Modulation of the Biosynthesis of Some Phenolic Compounds in *Olea europaea* L. Fruits: Their Influence on Olive Oil Quality

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The phenolic composition of olive fruits (*Olea europaea* L.) (cv. Picual, Villalonga, Alfafarenca, and Cornicabra) grown in different areas of Spain was studied by high performance liquid chromatography−mass spectrometry. Different levels of tyrosol, catechin, *p*-coumaric acid, rutin, luteolin, and oleuropein were observed in the different varieties analyzed. Treating the fruit with 0.3% Brotomax 50 days after anthesis had a beneficial effect on fruit size, oil content, levels of polyphenolic compounds, and Trolox-equivalent antioxidant activity (TEAC) in all the varieties analyzed.

Keywords: *Antioxidant activity; Brotomax; catechin; luteolin; oleuropein; olive fruit; p-coumaric acid; rutin; tyrosol*

INTRODUCTION

Phenolic compounds constitute a complex mixture in both olive fruit and their derived products (notably oil), and although some reports exist on the nature of these compounds (*1*−*4*), there have been few studies on the polyphenolic composition of Spanish varieties (*5*).

Virgin olive oil is obtained from the olive fruit by mechanical means (pressure, centrifugation, or selective filtration), and is really the juice of the fruit. Its oxidative stability, which is high because of the presence of triacylglycerols, is also related to the presence of antioxidants and other minor components. Among these, phenolic compounds are considered to be responsible for imparting specific organoleptic properties to the oil (*6*) and determine a greater resistance to auto-oxidation (*7*, *8*). The ability of polyphenolic compounds to act as antioxidants depends on the redox properties of their phenolic hydroxy groups and the structural relationships between the different parts of the chemical structures (*9*, *10*).

In previous studies we examined the effect of Brotomax on growth processes and on the synthesis and/or accumulation of flavanones and coumarins in *Citrus* (*11*, *12*), and it was thought it might be of interest to extend this study to other plant materials. The aim of the present investigation was to characterize the phenolic compounds in Spanish varieties (Picual, Villalonga, Cornicabra, and Alfafarenca) of olive and to evaluate the effect of Brotomax on fruit growth, oil content, and expression of these phenolic compounds in olive fruits.

MATERIALS AND METHODS

Plant Materials, Brotomax Treatments, and Measurement of Characteristics of Fruit. The study was carried out on olive fruits (*Olea europaea* L.*)* from four Spanish varieties grown on commercial plantations: Picual (Jaén), Villalonga (Valencia), Alfafarenca (Valencia), and Cornicabra (Alicante). Fifty days after anthesis, 10 trees of each variety were sprayed with an aqueous solution of 0.3% Brotomax using 5 L/tree (treated) and another 10 trees of each variety were left untreated (control). After 170 days, 500 fruit (50 per tree) were harvested in each case (treated and untreated), all from all around the middle part of the trees. From each lot, 50 fruit were chosen at random and the equatorial and longitudinal diameters (mm) and the fresh weight were measured using a digimatic caliper (Mitutoyo, Tokyo) and a precision balance, respectively.

Chemicals. The compound Brotomax was purchased from AGROMETODOS, S.A. (Madrid, Spain). The standard phenolic compounds rutin, catechin, *p*-cumaric acid, and luteolin were purchased from Sigma (St. Louis, MO). Tyrosol was from Aldrich (Madrid, Spain) and oleuropein from EXTRASYNTHÈSE, S. A. (Genay, France). ABTS was supplied by Aldrich (Madrid, Spain) and Trolox was supplied by Sigma.

Extraction, Identification, and Quantification of Phenolic Compounds. Three fruit per tree for each experiment (control and treated) were collected, mixed, and divided into three lots of 10 fruit. These were ground and shaken with dimethyl sulfoxide (DMSO) (150 mg of fresh weight/mL) for 1 h for extraction. The corresponding extracts were filtered through a 0.45-μm nylon membrane before analysis by HPLC with a Hewlett-Packard liquid chromatograph (model HP 1050) fitted with a diode array detector (range scanned, 220−500 nm). The stationary phase was a (250 mm × 4 mm i.d.) Sherisorb ODS-2 column with a particle size of 5 μm, thermostated at 30 °C. As solvent we used a mixture of acetonitrile (A) and water (B): 25 to 95% of A in 50 min. Eluent flow was 1 mL/min. The absorbance changes were recorded in the UV−V diode array detector at 280 and 353 nm. The amounts of the principal phenolic compounds were determined from the area given by the integrator using the response factor of the corresponding standards.

The principal phenolic compounds in these extracts were collected at the exit of the HPLC column with a fraction collector (Pharmacia LKB Biotechnology, Sweden) for identification by means of a Hewlett-Packard mass spectrometer (model 5989).

Extraction of Olive Oil. The olive fruits (fresh weight known) were ground in a grinder, and the paste was placed in an oven at 105 °C for 48 h until a constant mass was

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10.1021/jf9913108 CCC: $20.00
Published on Web 12/06/2000

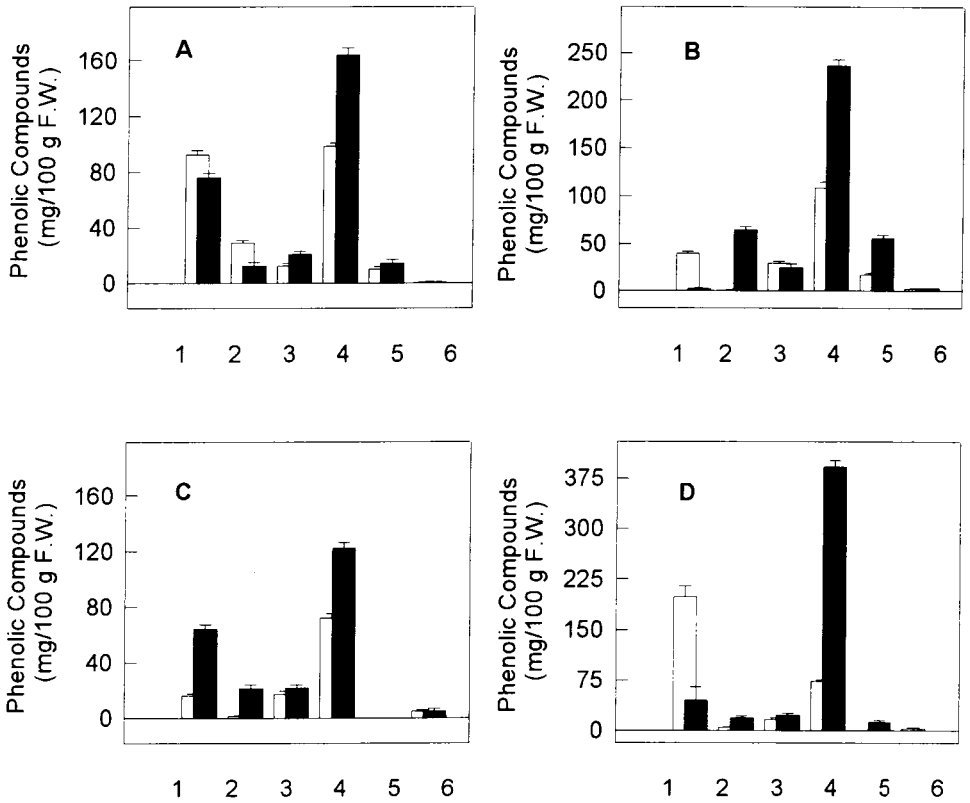

Figure 1. Effect of Brotomax on phenolic compound levels in mature fruit of Picual (A), Villalonga (B), Cornicabra (C), and Alfafarenca (D) varieties: (□) control and (■) treated fruits. Phenolic compounds: 1, tyrosol; 2, rutin; 3, catechin; 4, oleuropein; 5, luteolin; and 6, *p*-coumaric acid. The phenolic compounds levels (mg/100 g FW) represent mean values of three replicates ± SE.

reached. The oil was extracted in Soxhlet (25 g dry weight/300 mL of petroleum ether) at 70 °C for 3 h, and the oil content was computed as a percentage of the initial fresh weight.

Determination of Antioxidant Activity. The antioxidant activity of olive fruit extracts (750 mg fresh weight/5 mL DMSO) was determined spectrophotometrically, using the TEAC method (*13*), based on the abilities of different substances to scavenge the $ABTS^{\bullet+}$ radical cation compared with a standard antioxidant (Trolox) in a dose−response curve. $ABTS^{\bullet+}$ radical cation was prepared by passing a 5 mM aqueous stock solution of ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diamonium salt) from Aldrich through manganese dioxide (MnO_2) from Sigma on a Whatman no. 5 filter paper. The excess of MnO_2 was removed from the filtrate by passing it through a 0.4-μm nylon syringe filter. This solution was then diluted in 5 mM phosphate buffered saline (PBS) pH 7.4 with an absorbance of 0.70 (± 0.02) at 734 nm and preincubated at 30 °C prior to use. Fresh $ABTS^{\bullet+}$ radical cation solution was prepared each day.

2.5 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma) was prepared in PBS for use as stock standard. Fresh working standards were prepared daily by diluting 2.5 mM Trolox with PBS. All phenolic compounds were dissolved in DMSO to a concentration of 50 μM. After addition of 1 mL of $ABTS^{\bullet+}$ solution to aliquots of Trolox or phenolic compounds (1−100 μL, depending on the activity of the particular compound) the solutions were vortexed for exactly 30 s and the absorbance at 734 nm was taken, exactly 1 min after initiation of mixing, in a Unicam spectrophotometer at 30 °C. PBS blanks and DMSO blanks were run in each assay. The dose−response curve for Trolox was obtained by plotting the absorbance at 734 nm as a percentage of the absorbance of the uninhibited radical cation (blank) in triplicate determinations. The activity of the phenolic compounds in the olive extracts was assessed at four different concentrations determined to be within the range of the dose−response curve. Each extract was analyzed in triplicate at these four concentrations. By reference to the Trolox dose−response curve, the mean Trolox-equivalent antioxidant capacity (TEAC) value was derived for each olive extract.

RESULTS AND DISCUSSION

Levels of Phenolic Compounds in Fruits of *Olea europaea* L. The four varieties analyzed show the presence of tyrosol (t_R = 10.55 min, absorbance maximum at 277 nm), oleuropein (t_R = 23.83 min, absorbance maximum at 279 nm), catechin (t_R = 11min, absorbance maximum at 280 nm), *p*-coumaric acid (t_R = 17.07 min, absorbance maximum at 322 nm), luteolin (t_R = 19.21 min, absorbance maxima at 260 and 343 nm), and rutin (t_R = 18.27 min, absorbance maxima at 260 and 358 nm). These data are in agreement with those mentioned by other authors (*14*, *2*).

The mass spectra obtained for these compounds were identical to those obtained for the corresponding standards.

The levels of these compounds (tyrosol, oleuropein, rutin, catechin, luteolin, and *p*-coumaric acid) in each variety are shown in Figure 1. Tyrosol levels were highest in Alfafarenca (198 mg/100 g FW) followed by Picual (93 mg/100 g FW), Villalonga (39 mg/100 g FW), and Cornicabra (16.20 mg/100 g FW). However, oleuropein was most abundant in Villalonga (110 mg/100 g FW), followed by Picual (98.6 mg/100 g FW), whereas the lowest levels were found in Alfafarenca (74 mg/100 g FW) and Cornicabra (72.0 mg/100 g FW). The highest levels of catechin were detected in Villalonga (28 mg/100 g FW), with the levels in the other varieties analyzed being about 12−17%. *p*-Coumaric acid was not detected in Picual, and in Cornicabra, Villalonga, and

Table 1. Data of Physical Characteristics of Fruits from Different Varieties of *Olea europaea* L.: Fresh Weight (g) and Equatorial and Longitudinal Diameters (mm) Corresponding to Control and Treated (0.3 % Brotomax) Fruits[a]

	fresh weight		equatorial diameter		longitudinal diameter	
Spanish varieties	control	treated	control	treated	control	treated
Picual	2.4 ± 0.1 A	3.0 ± 0.2 B	13.4 ± 1.1A	16.6 ± 2.3 B	21.1± 1.4 A	22.4 ± 2.1A
Villalonga	3.6 ± 0.3 A	4.1 ± 0.4 B	15.9 ± 3.6A	17.9± 1.2 B	21.1± 1.3 A	21.8 ± 1.1A
Cornicabra	4.5 ± 0.1 A	6.3 ± 0.2 B	16.4 ± 1.4A	18.6 ± 1.1B	23.4 ± 1.9A	26.6 ± 1.6B
Alfafarenca	4.1 ± 0.2 A	4.9 ± 0.2 B	15.2 ± 0.8A	17.1 ± 1.5B	23.8 ± 1.3A	22.7 ± 1.6A

[a] Data represent mean values ± SE of 50 determinations. Values corresponding to control and treated fruit were compared by Duncan's multiple range test ($p = 0.01$).

Table 2. Oil Content (% per FW) of Fruits (control and treated with 0.3% Brotomax) of Different Varieties of *Olea europaea* L[a]

Spanish varieties	control	treated
Picual	20.7 ± 0.1 A	24.3 ± 0.2 B
Villalonga	20.5 ± 0.3 A	21.2 ± 0.2 A
Cornicabra	18.6 ± 0.2 A	20.2 ± 0.1 B
Alfafarenca	15.5 ± 0.1 A	16.6 ± 0.1 B

[a] Data represent mean values (%) ± SE of three determinations. Values corresponding to control and treated fruit were compared by Duncan's multiple range test ($p = 0.1$).

Alfafarenca the data were between 2.16 and 5.09 mg/100 g FW. Rutin was not detected in Villalonga; the highest rutin value observed was in Picual (29.0 mg/100 g FW), followed by Alfafarenca (4.10 mg/100 g FW), and Cornicabra (1.15 mg/100 g FW). Luteolin was not detected in Cornicabra or Alfafarenca, but Picual and Villalonga presented levels of 10.5 and 17.0 mg/100 g FW, respectively. Quantitative correlations were observed between the polyphenolic levels recorded in the Spanish cultivars studied and those in the Italian cultivars studied by Romani (*2*); for example, tyrosol levels of 118.6 mg/100 g FW were detected in Frantoio variety and 10.13 mg/100 g FW in Ciliegino variety. Oleuropein was present in substantial amounts (155 mg/100 g FW in Cuoricino variety), the same variety showing the highest levels of rutin (27 mg/100 g FW). However, luteolin levels were lower (about 3 mg/100 g FW) in Ciliegino, Cuoricino, Rossellino, and Frantoio varieties, and it was not detected in Grossolana variety (*2*).

Influence of Brotomax on Olive Fruit Growth and on the Oil Olive Content. Table 1 shows the mean fresh weight (g) and equatorial and longitudinal diameters (mm) corresponding to the control fruit and fruit treated with 0.3% Brotomax. The fresh weight values were significantly higher in the treated fruit with increases of 25% in Picual, 14% in Villalonga, 40% in Cornicabra, and 19% in Alfafarenca. The equatorial diameter of treated fruits was also significantly higher in treated fruit: 24% increase in Picual, 13% in Villalonga, Cornicabra, and Alfafarenca. However, the longitudinal diameter of treated fruits remained fairly close to the values of control fruits, with increases of between 2 and 6% in all varieties except Cornicabra, wich showed a significantly higher longitudinal diameter in treated fruit, with an increase of 14%.

Furthermore, the olive oil content (%) (Table 2) of fruits treated with Brotomax showed significantly higher values than the control in the Picual, Cornicabra, and Afafarenca varieties, with increases of 17%, 8.25%, and 7%, respectively. However, the increase observed for Villalonga variety (3%) was not significant.

Table 3. Antioxidant Activity (TEAC, mM) of Olive Fruits of Four Spanish Varieties

varieties	control	treated
Picual	3.73 ± 0.15	4.12 ± 0.15
Villalonga	5.89 ± 0.18	6.54 ± 0.15
Cornicabra	4.04 ± 0.25	9.60 ± 0.23
Alfafarenca	5.55 ± 0.09	6.63 ± 0.06

[a] Values represent the mean values ± SE of three determinations.

Influence of Brotomax on the Phenolic Composition and Antioxidant Activity of Olive Fruits. Figure 1 (A, B, C, and D) shows the concentrations of some phenolic compounds in Picual, Villalonga, Cornicabra, and Alfafarenca varieties (respectively) for both the control and treated fruits. As can be seen (Figure 1A), Brotomax increased the catechin, oleuropein, and luteolin levels by 76, 66, and 41%, respectively, in Picual, and the levels of luteolin and oleuropein in Villalonga olives (Figure 1B) were 229 and 116%, respectively, more concentrated in treated fruits than in control fruits. In the Alfafarenca variety, luteolin was not detected in control fruits but was clearly observable in the treated fruits, in which catechin levels increased by 34%, rutin increased by 363%, and oleuropein increased by 432% (Figure 1D). The results reported in Figure 1C show that Brotomax treatment increased tyrosol (295%), catechin (29%), rutin (1813%), and oleuropein (71%) levels in Cornicabra. Such differences in phenolic compound expression are probably due to the different extent to which the biosynthetic pathways of these compounds are affected. Significant quantitative differences in the phenolic content have been associated with several factors (type of cultivar, growing conditions, and time of ripening) in *Olea europaea* fruits (*15*−*17*), and in *Citrus* varieties (*18*). The increase in the levels of these polyphenolic compounds in *Olea europaea* L. brought about by Brotomax treatment agrees with the results obtained with Brotomax in *Citrus*, where an increased expression of flavanones (*11*) and coumarins (*12*) was observed.

In a previous paper we have described the antioxidant activity of these phenolic compounds in *Olea europaea* L. (*19*). The results described here confirm that Brotomax-treated fruit show greater antioxidant activity than untreated fruit (in the four varities studied). Thus, antioxidant activity (TEAC) increased (Table 3) by 10% in Picual, 11% in Villalonga, 137% in Cornicabra, and 128% in Alfafarenca. These increases in antioxidant activity are clearly related to the increases in polyphenol levels observed after Brotomax treatment. On the basis of the results obtained, it may be considered that the increase in phenolic compound levels reflects an important improvement in the quality of olive fruit and their derivative products, because of the beneficial effect on health of consuming products with a high phenolic

compound content and for the effect which such compounds have on the stability of olive oil (*20*, *21*).

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Received for review December 2, 1999. Revised manuscript received October 16, 2000. Accepted October 17, 2000.

JF9913108