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L-Phenylalanine ammonia-lyase activity and concentration of phenolics in developing olive (*Olea europaea* L cv Arbequina) fruit grown under different irrigation regimes

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Abstract: Changes in L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity and total phenolic, *ortho*-diphenolic and fat contents of olive flesh in response to different irrigation treatments applied to olive tree cv Arbequina were studied during fruit ripening. Results indicate that the fat content of olive flesh at harvest was not affected by irrigation, although olives from the most heavily irrigated treatment reached their final fat content (dry weight) earlier than those from other irrigation treatments. PAL activity and phenolic content, expressed on a dry weight basis, decreased during fruit development and were affected by irrigation, being lowered as the water supplied increased. Good correlations were established between PAL enzymatic activity and the polyphenol and *ortho*-diphenol contents of olive flesh, indicating that PAL is involved in the phenolic metabolism of olive fruit. The phenolic content of the oil depends on the PAL activity in the fruit, which varies with changes in water status.

Keywords: olive fruit; L-phenylalanine ammonia-lyase; phenolic compounds; Arbequina cultivar; irrigation

INTRODUCTION

Diet plays a major role in human physiology and pathology as indicated by the considerable amount of epidemiological evidence showing an association between diets rich in fresh fruits and vegetables and a lower risk of the degenerative diseases that accompany aging.[1] The protection provided against disease by fruits and vegetables has been attributed to various antioxidants present in these foods.[1,2] Owing to the climate, plants such as olives in the Mediterranean basin are subjected to prolonged exposure to sunlight. These plants accumulate antioxidant compounds for protection against ultraviolet light and subsequent oxidative damage.[3]

There is evidence that a regular intake of olive oil protects us against cardiovascular diseases and decreases the risk of breast cancer.[3] These beneficial effects are attributed to the high oleic acid percentage of olive oil and the presence of some less abundant antioxidants such as tocopherols, carotenoids, phenolic compounds, etc which also have organoleptic and nutritional properties. Numerous studies have highlighted the role of the polyphenolic components of higher plants as dietary antioxidants. Their antioxidant activity is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, they have a metal chelation potential avoiding lipid oxidation inside arteries and the subsequent accumulation of oxidised LDLs (low-density lipoproteins) in the arterial walls, a primary cause of coronary heart disease.[4]

For centuries, fruits of the olive (*Olea europaea* L) tree have been used for direct consumption and oil production. The polyphenol content in olive flesh is high compared with the majority of fruits, even reaching more than 70 mg caffeic acid g^{-1} olive flesh dry weight. Olive fruit phenols include different classes of compounds such as phenyl acids (hydroxycinnamic acid derivatives), flavonoids (flavonols, flavones and anthocyanins) and secoiridoids (oleuropein, demethyloleuropein, ligstroside and their derivatives).[5] Oleuropein, the main component producing bitterness in olives, is a heterosidic ester of elenolic acid and 3,4-dihydroxyphenylethanol.[6] As the fruit ripens, oleuropein progressively decreases[7,8] and phenolic

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compounds such as demethyloleuropein and 3,4-dihydroxyphenylethanol accumulate, as does the non-phenolic compound elenolic acid glucoside, which only corresponds to the secoiridoid part of oleuropein.[9] Other natural phenols that have been identified in olives are flavonol glycosides such as quercetin 3-rutinoside (rutin) and the flavones luteolin 7-glycoside, apigenin 7-glycoside and luteolin 5-glycoside.[5,10–12]

Understanding phenolic metabolism in plant cells and tissues requires knowledge of biosynthetic reactions and their regulation. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyses the reductive deamination of L-phenylalanine to form *trans*-cinnamic acid, the first step in the biosynthesis of plant phenylpropanoid compounds, which includes the formation of lignin, flavonoids and hydroxycinnamic acids. A high PAL activity is associated with the accumulation of anthocyanins and other phenolic compounds in tissues of several fruit species.[13–15] PAL activity varies with the development stage of the plant and cell and tissue differentiation.[16] It was shown that various stresses such as irradiation, wounding, nutrient deficiencies, herbicide treatment and viral, fungal and insect attacks increased either PAL synthesis or PAL activity in various plants.[17]

The accumulation of phenolic compounds varies widely with the physiological state of the fruit and is a result of a balance between biosynthesis and catabolism. Mineral nutrition has a major effect on phenolic accumulation. A limited nitrogen supply or boron deficiency seems to be associated with higher levels of phenolics in the plant.[18,19]

One of the major environmental constraints on phenolic production is light. Leaves from plants grown using an experimental set-up with a long day contain high levels of soluble phenolics. When transferred to short-day conditions, their ability to accumulate phenolics declines rapidly, as do the levels of PAL.[20] In mangoes, irradiation enhanced some phenolic acids and flavonoids.[21] Other environmental factors that are able to influence phenolic metabolism include ambient temperature and water availability. Lower temperatures tend to favour an increase in anthocyanin production. Acclimation of apple trees to cold climates was found to be associated with a seasonal accumulation of chlorogenic acid in apple fruit.[17] Studies of peach showed that PAL activity is affected by the soil water content,[22] and recent research on olive fruit showed that PAL activity and polyphenol concentration in oils decrease with the irrigation water applied to different olive tree cultivars.[23]

So far, variations in PAL activity during the development of the olive and their relationship with changes in phenolic compounds have not been reported. Our first studies showed that regulated deficit irrigation strategies applied to olive trees increased the polyphenol oil concentration, especially during the early stages of ripening.[24] The objective of the present study was to monitor PAL activity in order to study the cause of this variation and quantify changes in phenolic and *ortho*-diphenolic compounds in olive flesh during the development of the fruit (cv Arbequina) as well as to investigate the effect of different linear irrigation levels applied to olive (*Olea europaea* L) trees.

MATERIALS AND METHODS

Plant material

The trial was carried out in 1999 in a 6-year-old olive orchard (*Olea europaea* L cv Arbequina) planted on a predominantly clay loam soil located in the Segrià region (Catalonia, Spain) with trees spaced 6 m × 4 m. Annual rainfall for 1999 was 427 mm, being abundant during the spring and autumn and almost insignificant during the summer. Annual reference crop evapotranspiration (ET_0) was 1073 mm (see Fig 1).

The experimental irrigation was based on a linear irrigation design where the total applied irrigation water changed linearly with the effective crop coefficient (K_c) used when the water budget method proposed by the FAO[25] was applied to determine the crop water requirements (ET_c). This used the reference crop evapotranspiration (ET_0) from an agronomic weather station and the effective crop coefficient (K_c) ($ET_c = ET_0 \times K_c$). The water budget method calculates the irrigation requirements by subtracting the effective precipitation (P_{ef}) from ET_c. Owing to the fact that K_c for olive trees is almost constant throughout the year, this experimental design allows the relationship between the applied K_c and vegetative growth, olive and oil production and oil quality to be determined.

Seven irrigation treatments (T1–T7) were applied from the beginning of April to November, with estimated crop coefficients (K_c) of 0.25, 0.38, 0.50, 0.57, 0.64, 0.71 and 0.85 respectively. The trees were

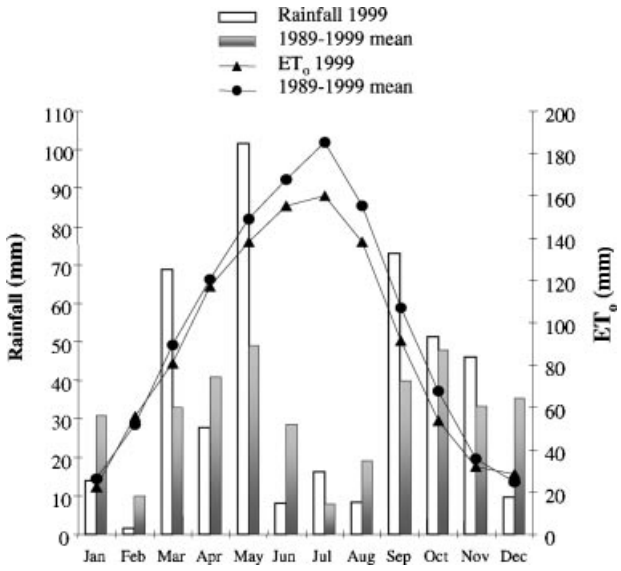

Figure 1. Rainfall and reference crop evapotranspiration (ET_0) for year 1999.

not irrigated in the remaining months because $ET_c - P_{ef}$ was negative.

ET_c was calculated from the modified Penman-determined reference crop water use (ET_0) for each irrigation treatment (using data from a weather station close to the experimental field). To adjust the predicted ET_c to the size of the canopy,[26] a reduction of 65% ($K_r = 0.35$) was imposed on irrigation treatments T1–T3 and a reduction of 60% ($K_r = 0.40$) on treatments T4–T7.

The experimental plot consisted of four blocks with seven irrigation levels in each of them. Each experimental unit (an irrigation level in a block) consisted of seven trees, with only the five central ones being monitored. Olive trees were irrigated daily with four 8 l h^{-1} drippers placed around the tree. A water meter was installed at the beginning of each line to verify that the water applied corresponded to each treatment.[27] Olive fruits from each irrigation treatment were randomly selected for sampling throughout ripening, from the immature stage to the normal harvest date, on the following dates: 30 September, 14 October, 4 November and 22 November (harvest period). Representative samples (60 olives) from each irrigation treatment/block (7 treatments × 4 blocks = 28 samples; four samples for each irrigation treatment and each picking date) were picked and brought to the laboratory for olive analyses.

Olive analyses

Ripening index

This was determined according to the proposals of the National Institute of Agronomical Research of Spain.[28] The procedure consisted of distributing a random sample of olives into eight groups according to the extent of pigmentation of the epicarp and mesocarp of the drupes: group 0, skin bright green; group 1, skin greenish-yellow; group 2, skin green with reddish spots; group 3, skin reddish-brown; group 4, skin black with white flesh; group 5, skin black with <50% purple flesh; group 6, skin black with >50% purple flesh; and group 7, skin black with 100% purple flesh. The index is given by $\sum(i\ n_i)/N$ where i is the number of the group, n_i is the number of olives in it and N is the total number of olives.

Water content

Samples of approximately 5 g of olive flesh from 20 olives were weighed, then dried for 24 h at 105 °C, cooled for 30 min in a desiccator and reweighed.

Fat content

Dried samples of olive flesh (approximately 2 g) were subjected to Soxhlet extraction using petroleum ether as solvent. After solvent evaporation the flask containing the fat was dried at 105 °C, cooled in a desiccator and reweighed. The results are expressed as g fat kg^{-1} dry weight olive flesh (g kg^{-1} dw).

Extraction and assay of L-phenylalanine ammonia-lyase (PAL) activity

For every sample, after prior freezing with liquid nitrogen, olive flesh from 20 olives was ground to a powder and used for biochemical analysis. The frozen powder (1.0 g) was homogenised for 30 s in chilled 0.05 M potassium phosphate buffer (25 ml; pH 6.6) with 0.2 g of Triton X-100 using a Polytron homogeniser. Polyvinylpolypyrrolidone (PVPP) (25 mg) was added and the suspension was centrifuged at 4 °C for 15 min at 13 000 rpm. The supernatant, stored on ice, was filtered through glass wool and used as a source of crude enzyme.

PAL activity in the crude enzyme extracts was assayed by an adaptation of the method of Zucker[29] as reported by McCallum and Walker.[30] The assay mixture consisted of 0.06 M sodium borate buffer (4.1 ml; pH 8.8) and crude enzyme (0.4 ml) and the reaction was initiated by the addition of 1 ml of a solution of L-phenylalanine (10 mg ml^{-1}; final concentration 11 mM). Tubes were incubated at 37 °C for 1 h. The reaction was stopped by the addition of 35% (w/w) trifluoroacetic acid (TFA) (0.5 ml) and tubes were centrifuged for 5 min at 5000 × g to pellet the denatured protein. The cinnamic acid yield was estimated by measuring the absorbance at 290 nm (A_{290}) of the supernatant in 1 cm quartz cuvettes. Triplicate assays were performed for each extract, both with and without substrate in order to compensate for increases in absorbance even in the absence of added L-phenylalanine. PAL enzymatic activity was expressed in μmol cinnamic acid liberated g^{-1} dry weight olive flesh h^{-1} (μmol cinnamic acid g^{-1} dw h^{-1}).

Extraction and quantification of phenolic compounds

The methodology to extract total polyphenols followed an adaptation of the method of Fantozzi and Montedoro[31] as reported by Chimi and Atouati.[32] Approximately 1 g of ground olive flesh from 20 olives was mixed in duplicate with 40 ml of hexane and agitated for 4 min; the upper phase was recovered and the extraction was repeated twice successively with the lower phase to allow removal of pigments and most of the lipids. Phenolic compounds were extracted with 80 ml of 80% (v/v) methanol containing 400 ppm sodium metabisulphite. The mixture was homogenised for 30 s using a Polytron homogeniser and then centrifuged for 5 min at 3000 rpm to separate the hydromethanolic phase. This procedure was repeated twice. The hydromethanolic phases were combined and filtered.

Aliquots of this extract were assayed for total polyphenol content using Folin–Ciocalteau reagent according to the method of Vázquez Roncero *et al.*[33] The polar fraction solution (2 ml) was transferred to a 50 ml volumetric flask and Folin–Ciocalteau reagent (2.5 ml) was added. After 3 min, 5 ml of saturated sodium carbonate solution was added and the flask was made up with distilled water and stored in the dark for 1 h. The absorbance of the solution was measured

at 725 nm. A calibration curve was prepared using concentrations of the standard ranging from 30 to 400 μg caffeic acid per 50 ml of solution. The total polyphenol content was expressed as g caffeic acid-kg^{-1} dry weight olive flesh (g caffeic acid kg^{-1} dw).

The concentration of *ortho*-diphenolic compounds in the methanolic extract was determined by the method of Maestro Durán *et al.*[34] A 1 ml volume of 5% (w/v) sodium molybdate in 50% ethanol was added to the aliquots of the hydromethanolic extract (5 ml). The contents were mixed for 1 min and the absorbance was measured after 15 min at 370 nm against a blank reagent prepared by adding 1 ml of 50% ethanol to the aliquot of the extract instead of sodium molybdate solution. Caffeic acid served as a standard to prepare a calibration curve in the 10–200 μg range per 5 ml of solution. The *ortho*-diphenolic content was expressed as g caffeic acid kg^{-1} dry weight olive flesh (g caffeic acid kg^{-1} dw).

Statistical analysis

Data were analysed by the General Linear Model procedure to determine the effects of picking date on each parameter using the version 6.12 SAS System package (SAS Institute Inc, Cary, NC, USA). Where appropriate, differences between picking date means were compared using least significant difference (LSD) at the 95% level. A regression procedure was used to establish the effects of irrigation treatments (K_c) on each parameter for every picking date and to demonstrate trends.

RESULTS AND DISCUSSION

Effect of picking date

From the first to the last picking date the ripening index increased in fruits from all irrigation treatments and reached values from 2 (green with red-spotted epicarp) to 3 (reddish-brown epicarp) at the time of harvest (22 November) (Table 1). Olives from the most extreme irrigation treatments, T1 and T7, reached a higher degree of ripening earlier than than those from the other irrigation treatments. The main increase in the ripening indices of olives from the T1 and T7 treatments was observed at the first picking dates, between 30 September and 14 October, while in the other irrigation treatments this increase was observed on 22 November, coinciding with the harvest period.

Although there were no statistical differences in fruit weight between the four picking dates in all irrigation treatments, it was observed that olive fruit weight showed a slight increase until it reached its maximum on 4 November, followed by a slight decrease in the last sampling as a consequence of the decrease in water content of the olive flesh.

The water content of olive flesh followed the same evolution in all irrigation treatments applied (Table 1). Between 30 September and 4 November, water content became practically constant, while it decreased significantly during the last sampling period, coinciding with harvest. This marked decrease in water content of the olive flesh could be related to fruit ripening and to the decrease in air temperature because of root hydraulic resistance in response to cold stress.[35]

The fat and phenolic contents and PAL activity

Table 1. Effects of picking date and irrigation treatment (K_c) on maturity index, weight, water content and fat content of olive (values are mean ± SE)

	Picking date[a]	Irrigation treatment (K_c)[b]							
		T1 (0.25)	T2 (0.38)	T3 (0.50)	T4 (0.57)	T5 (0.64)	T6 (0.71)	T7 (0.85)	
Maturity index	30/09/99	0.99 ± 0.04a	1.07 ± 0.13a	0.97 ± 0.08a	0.98 ± 0.01a	0.98 ± 0.01a	1.01 ± 0.01a	1.05 ± 0.03a	NS
	14/10/99	1.95 ± 0.32b	1.54 ± 0.26a	1.25 ± 0.19a	1.45 ± 0.04ab	1.24 ± 0.15a	1.13 ± 0.07a	2.08 ± 0.24b	NS
	04/11/99	2.33 ± 0.33bc	2.00 ± 0.32a	1.50 ± 0.06a	1.93 ± 0.30b	1.45 ± 0.21ab	1.44 ± 0.27ab	2.63 ± 0.01b	NS
	22/11/99	2.92 ± 0.07c	3.01 ± 0.38b	2.19 ± 0.24b	3.07 ± 0.41c	1.96 ± 0.14b	1.91 ± 0.29b	2.82 ± 0.47b	NS
		**	**	**	**	**	*	*	
Weight (g per fruit)	30/09/99	1.27 ± 0.11	1.08 ± 0.08	1.14 ± 0.02	1.20 ± 0.08	1.18 ± 0.06	1.12 ± 0.08	1.32 ± 0.06	NS
	14/10/99	1.45 ± 0.15	1.25 ± 0.12	1.33 ± 0.01	1.40 ± 0.08	1.31 ± 0.04	1.28 ± 0.09	1.51 ± 0.13	NS
	04/11/99	1.61 ± 0.16	1.31 ± 0.11	1.36 ± 0.06	1.56 ± 0.14	1.47 ± 0.09	1.39 ± 0.08	1.66 ± 0.15	NS
	22/11/99	1.44 ± 0.12	1.29 ± 0.08	1.33 ± 0.02	1.43 ± 0.11	1.22 ± 0.06	1.30 ± 0.06	1.53 ± 0.12	NS
		NS	NS	NS	NS	NS	NS	NS	
Water content (%)	30/09/99	56.43 ± 2.11a	55.17 ± 2.30a	57.84 ± 2.27a	57.32 ± 0.11a	57.33 ± 1.03a	60.96 ± 0.32a	61.54 ± 1.29a	**
	14/10/99	56.88 ± 0.56a	56.34 ± 0.39a	58.21 ± 1.20a	57.39 ± 0.87a	56.92 ± 1.36a	58.87 ± 0.25b	59.72 ± 0.64a	*
	04/11/99	55.39 ± 1.92a	53.22 ± 1.52a	55.97 ± 1.61a	56.22 ± 1.11a	55.86 ± 0.96a	58.08 ± 0.72b	59.13 ± 1.48a	**
	22/11/99	47.17 ± 0.76b	46.76 ± 0.36b	48.79 ± 2.08b	47.46 ± 1.42b	47.22 ± 1.44b	48.51 ± 0.68c	50.71 ± 1.42b	NS
		**	**	*	**	**	**	**	
Fat content (g kg^{-1} dw)	30/09/99	39.32 ± 0.49a	39.96 ± 0.22a	41.10 ± 0.19a	40.88 ± 0.49a	41.06 ± 0.66a	41.21 ± 0.23a	42.74 ± 0.46	**
	14/10/99	42.54 ± 0.74b	41.52 ± 0.98ab	42.73 ± 0.92ab	43.13 ± 0.29b	42.16 ± 0.39ab	43.53 ± 0.39b	43.74 ± 0.61	NS
	04/11/99	44.15 ± 0.46b	42.56 ± 0.46bc	42.80 ± 0.27ab	44.22 ± 0.64bc	42.97 ± 0.05bc	44.12 ± 0.26bc	43.80 ± 0.54	NS
	22/11/99	44.79 ± 0.99b	44.34 ± 0.60c	44.09 ± 0.04b	45.05 ± 0.33c	44.27 ± 0.64c	44.55 ± 0.28c	44.18 ± 0.45	NS
		**	**	*	**	*	**	NS	

[a] Different letters within the same column indicate a significant difference by picking date.

[b] Significance level of the model by row.

NS, not significant ($p > 0.05$);* $p < 0.05$; ** $p < 0.01$.

of olive flesh are expressed on a dry weight (dw) basis to eliminate variations in water content between samplings and irrigation treatments. Olives from irrigation treatments T1–T6 showed a significant increase in fat content (dw) between the first and last picking dates (Table 1). Olives from the most heavily irrigated treatment, T7, seemed to have reached their final fat content on 30 September, while those from the other irrigation treatments reached that point in mid-October, remaining practically unchanged from then on. This observation is in agreement with a study by García *et al*,[36] who found that lipid biosynthesis occurs mainly during cell growth of the olive fruit and stops when ripening begins.

Enzymatic PAL activity significantly decreased with fruit ripening in the T1–T4 irrigation treatments, reaching low levels at harvest time (22 November) (Table 2). Although this decrease is not statistically significant, in olives from the T5–T7 irrigation treatments the same trend could be observed. No relationship was found between K_c and the ripening index; therefore the difference in the level of PAL activity at the first sampling date cannot be ascribed to different degrees of fruit ripening under our experimental conditions. The higher PAL activity observed in the less irrigated trees could be a response to a stress situation accentuated by the climatic conditions of the summer (low rainfall and high ET_0). In autumn the abundant rainfall contributed to restoring the water conditions of the olive trees (Fig 1). Variations in PAL activity have been extensively studied during the growth and maturation of various fruits.[5] In numerous fruits (grapes, cherries, pears, grapefruit, etc), PAL activity is at its maximum in very young fruits and then falls very rapidly during growth. In contrast, in tomatoes, PAL activity reaches its maximum only at the end of growth, just before the fruits change colour, and then decreases. PAL increases again during maturation in fruits where anthocyanins accumulate, such as grapes, peaches, cherries and strawberries. Owing to the typical climatic conditions of the area where the experimental plot is located (Lleida, Catalonia, Spain), which is characterised by long, cold winters with persistent fogs, olives do not reach a ripening index superior to 3 or 4 at harvest, so the increase in PAL activity with the accumulation of anthocyanins in fruit flesh was not observed in our study. In this region, autumn frosts are very common. Consequently, harvest is not delayed, this covering the period from November to December.

During the sampling period there was a significant decrease in the total polyphenol and *ortho*-diphenol contents of olives subjected to all irrigation treatments, similar to that observed in PAL activity (Table 2). This notable decrease in phenolic substances during olive ripening has been previously reported by other authors.[5,37,38]

Effect of linear irrigation strategy

Some of the parameters analysed were affected by the linear irrigation strategy applied to the olive trees. The relationships between these and K_c, which determines the amount of water applied to the olive trees, were obtained by regression analyses and are shown in Table 3.

There was no statistical evidence that an increase in the irrigation water applied to the olive trees affected either the ripening index or the weight of the olives at any of the picking dates (Table 1).

At the first three picking dates a positive linear relationship between K_c and the water content of olive flesh was found (Table 3). An increase in K_c was associated with an increase in water content. Comparing the three independent linear regressions, it was found that their slopes and ordinates were not statistically different. Thus the effect of irrigation

Table 2. Effects of picking date and irrigation treatment (K_c) on PAL activity, polyphenol content and *o*-diphenol content in flesh olive (values are mean ± SE)

	Picking date[a]	Irrigation treatment (K_c)[b]							
		T1 (0.25)	T2 (0.38)	T3 (0.50)	T4 (0.57)	T5 (0.64)	T6 (0.71)	T7 (0.85)	
PAL activity	30/09/99	19.98±0.88a	20.13±1.41a	20.52±0.82a	16.11±0.72a	16.60±0.84	16.41±1.49	14.29±0.05	**
(μmol g^{-1}	14/10/99	19.73±1.59a	19.78±0.59a	19.34±0.58a	16.20±0.78a	15.04±0.91	14.55±0.67	12.30±1.01	**
dw h^{-1})	04/11/99	18.90±0.88a	15.71±1.63b	16.17±0.16b	13.73±1.30ab	14.20±0.68	14.89±0.64	13.18±1.61	**
	22/11/99	15.54±1.62b	14.68±0.76b	14.91±0.61b	12.24±0.08b	13.26±0.26	12.84±0.52	9.94±1.16	**
		*	*	**	*	NS	NS	NS	
Polyphenol	30/09/99	100.90±4.42a	95.38±1.94a	96.17±6.48a	98.97±4.61a	92.84±7.30a	86.91±1.10a	85.81±7.35a	*
content	14/10/99	100.76±4.52a	88.42±7.45a	88.29±4.19ab	80.25±2.44ab	82.76±4.64a	79.81±6.36b	79.49±3.91a	**
(g kg^{-1} dw)	04/11/99	89.54±1.98ab	82.06±5.24ab	81.19±1.79bc	83.61±7.24bc	80.04±1.62a	78.62±4.31b	76.08±4.62ab	*
	22/11/99	76.74±5.21b	71.40±2.87b	72.58±2.57c	63.28±7.16c	66.58±0.86b	66.24±0.36b	60.35±2.83b	**
		**	*	*	**	*	*	*	
o-Diphenol	30/09/99	5.93±0.43a	4.85±0.59a	4.54±0.18a	4.16±0.60a	4.14±0.18a	3.44±0.12a	3.32±0.15a	**
content	14/10/99	4.33±0.19b	4.25±0.41ab	3.63±0.18b	3.45±0.42a	3.24±0.18ab	2.90±0.06b	2.83±0.14ab	**
(g kg^{-1} dw)	04/11/99	4.12±0.30b	3.14±0.38bc	3.11±0.26b	3.48±0.36a	3.13±0.47b	2.48±0.15b	2.24±0.18bc	**
	22/11/99	2.66±0.34c	2.14±0.27c	2.23±0.14c	1.98±0.23b	2.15±0.14c	1.76±0.25c	1.62±0.27c	**
		**	**	**	*	**	**	**	

[a] Different letters within the same column indicate a significant difference by picking date.

[b] Significance level of the model by row.

NS, not significant ($p > 0.05$);* $p < 0.05$; ** $p < 0.01$.

Table 3. Regression equations showing effects of irrigation treatment (K_c) on water, fat, polyphenol and *o*-diphenol contents and PAL activity of olive flesh

Picking date	*Slope*	*Intercept*	R^2
Water content (%)			
30/09/99	9.85 ± 1.81	52.6 ± 3.08	0.73
14/10/99	4.75 ± 0.99	55.1 ± 1.69	0.62
04/11/99	7.78 ± 1.58	51.9 ± 2.69	0.68
Fat content (g kg^{-1} dry weight)			
30/09/99	5.06 ± 0.49	3.81 ± 0.83	0.90
PAL activity (μmol cinnamic acid g^{-1} dw h^{-1})			
30/09/99	−10.5 ± 1.32	23.6 ± 2.35	0.76
14/10/99	−13.8 ± 1.16	24.4 ± 1.97	0.89
04/11/99	−7.68 ± 1.41	19.5 ± 2.35	0.75
22/11/99	−8.56 ± 1.05	18.1 ± 1.78	0.81
Polyphenol content (g caffeic acid kg^{-1} dw)			
30/09/99	−24.49 ± 9.73	107.50 ± 5.72	0.74
14/10/99	−33.81 ± 9.56	104.52 ± 5.62	0.79
04/11/99	−19.06 ± 7.74	92.21 ± 4.55	0.82
22/11/99	−25.45 ± 7.18	82.35 ± 4.22	0.82
o-Diphenol content (g caffeic acid kg^{-1} dw)			
30/09/99	−4.27 ± 0.41	6.71 ± 0.70	0.94
14/10/99	−2.88 ± 0.28	5.12 ± 0.48	0.95
04/11/99	−2.69 ± 0.38	4.60 ± 0.65	0.77
22/11/99	−1.53 ± 0.26	2.93 ± 0.45	0.84

treatment on the water content of olive flesh is independent of the stage of ripening (Table 3). This positive linear relationship disappeared in the period prior to harvest (22 November), probably because low temperatures would have affected the water uptake of the olive trees as a result of a decrease in the hydraulic conductivity of the roots.[35]

The fat content of the olives only increased significantly ($p < 0.01$) with the amount of water applied to the olive tree in the early stages of ripening (30 September); the effect of irrigation disappeared with fruit ripening, and at harvest there were no differences in the fat content of olives from different treatments (Table 1).

The L-phenylalanine ammonia-lyase (PAL) activity of olive flesh varied depending on the irrigation treatment throughout the sampling period (Table 2). No statistical differences were found between the four slopes of the linear regressions between K_c and PAL activity (Table 3), showing that irrigation affected PAL activity in the same way whatever the development stage of the olive. At the last two picking dates, although PAL activity decreased at the same rate as at the previous picking dates, the values were lower. Activities were lower than those reported by Patumi *et al*[23] in Italian olive cultivars (Kalamata, Ascolana Tenera and Nocellara del Belice); however, the pattern was similar. PAL activity was higher in the rain-fed control treatment than in those treatments irrigated daily, supplying different percentages of crop evapotranspiration. The results also agree with studies on peaches, which showed that PAL activity is affected by soil water content.[22]

A linear relationship between K_c and the polyphenol content of olive flesh throughout the whole sampling period was also found, showing that as the water applied to olive trees increased, the content of these compounds decreased (Table 2). Compared with PAL activity, the effect of irrigation treatments on the polyphenol content of olive flesh was not dependent on the picking date. The stage of olive development only affected the absolute level of these compounds, being lower as the fruit ripened, as can been observed by the intercept value of the regression equations (Table 3).

A decreasing *ortho*-diphenol content of olive flesh was shown for all picking dates as K_c increased (Table 2). Comparing the four independent linear regressions obtained between K_c and the *ortho*-diphenol content, one for each picking date (Table 3), a difference was found in relation to the pattern followed by PAL activity and the polyphenol content. In the case of the *ortho*-diphenol content at the last picking date, besides the level of these compounds being inferior to that at the first picking date, the rate at which they decreased as K_c increased was less accentuated. As the fruit ripened, the effect of irrigation treatments on the *ortho*-diphenol content of the olive flesh was not so marked.

Correlation of PAL enzymatic activity with polyphenol and *ortho*-diphenol contents of olive flesh

There was a significant correlation between PAL activity and the polyphenol and *ortho*-diphenol contents of olive flesh ($r = 0.65$, $p < 0.01$ and $r = 0.75$, $p < 0.01$ respectively). PAL activity is closely related to the physiological or developmental status of a plant increasing levels of it and concomitant increasing levels of various fruits.[16,39,40] This study confirmed that there is a probable correlation between changing enzyme activities and changing levels of phenolic compounds. It also suggested the role of this enzyme, as the final concentration of phenolic compounds in the oil depends on the activity of this enzyme in the fruit, which varied as a result of changes in water status.

With the results observed in this study, it is possible to conclude that the fat content of olive flesh at harvest was not affected by irrigation, although olives from the most heavily irrigated treatment reached their final fat content (dw) before those from the other irrigation treatments. PAL activity and the polyphenol and *ortho*-diphenol contents of olive flesh decreased with fruit ripening and were affected by irrigation, all three becoming lower as irrigation increased. The significant correlation observed between PAL activity and the phenolic content of olive flesh may be evidence that this enzyme could condition the phenol content of virgin olive oil.

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