moisture of 57.60% were measured.

Seville has a mild Mediterranean climate due to oceanic influences. During harvesting season (from mid-June to mid-December), the temperature oscillated between a minimum of 0.2 °C and a maximum of 43 °C, the accumulated rainfall was 67 mm and the mean moisture was 49.1%.

Twenty trees of each variety were selected in Lleida, and five were selected in Seville. The experiment started 15 days after bloom. The samples were harvested regularly every 7 days until November 11 in Lleida and every 15 days until December 14 in Seville. For each sample, approximately 500 g of fruits was collected from each olive tree variety. The samples were hand-picked between 10 a.m. and 11 a.m. For each orchard type, 22 samples were collected in Lleida, and 14 samples were collected in Seville. For each ripening stage investigated in this study, a representative sample of 100 fruits was chosen according to evolution of their weight and color. The evaluations were conducted using homologous stages of the developed fruit. The weight of the fruit in each sample collection was higher than or equal to the inferior stage, and the color changed gradually through the sequence from intense green skin to green skin, light skin, green-yellow skin, purple skin and, finally, to black.

Moisture Measurement. The moisture level was determined in a moisture analyzer (Ohaus MB35, Nänikon, Switzerland). The analysis conditions were 5 g of fruit and a drying temperature of 120 °C. The device stops automatically once the weight of the dehydrated sample reaches a constant value. The analyses were performed in triplicate.

Chemicals and Regents. High-performance liquid chromatography (HPLC)-grade ethanol was purchased from Romil Chemical, Ltd. (Heidelberg, Germany), and *n*-hexane was purchased from Prolab (Leuven, Belgium). The standard *p*-hydroxy phenyl acetic acid was purchased from Sigma-Aldrich (St. Louis, MO). The tyrosol, hydroxytyrosol, and oleuropein standards were purchased from Extrasynthese (Genay, France) for the HPLC analysis. The 3,4-DHPEA-EDA (dialdehydic form of elenolic acid linked to hydroxytyrosol), *p*-HPEA-EDA (dialdehydic form of elenolic acid linked to tyrosol), and *p*-HPEA-EA (aldehydic form of elenolic acid linked to tyrosol) were not available commercially and were isolated from virgin olive by semipreparative HPLC.[5] The 3,4-DHPEA-EA (oleuropein aglycone) was obtained from the *β*-glucosidase enzymatic treatment of oleuropein[6] and subsequent purification by semipreparative HPLC.[5] The standard stock solutions of each compound (50 mg/L) and a solution of *p*-hydroxyphenyl acetic acid (internal standard, IS) (150 mg/L) were prepared weekly in methanol and stored in a dark flask at 4 °C. Methanol (supragradient HPLC grade) and formic acid were provided by Scharlau Chemie (Barcelona, Spain). Water was of Milli-Q quality (Millipore Corp, Bedford, MA, USA). Sodium borate, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), polyvinylpolypyrrolidone (PVPP) and *p*-nitrophenyl-*β*-D-glucopyranoside (pNPG) were purchased from Sigma-Aldrich.

Extraction of Phenolic Compounds. To prepare the lyophilized pulp, the olive pulp was cut into pieces of 8−9 mm immediately after harvesting and frozen with liquid nitrogen. Then, the frozen pieces were kept at −32 °C before freeze-drying. The freeze-drying process was performed in a Heto-FD3 freeze-drier (Allerod, Denmark) at −54 °C and 0.066 mmHg for at least 48 h. The drying processes ended when the weight of the samples remained constant.

The extraction of the phenolic compounds from the lyophilized pulp was performed following the method proposed by Ríos and Gutiérrez-Rosales.[7] In summary, the method consists of adding 15 mL of an ethanol−water mixture (80:20 v/v) and 1 mL of an IS solution to 1 g of lyophilized pulp. The mixture was homogenized with an Ultra-Turrax blender for 4 min at 2561*g* and a low temperature (0−4 °C). The extract that was obtained was centrifuged at 0 °C for 15 min at 6386*g* and then vacuum filtered. Any residual ethanol was removed by evaporation. The aqueous extract was rinsed with *n*-hexane to eliminate lipids and pigments. The *n*-hexane was subsequently removed, and a water−methanol mixture (70:30 v/v) was added to the solution to a

final volume of 25 mL. The samples were stored in liquid nitrogen until they were analyzed.

Identification and Quantification of Phenols by HPLC/Electrospray Ionization (ESI)/Tandem Mass Spectrometry (MS/MS). The HPLC analysis of the phenolic extracts was conducted using the method published in a previous study[4] using a SunFire C_{18} colum (4.5 mm × 150 mm, 3.5 μm) (Waters, Milford, MA). The HPLC was coupled to a photodiode array (PDA) detector AcQuity UPLC and a tandem quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA). The wavelengths in the PDA detector were set at 278 and 339 nm. The MS/MS analyses were carried out on a TQD equipped with a Z-spray electrospray interface. The analyses were performed in negative mode, and the data were acquired in selected reaction monitoring (SRM).

A fitted linear regression model ($y = ax + b$) was used to calculate the relationship between the compound/IS peak abundance ratio (y) and the compound concentration (x). The concentrations of the phenolic compounds in the samples were estimated by substituting their compound/IS peak abundance ratios in the equation. Oleuropein and tyrosol were quantified using the above equation on the basis of standards obtained from commercial suppliers. The aglycone oleuropein (3,4-DHPEA-EA), *p*-HPEA-EA, and *p*-HPEA-EDA were quantified by using the calibration curve of 3,4-DHPEA-EDA standard obtained by semipreparative HPLC.[5] Elenolic acid, 7-ketologanin, oleoside 11-methyl ester, elenolic acid-glucoside, ligstroside, 7-β-1-D-glucopyranosyl-11-methyl oleoside were determined with the calibraton curve of oleuropein. For each compound, the calibration curve was obtained by analyzing five different concentration levels, and three compound solutions were prepared for each concentration. *p*-Hydroxyphenylacetic acid was used as an IS for the quantification. To normalize the data, the results for each individual compound were expressed in μmol/g of dry pulp.

Extraction and Measurement of β-Glucosidase Activity. The method proposed by Minguez-Mosquera et al.[8] was used to prepare the protein precipitate, and the method proposed by Romero-Segura et al.[9] was used with slight modification to determine the β-glucosidase activity.

Preparation of the Protein Precipitate. The pitted and sliced fruits (25 g) were titrated with 20 vol of acetone at −20 °C (500 mL). After macerating for 15 min in a freezer at −20 °C, the supernatant was removed by decantation, and the residue was treated again with 8 vol of acetone (200 mL). This procedure was repeated until the supernatant was colorless (4 washes were generally sufficient). Finally, the precipitate was collected by vacuum filtration and left to dry at ambient temperature. From each gram of fruit, approximately 0.12 g of powder was obtained. To obtain the raw extract, 0.25 g of the protein precipitate was extracted with 17.5 mL of 0.1 M borate buffer (pH 9) containing 5 mM EDTA, 0.25% DTT (w/v), and 1 mM/L PMSF.

Measurement of β-Glucosidase Activity. The enzyme activity was determined by measuring spectrophotometrically at 405 nm the *p*-nitrophenol released as consequence of the hydrolysis of the synthetic substrate *p*-nitrophenyl-β-D-glucopyranoside (pNPG) catalyzed by β-glucosidase. The reaction mixture consisted of 100 μL of raw extract, 1 mL of 50 mM phosphate buffer (pH 5.5), and 15 mmol/L pNPG. The pNPG concentration was quantified by its molar extinction coefficient (ε), whose value was determined to be 630.8 M^{-1} cm^{-1}. The results are expressed in μkat/g of acetonic powder.

The optima pH and temperature values for maximal activity were previously evaluated. The pH was considered in a range of 4.5−7.0 with 50 mM sodium phosphate buffer, and the temperature was studied in the range 25−75 °C. The maximum enzyme activity was obtained at pH 5.5 and 50 °C.

Finally, the effect of oleuropein as substrate of β-glucosidase was evaluated. In this case, the enzyme activity was measured as the rate of decrease of the oleuropein concentration. The incubations were carried out with increasing concentrations of substrate in the range 0.02−20 mM. Figure 3 shows the effect of the enzyme activity on the oleuropein substrate. A typical Michaelis−Menten curve was obtained, and the kinetic parameters K_m and V_m were determined using the Lineweaver−Burk plot (Figure 3). The K_m for oleuropein was 2.78 mM, which indicates that the affinity of β-glucosidase for this substrate is high, as it has been previously reported[6,9]

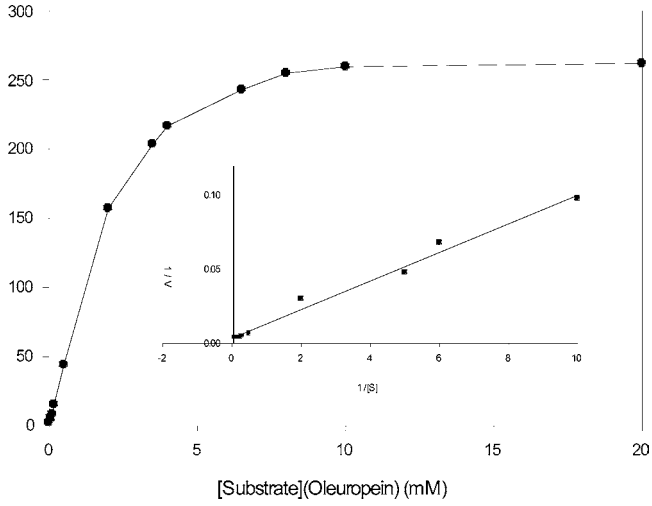

Figure 3. Enzyme kinetic curve and Lineweaver−Burk transformation plot for β-glucosidase activity with oleuropein. K_m was 2.78 ± 0.04.

Statistical Analysis. All values were expressed as the mean of three determinations ± SD. An analysis of variance (ANOVA) was used for comparisons, and the identification of significant differences between results was performed by applying a Tukey test. The level of statistical significance was set to $P \leq 0.05$. The STATISTICA computer software (version 6.0, Statsoft, Tulsa, USA) was used for the statistical analysis of the results.

RESULTS AND DISCUSSION

Fruit Development and Seasonal Changes in Moisture Content. The changes in the moisture content and the weight of the fruit and the stone as a function of the harvesting date are shown in Figure 4. In Lleida, the initial weight of the fruit was 0.07 g, both in the organic and conventional orchards, and the weight increased steadily until it reached 2.04 g in the organic orchards and 1.87 g in the conventional orchards. In Seville, the initial weight of the fruits (0.3 g) was also similar for both orchard types. At the end of their development, the fruits registered a weight of 2.18 g in the organic orchards and 0.99 g in the conventional orchards. When the weights of the fruits from both regions (Lleida and Seville) within the same orchard type were compared, it was observed that the weights of the fruits from the organic orchards were similar for both regions, whereas the fruits from the conventional orchards in Lleida had approximately twice the weight of the fruits from Seville. The scarce fruit development in the conventional orchards from Seville might be related to the lack of rain and to the high temperatures that occurred during the summer (average temperature = 37 °C). These conditions may not have affected the organic orchards due to their low water requirements. Therefore, in both regions the weight of the fruits was higher in the organic orchards than in the conventional orchards.[10,11]

Throughout the studied period the fruits in Lleida compared to Seville underwent different physiological processes. In Lleida, the fruit exhibited slow growth and a fast ripening process, whereas in Seville, the fruit exhibited fast growth and a slow ripening process.

Concerning the weight of the stone, in fruit from Lleida was absent from the first four samples when the fruit was still an intense green bud. Once the lignification started, the stone reached a weight of 0.16 g and increased to a weight of 0.27 and 0.25 g of the two orchard types. In Seville, the stone was absent

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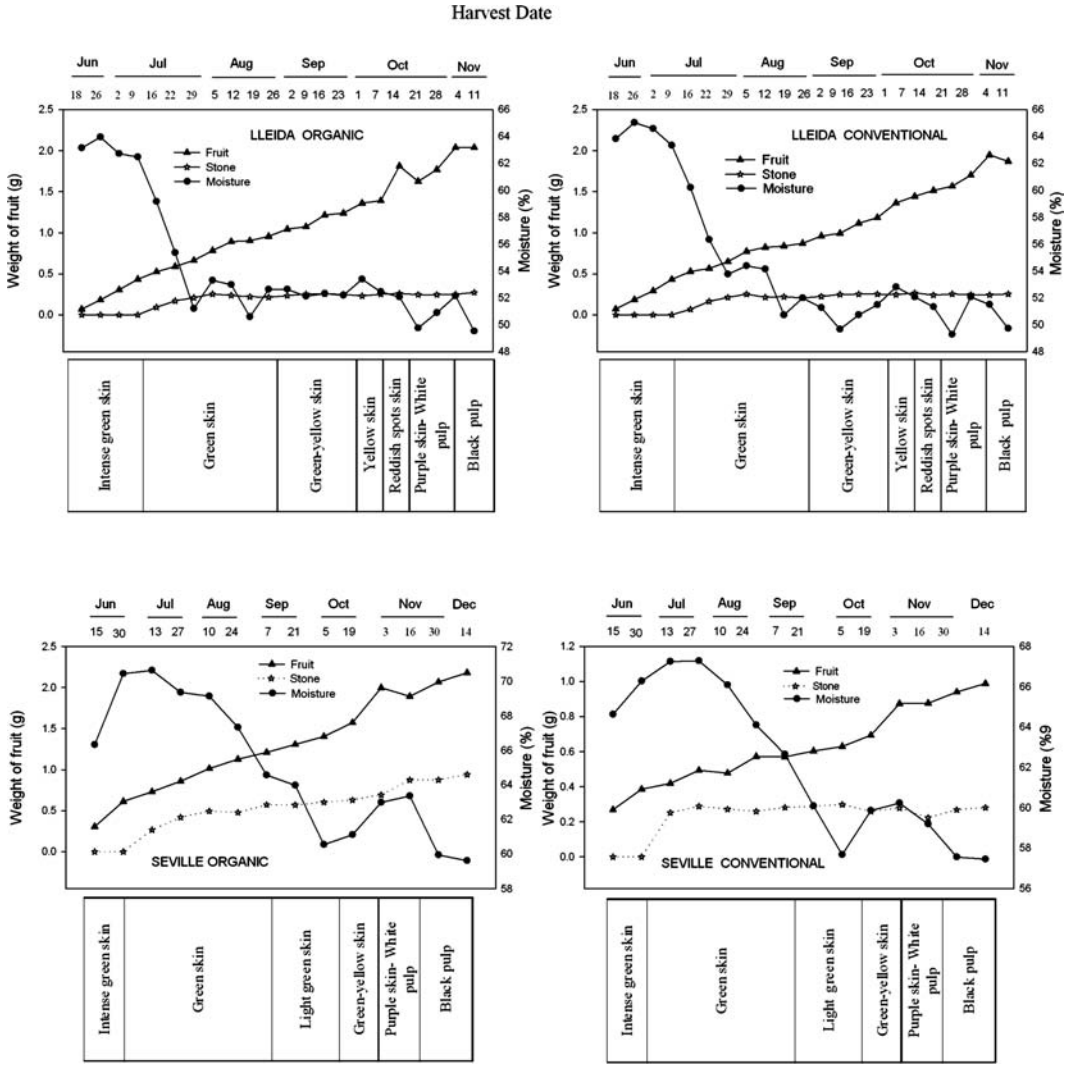

Figure 4. Changes in both the weight of the fruit and stone (g) and the moisture content (%) during different ripening stages of the Arbequina samples from Lleida and Seville.

from the first two samples, and it subsequently evolved in a different way in each orchard type. Whereas in the organic orchard registered a maximum weight of 0.94 g, the weight in the conventional orchard did not exceed 0.28 g from the time the fruit was green until the pulp turned black.

Regarding the moisture of the fruits, no differences were registered between the two orchard types in Lleida, where the initial moisture was approximately 63%, and the moisture declined steadily to 50−52% by the end of the maturation stage. During a period of 20 days (from July 9 to July 29), there was a sharp decrease in the moisture content of the fruits (loss of 19% and 16% of the initial moisture).

In Seville, the maximum moisture content of the fruit was observed on July 13 (71% and 67% in the organic and the conventional orchards, respectively). In both orchards, the moisture also declined sharply during a period of more than two months (from July 27 to October 5) to 60% and 57%, respectively (representing a 15% loss for both orchard types). The moisture decline in Seville was less pronounced than that registered in Lleida, and it took place over a time period that was three times longer. The maximum temperatures were twice as high in Seville as in Lleida. In spite of these differences, the fruits grown in Seville maintained higher moisture content during a longer period of time, regardless of the fact that the temperature was twice as high as that in Lleida.

Metabolic Changes during the Accumulation of Oleuropein. Figure 5 shows the extracted ion chromatograms of the main phenolic compunds quantified in olive fruit by HPLC−MS/MS. The intermediary precursors involved in the accumulation of oleuropein and its transformation products by β-glucosidase (Figure 2) were monitored during the growth, development, and ripening of the fruits (Figures 6 and 7), and their concentrations are expressed in μmol/g of dry pulp. This study investigated 7-ketologanin, oleoside-11-methyl ester, 7-β-1-D-glucopyranosil-11-methyl oleoside, tyrosol, ligstroside, oleuropein, and the enzymatic hydrolysis products derived from β-glucosidase activity (Figure 2). The latter compounds include oleuropein aglycone (3,4-DHPEA-EA), its dialdehydic form (3,4-DHPEA-EDA), and the aglycones corresponding to ligstroside (p-HPEA-EA and p-HPEA-EDA) and elenolic acid. Trace concentrations of the biosynthetic intermediates 7-ketologanin, oleoside-11-methyl ester, 7-β-1-D-glucopyranosil-11-methyl oleoside, and tyrosol were measured in olive fruits from the four orchards studied, suggesting that the early synthesis of ligstroside allows for the rapid formation of oleuropein, which is its immediate precursor (Figures 1 and 2). This finding

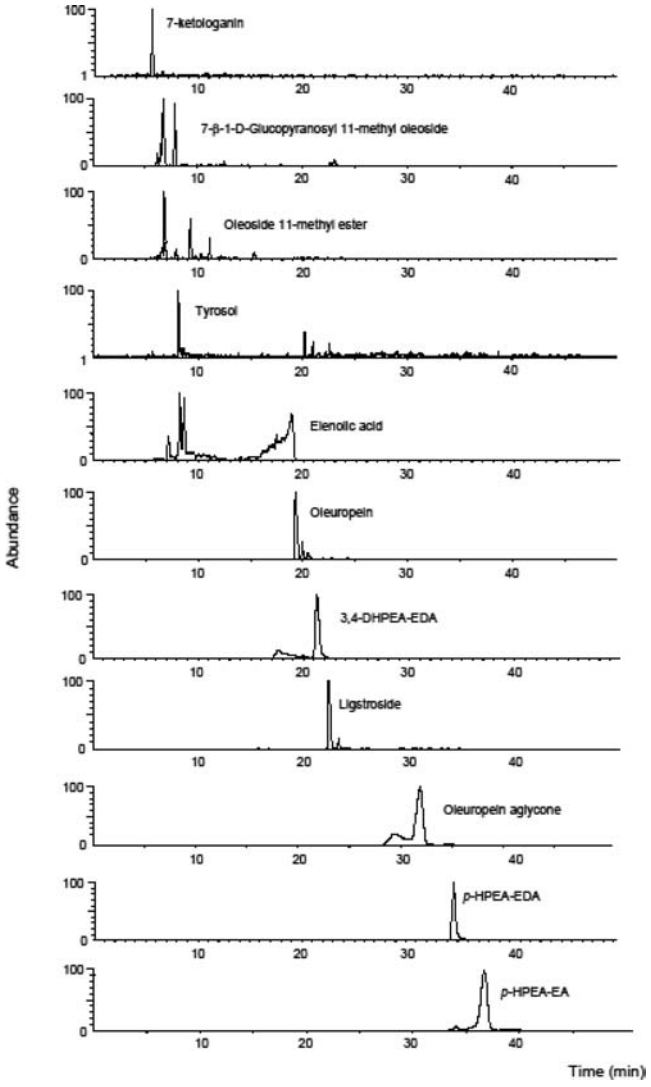

Figure 5. Extracted ion chromatograms of the main phenolic compunds quantified in olive fruit by HPLC−MS/MS.

is in accordance with the high levels of ligstroside and oleuropein recorded in the immature fruits of the four olive groves. In Lleida (Figure 7), the ligstroside concentration of the sample of June18 was 208 μmol/g in the organic orchard and 215 μmol/g in the conventional one. In the sample of July l2, the concentration of ligstroside abruptly decreased, resulting in a net increase in the oleuropein concentration of 22.6 and 52.8 μmol/g in the organic and conventional orchards, respectively, that leads to peak concentrations (255 μmol/g in the organic orchard and 277 μmol/g in the conventional one) in the immature fruit. From this time, the oleuropein became the most abundant phenol compound, reaching concentrations that ranged between 255 and 203 μmol/g in the organic orchard and between 277 and 183 μmol/g in the conventional orchard (from June 26 to July 9). However, oleuropein was not the most abundant phenol during all the physiological processes of the fruit. At the green fruit stage, the concentration of oleuropein decreased and remained practically constant between 153 and 132 μmol/g for the organic orchard and about 180 μmol/g for the conventional orchard for a period of 4 weeks (from July 16 to August 5). After this stage, a loss in the net concentration of oleuropein of 81 and 107 μmol/g was measured for the organic and the conventional orchards, respectively. In the conventional grove, the net loss was prolonged until September 2. At the end of the ripening stage, when the fruit had a purple color, the oleuropein concentration was approximately 0.3−0.1 μmol/g in both orchards.

In Seville's groves, the reactions involved in the biosynthetic route followed the same seasonal pathway described for Lleida's groves. The highest ligstroside concentrations were measured for the sample of the June 15 to be 76.3 μmol/g in the organic orchard and 100 μmol/g in the conventional orchard. Starting in the sample June 30, the ligstroside concentration decreased, and a minimum value of 16 and 17.6 μmol/g in the organic and conventional orchards, respectively, was observed in the sample July 27. These decreases had an impact on the oleuropein formation, which reached its highest concentration (93.6 μmol/g in the organic orchard and 137 μmol/g in the conventional orchard, corresponding to increases of 38 and 93 μmol/g, respectively) in the June 30 sample (Figure 7). Subsequently, the concentration of oleuropein decreased to values ranging from 85 to 35 μmol/g in the organic grove and from 112 to 60 μmol/g in the conventional grove. These concentrations were maintained until the sampling August 24, after which the concentrations decreased constantly and then remained at approximately 20−23 μmol/g in the organic orchard and 40−50 μmol/g in the conventional orchard from August 24 until October 5. After this stage, the net concentration loss was 17 and 13 μmol/g in the organic and the conventional orchards, respectively. At the end of the ripening stage, when the fruit skin had a purple color, the oleuropein concentration was approximately 0.2−0.3 μmol/g in both orchards. The higher abundance of oleuropein in the orchards of Lleida than in those of Seville until nearly the end of the biological process is thought to be related to the higher biosynthetic capacity of the former orchards.

In summary, in both types of farming the differences in the ligtroside and oleuropein concentrations were lower than those among geographical origins. These results reveal the crucial influence of the climatic and environmental factors on the biosynthetic rate of olive secoroids. When comparing the cultivar's origin, the concentration of ligstroside and oleuropein found in the Lleida samples is approximately two times higher than those from Seville.

The information regarding the presence of oleuropein in different olive varieties is restricted to selective data within a relatively narrow range of values. Amiot et al.[12] measured values that were very similar to ours for different olive varieties during their development and ripening stages (approximately 46.3 μmol/g for the Luques and Richoline varieties and 45.2 μmol/g for the Solonenque variety). In a study of immature green fruits in 1999, Ryan et al.[13] determined the maximum oleuropein concentration to be 44.4 μmol/g. Regarding the investigations conducted in Spain, Ortega-García and Peragón[14] reported that the concentration of oleuropein in green fruits was 69.9 and 54.7 μmol/g for the Picual and the Arbequina varieties, respectively. Concerning the olive trees grown in Córdoba (Spain), Gutiérrez-Rosales et al.[4] measured a maximum concentration of 20 μmol/g in the green fruits of the Hojiblanca variety and a maximum concentration of 40 μmol/g for the Arbequina variety. Therefore, the reported mean value ranges from 20 to 70 μmol/g.

Ortega-García and Peragón[15] investigated the presence of oleuropein in the stems and roots of *Olea europaea* L. cv. Picual during ripening and found that the oleuropein concentration in

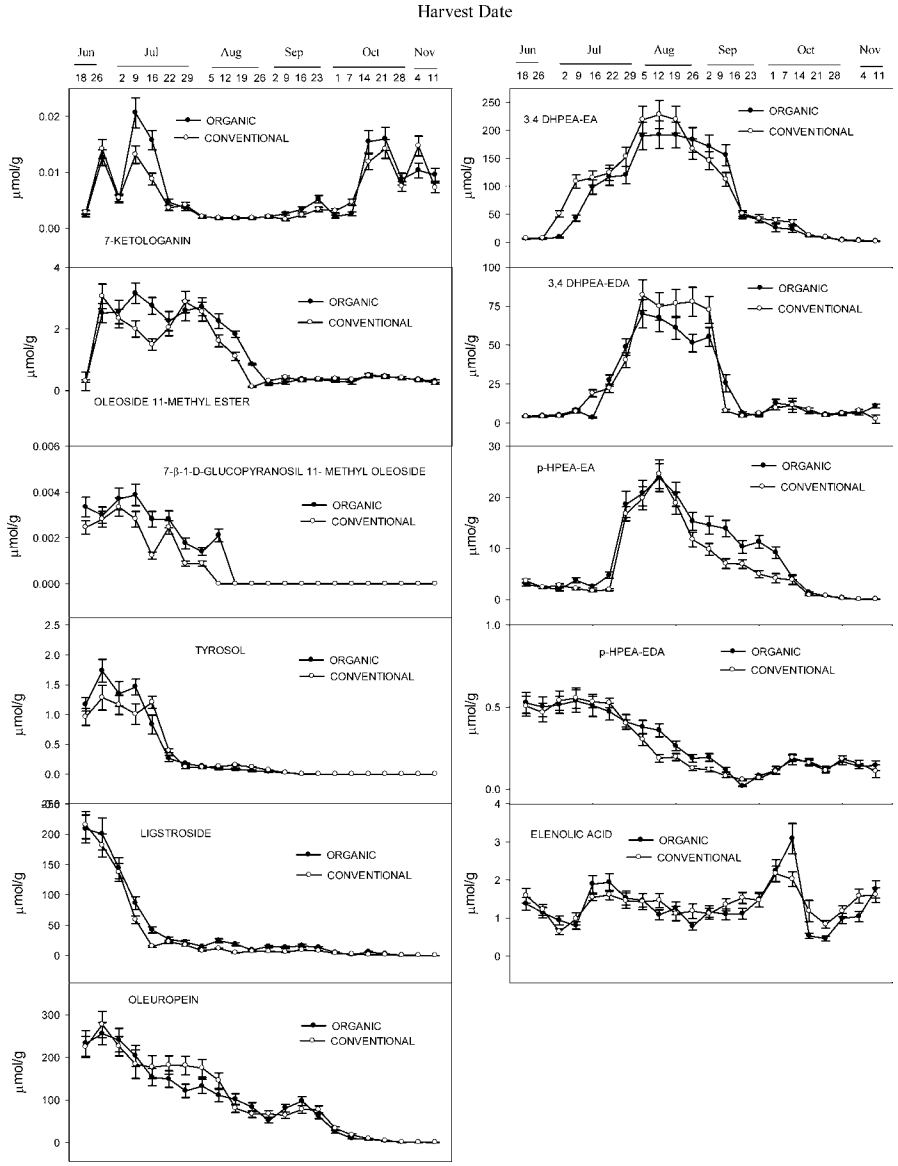

Figure 6. Changes in the concentration of polyphenols (μmol/g dry pulp) integrated in the synthesis and degradation pathways of oleuropein during the vegetative cycle of the Arbequina samples from Lleida. Each value is expressed as the mean of three determinations ± SD; RSD < 15%.

the stems increased from mid-September until the end of November (from 37 to 111 μmol/g), whereas the levels of oleuropein in the roots declined during fruit ripening from 11 to 0.9 μmol/g.

The maximum oleuropein concentration measured in the Seville orchards in this study are of the same magnitude as those reported by Ortega-García and Peragón[15] in the stems, whereas the values measured in Lleida's olive buds were clearly higher (although this physiological stage had not been studied elsewhere).

The influence of the farming practices (organic and conventional) on the fruit composition (polyphenols, vitamin C, β-carotene, etc.) has been studied in different species,[16−18] with contradictory results. Thus, Dani et al.[16] reported that total phenolics and resveratrol contents in white and black grapes were higher (P < 0,05) in juices from fruits cultivated with organic farming practices in comparison with fruits from the conventional one. On the other hand, Lombardi-Boccia et al.[17] carried out a comparative study of different nutrients and antioxidant in yellow plums, finding that the polyphenols contents were higher in conventional plums. Nevertheless, Devanand et al.,[18] who investigated the influence that organic and conventional farming practices exerted on the total phenolic content in eggplant pulp from two origins (USA and Japan), concluded that multiple repetitive analyses of plant products collected from different plants grown over different time periods (seasons) at different locations should always be carried out to unambiguously prove the impact of growing conditions on phenolic content or antioxidant.

Involvement of β-Glucosidase in Oleuropein Degradation. β-Glucosidase (3.2.1.21) is the enzyme responsible for the hydrolysis of the glucosides oleoside-11-methyl ester, oleuropein and ligstroside, generating elenolic acid and the corresponding oleuropein (3,4-DHPEA-EA, 3,4-DHPEA-EDA) and ligstroside (p-HPEA-EA, p-HPEA-EDA) aglycones. As can be observed in Figure 8 the measured activity of the enzyme exhibited a clear development pattern depending on the orchard. In Lleida (Figure 8A), the enzyme activity increased from July 9 to September 2, reaching respective values of 1.3 and 2.7 μkat/g in the organic orchard and 1.4 and 3.0 μkat/g in

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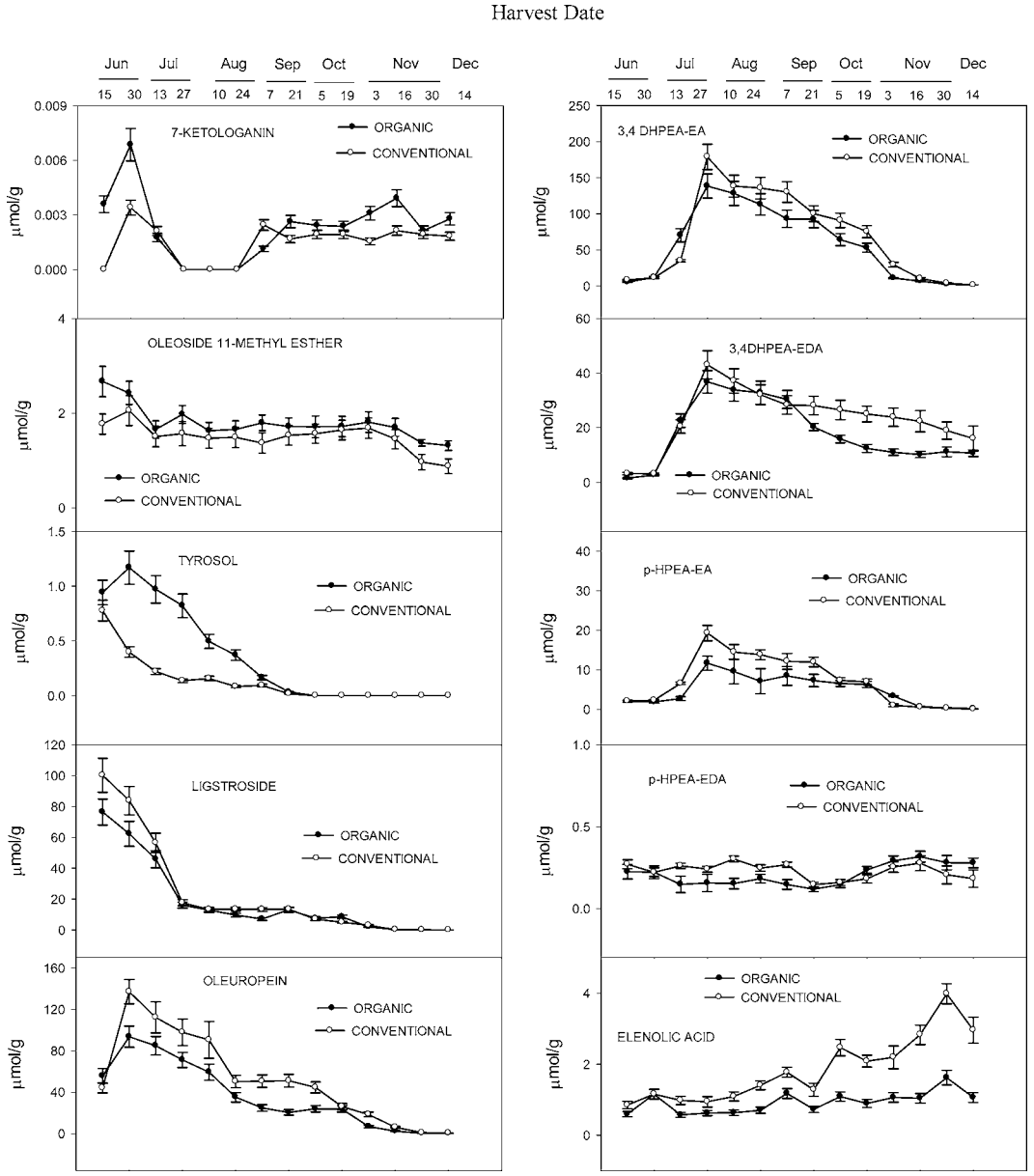

Figure 7. Changes in the concentration of polyphenols (μmol/g dry pulp) integrated in the synthesis and degradation pathways of oleuropein during the vegetative cycle of the Arbequina samples from Seville. Each value is expressed as the mean of three determinations ± SD; RSD < 15%.

the conventional orchard. In Seville, the enzyme activity decreased from July 27 until September 21, with respective values of 2.9 and 1.8 μkat/g in the organic grove and 3.8 and 2.6 μkat/g in the conventional grove (Figure 8B).

Notably, in the four orchards, the maximum β-glucosidase activity was concomitant with the emergence of the aglycones. Therefore, the highest concentrations of oleuropein were measured when the β-glucosidase activity was absent. The maximum oleuropein aglycone concentrations in fruits were observed during the period of maximum enzymatic activity, and they remained the most abundant compounds during the rest of the fruit's vegetative cycle. This observation suggests that the *de novo* synthesis of oleuropein begins in the young fruits. Given that the concentration of oleuropein was always higher in Lleida, it can be stated that the synthesis of this compound depends more on the geographical origin of the fruit than on the farming practice, an aspect that supports what we commented on in the above section.

The stability of oleuropein content, in both the Lleida and Seville groves, indicates that the oleuropein biosynthetic route was still active, and it was balanced by the catabolic route of β-glucosidase with which it competes. It should be noted that in Lleida, as the fruit ripened and turned yellow-green, there was a second maximum in the concentration of oleuropein (September 16) of 97 and 77 μmol/g in the organic and conventional orchards, respectively. This maximum occurred at the same time as the maximum observed in a previous study conducted in Seville[4] in which the oleuropein synthesis was lower (40 μmol/g). These maxima can be explained by a lower concentration of β-glucosidase activity, which results in a reduced modification of the substrate *in vivo* and, therefore, a decrease in the formation of aglycones that would prevent the oleuropein increase. The recorded activity of this enzyme on September 16 was 0.68 μkat/g in the organic orchard and 0.83 μkat/g in the conventional orchard. In the previous study,[4] the recorded activity of β-glucosidase at the time of the

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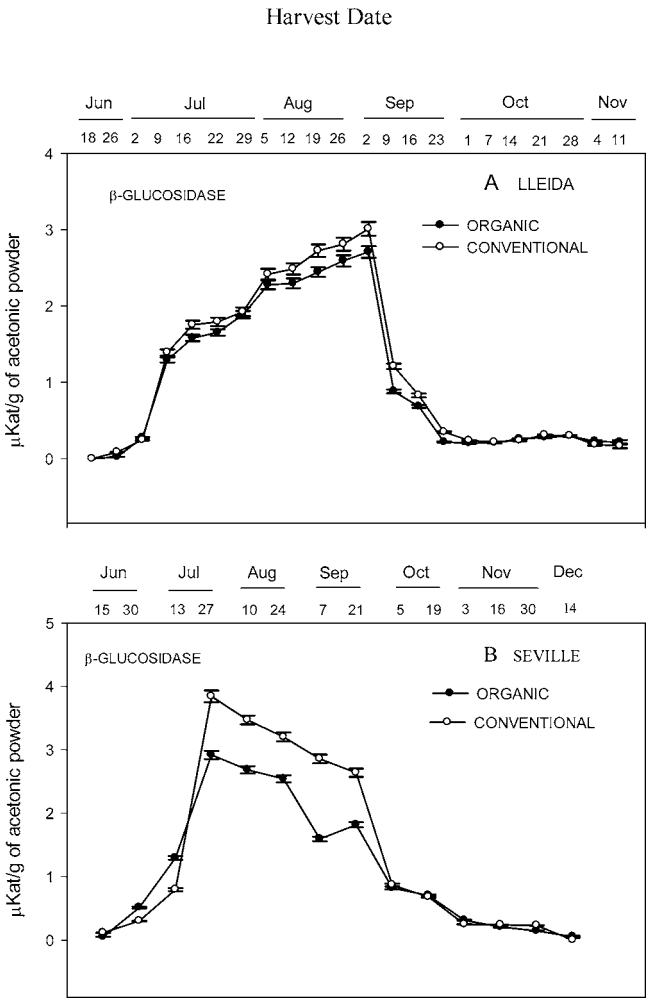

Figure 8. Changes in β-glucosidase activity during the vegetative cycle of the fruit (μkat/g acetonic powder) of the Arbequina samples from Lleida (A) and Seville (B). Each value is expressed as the mean of three determinations ± SD; RSD < 5%.

maximum oleuropein concentration was much higher (1.99 μkat/g), and consequently, the oleuropein concentration was much lower. This result suggests that the maximum reported in this study[4] corresponds to a degradation stage and that the massive synthesis stage could not be observed because of a faster development of the fruit and a 2.4-fold higher (1.99/0.83) β-glucosidase activity.

In Tables 1 and 2 we show the theoretical oleuropein synthesis in absence of β-glucosidase (estimated as sum of concentrations of oleuropein and its aglycones 3,4-DHPEA-EA and 3,4-DHPEA-EDA). In Lleida (Table 1), a higher biosynthetic capacity for both orchards was observed between August 5 and September 2, coinciding with the maximum values of the β-glucosidase activity (2.2−2.7 μkat/g in the organic orchard and 2.4−3.0 μkat/g in the conventional orchard). Consequently, during this period, there is a higher modification of the substrate that leads to maximum concentrations of the oleuropein aglycones. This maximum concentration translates into a maximum biosynthetic capacity of 392.1 μmol/g in the organic orchard and 475.31 μmol/g in the conventional orchard on August 5.

In the Seville orchards (Table 2), the biosynthetic capacity of both orchards is significantly lower than that in Lleida. The maximum biosynthetic capacity was 246.2 μmol/g in the organic grove and 319.6 μmol/g in the conventional grove. This maximum value was recorded July 27 and coincided with the maximum activity for β-glucosidase (2.9 and 3.8 μkat/g in the organic and conventional groves, respectively).

These maxima were not observed for oleuropein (Figures 6 and 7) because they were mediated by the activity of β-glucosidase, which hydrolyzes oleuropein to its corresponding aglycones. At this stage, the accumulation of oleuropein and,

Table 1. Theoretical Oleuropein Production in the Absence of β-Glucosidase (Estimated as Sum of Oleuropein and Its Aglycones) (μmol/g dry pulp) in Lleida Orchards[a]

harvest date	organic	conventional
June 18	243.02 ± 34.02a	235.07 ± 26.03a
June 26	266.19 ± 35.93b	288.30 ± 38.06b
July 2	254.06 ± 33.03c	280.89 ± 37.08c
July 9	254.30 ± 34.10d	299.01 ± 44.25d
July 16	254.83 ± 33.89e	310.82 ± 41.03f
July 22	292.86 ± 41.04g	327.88 ± 43.28g
July 29	289.63 ± 39.75h	373.73 ± 49.33i
August 5	392.08 ± 53.45j	475.29 ± 62.74k
August 12	370.10 ± 50.00l	448.18 ± 62.75m
August 19	353.70 ± 49.73n	376.05 ± 52.65n
August 26	317.16 ± 41.51o	311.27 ± 43.58o
September 2	277.83 ± 36.67p	285.35 ± 39.95p
September 9	261.90 ± 37.86q	183.41 ± 25.68r
September 16	151.32 ± 19.97s	132.68 ± 18.58s
September 23	107.33 ± 14.17t	124.82 ± 17.47t
October 1	63.80 ± 8.42u	81.75 ± 10.87v
October 7	43.50 ± 5.74x	64.67 ± 8.60y
October 14	25.59 ± 3.38w	29.38 ± 3.91w
October 21	16.97 ± 2.24z	17.87 ± 2.38z
October 28	11.03 ± 1.46α	9.46 ± 1.26α
November 4	9.74 ± 1.29β	9.84 ± 1.31β
November 11	13.02 ± 1.72μ	3.63 ± 0.48μ

[a]Each total value represents the mean of three determinations ± SD. Values in different columns for organic and conventional cultivars followed by different letters are significantly different (Tukey's multiple range test $P \leq 0.05$).

Table 2. Theoretical Oleuropein Production in the Absence of β-Glucosidase (Estimated as Sum of Oleuropein and Its Aglycones) (μmol/g dry pulp) in Sevilla Orchards[a]

harvest date	organic	conventional
June 15	62.43 ± 8.24a	55.60 ± 7.78a
June 30	108.33 ± 14.30b	151.55 ± 21.22b
July 13	177.71 ± 23.46c	167.68 ± 23.48c
July 27	246.23 ± 32.50d	319.58 ± 42.19e
August 10	221.43 ± 29.23f	265.99 ± 35.11f
August 24	180.73 ± 23.86g	217.83 ± 28.75g
September 7	148.13 ± 20.74h	208.84 ± 20.74i
September 21	133.22 ± 18.65j	179.27 ± 23.62k
October 5	103.79 ± 14.53l	161.88 ± 22.66m
October 19	89.07 ± 12.47n	126.18 ± 17.66o
November 2	28.61 ± 3.78p	71.55 ± 3.78q
November 16	19.02 ± 2.51r	38.72 ± 2.51s
November 30	13.71 ± 1.81t	23.40 ± 3.28u
December 14	12.11 ± 1.60v	17.85 ± 1.60x

[a]Each total value represents mean of three determinations ± standard deviation. Values in different columns for organic and conventional cultivars followed by different letters are significantly different (Tukey's multiple range test $P \leq 0.05$).

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consequently, the concentration of β-glucosidase were significantly higher in the conventional orchards than in the organic orchards ($P \leq 0.05$). The ratio between the maximum values observed in Lleida and Seville (Tables 1 and 2) was 1.5, indicating that the biosynthetic capacity is higher in fruits from Lleida. These results suggest that the geographical origin is more relevant than the farming practice. The oleuropein decreased as the fruit developed and ripened. This decrease occurred during a period in which the biosynthetic route of oleuropein competes with the catabolic route of β-glucosidase.

As mentioned before, the aglycone concentration increased concommitanly with the enzyme activity. This result confirms the endogenous oleuropein hydrolysis *in vivo* by β-glucosidase because as the concentration of oleuropein decreased, the rate of transformation of the substrate increased. During this period, the aglycones reached their maximum concentration, being the most abundant phenols, and then sharply declined. This decline suggests that these compounds can react to form other types of compounds that were not analyzed in the present study.

In the Lleida groves (Figure 6), the oleuropein aglycone (3,4-DHPEA-EA) from August 5 to September 2 was the most abundant compound among the studied polyphenols (range concentration 155−192 μmol/g in the organic orchard and 145−228 μmol/g in the conventional orchard). The dialdehydic form of the elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) also exhibited its maximum concentrations (55−70 and 72−82 μmol/g in the organic and the conventional orchards, respectively) during this period. These maximal concentrations of the oleuropein aglycones coincide with the maximum β-glucosidase activity in the organic (2.3−2.7 μkat/g) and the conventional (2.4−3.0 μkat/g) orchards.

From September 9 until the end of the harvest period, the concentrations of both aglycones (3,4-DHPEA-EA and 3,4-DHPEA-EDA) significantly decreased. Specifically, the concentration of 3,4-DHPEA-EA was 47−2.1 μmol/g in the organic orchard and 50−1.0 μmol/g in the conventional orchard, and the 3,4-DHPEA-EDA concentration ranged from 8.0−6.0 μmol/g in the organic grove and from 4.3−2.5 μmol/g in the conventional grove. Despite the decrease, these aglycones remained the most abundant compounds during the entire fruit life cycle. In this study, the aglycones were observed throughout the β-glucosidase activity cycle.

In the Seville orchards (Figure 7), the oleuropein aglycones from August 5 until September 2 were also the most abundant compound, which is similar to what was observed in the orchards in Lleida. Between July 27 and September 21, the concentration of the oleuropein aglycone (3,4-DHPEA-EA) was determined to be 138−92 μmol/g in the organic orchard and 178−100 μmol/g in the conventional orchard. During this period, 3,4-DHPEA-EDA also experienced its maximum concentrations (37.0−20.0 and 43.0−28.0 μmol/g in the organic and conventional groves, respectively). These maxima in the oleuropein aglycone concentration coincided with the maximum β-glucosidase activity in the organic (2.9−1.8 μkat/g) and conventional (3.8−2.7 μkat/g) orchards. After October 19, the 3,4-DHPEA-EA concentrations decreased to 11.0 μmol/g in the organic orchard and 29.4 μmol/g in the conventional orchard. This concentration continued to decrease to 1.3−1.4 μmol/g in both orchards by the end of the study. However, the concentration of 3,4-DHPEA-EDA experimented a smooth decline in both groves, decreasing to concentrations of 10.6 μmol/g in the organic orchard and 16 μmol/g in the conventional orchard by the end of the study.

Notably, once the initial stage of oleuropein accumulation is complete, the accumulated oleuropein is hydrolyzed by β-glucosidase even though the biosynthetic route is still active. This finding supports the results obtained in Lleida and the idea of two competing metabolic routes (anabolic and catabolic) during this growth period. The aglycone concentration was higher in the conventional orchards[17] in which the β-glucosidase activity reached its maximum in both Lleida and Seville. This observation was more pronounced in Seville where the β-glucosidase activity in the conventional orchard was higher than that observed in Lleida (3.8 and 3.0 μkat/g, respectively).

The low concentration of ligstroside aglycones (*p*-HPEA-EA and *p*-HPEA-EDA) in both Lleida and Seville, which was caused by the action of β-glucosidase, coincides with the disappearance of ligstroside, which was consumed in the biosynthetic route of oleuropein.

The increase in the concentration of elenolic acid (Figures 6 and 7) after July 9 in the Lleida orchards and July 27 in the Seville orchards could be associated with the action of β-glucosidase on oleoside-11-methyl ester, coinciding with a stabilization of the oleuropein concentration. In the second stage of olive growth, the presence of elenolic acid was associated with the degradation of oleuropein by the combined action of esterase and β-glucosidase,[19] which generated the oleoside-11-methyl ester that could be transformed into elenolic acid by the action of the β-glucosidase. Consequently, the formation of elenolic acid could be associated with the action of β-glucosidase via the intermediate oleoside-11-methyl ester until September 2 in the Lleida orchards and September 7 in Seville. During the rest of the studied period, the presence of elenolic acid was alternatively associated with the activity of β-glucosidase and esterase, both of which significantly modify the synthesis of oleuropein (Figure 2).

It should be noted that the formation and transformation of elenolic acid was analogous in the four orchards and followed the pathways described by Damtoft et al.[2] and Gutiérrez-Rosales et al.,[4] in which β-glucosidase participates. The higher abundance of oleuropein in the Lleida orchards appears to be related to the lower abundance of β-glucosidase, which is translated into a higher biosynthetic capacity of oleuropein. Therefore, the phase of accumulation of oleuropein depends on the geographical origin and the climate of the orchard. The lower relative moisture content in olive fruits from Lleida orchards might have had an influence on the *in vivo* of β-glucosidase activity, leading to a higher enzyme activity in the Seville groves.

To conclude, we are able to confirm that when β-glucosidase is absent, the Damtoft pathway is still present for fruits from Lleida and Seville. Once the first phase is finished and the β-glucosidase pathway is activated, oleuropein's biosynthetic pathway competes with β-glucosidase's catabolic pathway, remaining stable or decreasing the oleuropein concentration depending on whether both pathways are compensated or the catabolic pathway is dominant. The results of the study indicate a greater effect of the geographical origin than cultivation method of the olive tree.

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Notes

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