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Quantitative Changes in Phenolic Content during Physiological Development of the Olive (*Olea europaea*) Cultivar Hardy's Mammoth

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This investigation was designed to characterize phenolic metabolism of the olive cultivar, Hardy's Mammoth, by examining its constitutive tissues. The phenolic profiles of pulp, seed, stone, and new and old season leaves were monitored over two fruiting seasons, to investigate possible relationships between tissues and phenol content and to determine the impact of alternate fruit bearing. No major qualitative differences in phenolic composition were found between the various tissues; however, distinct differences between the tissues with respect to quantifiable phenols were established. Relationships between 2-(3,4-dihydroxyphenyl)ethyl (3*E*,4*E*)-4-formyl-3-(2-oxoethyl)hex-4-enoate ester, oleuropein, and hydroxytyrosol in pulp and leaf were identified and found to be related to alternate bearing. Concentrations of 5-caffeoylquinic acid in old season leaves differed dramatically between seasons, confirming earlier studies.

KEYWORDS: Phenol; olive; alternate bearing; oleuropein; pulp; seed; stone; leaf

INTRODUCTION

The nature of phenolic metabolism in higher plants is complex and not well-understood. Many phenomena and roles are attributable to the secondary metabolites. Phenols have been associated with plant and tissue maturation processes, defense mechanisms (*1*), and sensory characterization of plant-derived food products (*2*). Furthermore, phenols are chemically sensitive compounds (*3*, *4*); hence, the very nature of tissue sampling and subsequent extraction may significantly impact the distribution and content of these metabolites.

In *Olea europaea*, metabolic studies concerning phenolic compounds have focused on fruit pulp (*5*−*7*), presumably due to its importance in the food industry. In particular, the dynamics of oleuropein and its catabolic products (*6*−*8*) have received much attention due to its high concentration in green olive pulp (*6*). Other tissues, however, such as leaves and seeds have received little attention, and very rarely has the spectrum of olive tissues been investigated concurrently, as a function of sites of synthesis and translocation. Thus, many questions about the sites of biosynthesis and form of translocation of phenols between tissues in *O. europaea* remain unanswered. It is also not clear yet if individual tissues display a unique pattern of phenolic biosynthesis and catabolism. Furthermore, the involvement of phenolic compounds in fruit maturation and aging in olives is also not yet established. In the present study, we have investigated some of these issues.

The novel cultivar, Hardy's Mammoth, was selected for this investigation into phenolic metabolism since it is a uniquely Australian cultivar, and from preliminary results using randomly amplified polymorphic DNA, it is unrelated to any established cultivars (unpublished data). Phenolic profiles of the various tissues of cv *Hardy's Mammoth* have been previously presented (*9*). In this study, information concerning phenolic metabolism was gained by studying specific relations between structurally related phenols. These relationships were determined concurrently within different tissues and different developmental stages. Quantitative data are presented for 12 selected phenolic compounds: tyrosol, hydroxytyrosol, 2-(3,4-dihydroxyphenyl)-ethyl (3*S*,4*E*)-4-formyl-3-(2-oxoethyl)hex-4-enoate ester (3,4-DHPEA-DEDA), oleuropein, caffeic acid, 5-caffeoyl-quinic acid, verbascoside, oleuroside, luteolin-4-glucoside, luteolin-7-glucoside, a luteolin glucoside isomer, and nüzhenide in Hardy's Mammoth tissues over two fruiting seasons.

MATERIALS AND METHODS

Samples. Samples of fruit (cv. Hardy's Mammoth) were taken from an established grove in Yanco in southwestern New South Wales during the 1999 and 2000 harvest seasons (**Table 1**). Four trees from the same orchard row were selected based upon similarity of tree size, number of branches, and high fruit yields. On each tree, a scaffold, or major branch, was selected for sampling purposes. Each scaffold was facing northeast so as to minimize environmental variability.

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Table 1. Developmental Stage of Olive Fruit at Each Sampling Date

	degree of fruit maturation (based on fruit size and skin color)			
sampling date	tree 6	tree 8	tree 12	tree 14
		season 1		
1 Feb 1999	green	green	green	green
11 Feb 1999	green	green	green	green
8 Mar 1999	green	green	green	green
23 Mar 1999	green maturation	green maturation	green maturation	green maturation
6 Apr 1999	green–straw	green–straw	green–straw/red	green–straw
20 Apr 1999	green–red	green–red	green–red	green–red
3 May 1999	green–red	green–red/purple	green–red/purple	green–red
18 May 1999	reddish purple	reddish purple	reddish purple	green–red
31 May 1999	purple	purple	purple	purple
15 Jun 1999	black maturation	black maturation	black maturation	black maturation
		season 2		
30 Dec 1999	green	green	green	green
14 Feb 2000	green maturation	green maturation	green maturation	green maturation
24 Jul 2000	no fruit available	no fruit available	black maturation	black maturation

In addition to fruit, old and new season leaves were sampled. New season leaves are defined as those that grow above the fruiting zone and toward the extreme tip of the selected shoot. These leaves have not reached full cuticular development and are still soft. Old season leaves, however, encompass those leaves that grow between and beyond the fruiting zone toward the tree trunk.

Sample Pretreatment. After they were sampled, fruit and leaf samples were immediately frozen in liquid nitrogen and were subsequently freeze-dried using a Martin Christ Alpha 1-4 freeze-drier with LDC-1M controller. The freeze-dried olive pulp was removed from the olive stone using a scalpel and diced into small pieces (approximately 8−9 mm^3). The intact olive seed was removed from the stone by crushing. The stone was then macerated into small pieces (approximately 8−9 mm^3) using a hammer. Finally, dried leaves (both old and new season) were cut into small pieces (approximately 4−9 mm^2). All samples were placed in separate airtight, screw top, plastic jars and stored at −18 °C prior to analysis.

Chemical Reagents. Reagents from the following sources were used without further purification: acetic acid and acetonitrile (Ajax Chemicals), hexane (AlliedSignal), and methanol (EM Science). Phenolic standards were obtained as follows and were used without further purification: luteolin, luteolin-4-glucoside, luteolin-7-glucoside, and oleuropein from Extrasynthese; caffeic, chlorogenic, *o*-coumaric, *p*-coumaric, ferulic, and gallic acids from Sigma Chemical Company; and tyrosol from Aldrich Chemical Co. All standards were prepared in methanol:water (50:50 v/v) to the desired concentration and filtered through 0.45 μm plastic, nonsterile filters prior to chromatographic analysis. Grade 1 water (ISO3696) purified through a Milli-Q water system was used for all chromatographic analyses, sample and standard preparation.

Phenolic Extraction. Dried olive matter (0.25 g leaf, pulp, seed, or stone) was blended with methanol:water (5 mL; 50:50 v/v) for 20 s using an Ultra Turrax blender. The solution was left to stand for 30 min at ambient temperature and filtered using a Buchner funnel apparatus. The solid mass was recovered and reextracted as before; however, the solution was left to stand for 15 min prior to filtering. The filtrates were combined and washed with hexane (5 mL). The hexane was discarded, and the aqueous phase was filtered with GF/F filter paper using a Buchner funnel apparatus, followed by 0.45 μm plastic, nonsterile filters prior to high-performance liquid chromatography (HPLC) analysis.

HPLC. HPLC analyses were performed using a Perkin-Elmer binary LC-250 pump equipped with a 20 μL loop injector. A Perkin-Elmer LC-235 diode array detector (280 and 240 nm) and a Perkin-Elmer LC-240 fluorescence detector (excitation 280 nm, emission 340 nm) connected in series served to monitor the column eluent. Separation was achieved on a 150 mm × 4.6 mm i.d., 5 μm Alltech Alltima C18 column with gradient elution. The HPLC system was operated using Varian Star (version 4.5) software. The mobile phases were degassed under vacuum using Alltech Nylon 66 membranes and continuously sparged with high-purity helium during analysis to prevent resaturation by air. The gradient elution program employed water:acetic acid (100:1 v/v) as solvent A and methanol:acetonitrile:acetic acid (95:5:1 v/v/v) as solvent B, respectively, and a flow rate of 1 mL/min was used. A stepwise linear gradient commencing with 10% solvent B was employed. This was increased to 30% at 10 min, isocratic to 15 min, and then increased to 40% at 25 min, followed by further increases to 50 and 100% at 40 and 50 min, respectively, with a 5 min isocratic run followed by return to initial conditions at 80 min. This system allowed resolution and identification of the major phenols although routine monitoring at 280 nm did not differentiate hydroxytyrosol and its glucoside (*9*); they are therefore reported as "hydroxytyrosol" in **Table 2**.

RESULTS AND DISCUSSION

Sampling Strategy. As *O. europaea* is a known alternate bearer, i.e., high fruit loads occur every second season, tissue sampling was conducted over two seasons. This allowed investigation of metabolic data where the physiology of the tree suggested that large differences in metabolism were occurring. Sampling in the first fruiting season started with immature green fruit on 1 February 1999 and continued approximately fortnightly until the fruit had reached full black maturation on 15 June. Season 1 had a high fruit yield, and this facilitated more intense tissue sampling. Season 2 was a low-yield season, as expected; hence, samples were collected less frequently due to the limited amount of fruit. Only three sample collections were possible, but these were timed to correspond with the major developmental stages of the fruit, e.g., immature green fruit, 30 December 1999; mature green fruit, 14 February 2000; and mature black fruit, 24 July 2000. It should be noted that fruiting in season 2 began earlier than in season 1. Thus, the mature green fruit stage occurred on 8 March 1999 and 14 February 2000 in seasons 1 and 2, respectively.

Identification of phenolic compounds from the various tissues was carried out as previously reported (*9*). Some 55 individual compounds were detected in the tissues, and most compounds were found in all tissues (*9*). The levels of 12 selected phenols were determined in the various tissues on each date of sampling (**Table 2**). These phenols could be readily quantified from chromatographic data generated by monitoring at 280 nm. Calibration curves have been reported previously (*10*), and quantities of phenols are reported as tyrosol equivalents (mg/g dry tissue weight as tyrosol) since standards for all compounds were not available. Calculated phenolic concentrations in each individual plant tissue, from each of the four trees on a single

Table 2. Phenolic Content of Various Olive Plant Tissues Measured over Two Seasons[a]

		concentration (mg/g dry tissue weight as tyrosol)												
		season 1										season 2		
tissue	phenol	Feb 1	Feb 11	Mar 8	Mar 23	Apr 6	Apr 20	May 3	May 18	May 31	Jun 15	Dec 30	Feb 14	Jul 24
pulp	tyrosol	17	19	12	5	6	5	12	7	8	6	15	18	3
	hydroxytyrosol	38	31	55	27	37	30	75	38	56	127	52	40	86
	3,4-DHPEA-DEDA	61	45	57	37	48	50	17	33	18	5	65	39	3
	oleuropein	3	4	2	2	3	3	15	2	24	20	444	27	14
	caffeic acid	1	2	4	1	3	2	3	nd	2	2	1	nd	1
	5-caffeoylquinic acid	1	2	2	nd	2	3	4	3	3	3	2	5	1
	verbascoside	5	6	17	5	7	10	12	12	9	16	2	4	3
new season leaves	tyrosol	4	4	3	5	4	4	3	4	4	5	2	3	3
	hydroxytyrosol	17	13	10	15	29	18	15	25	31	36	31	19	29
	3,4-DHPEA-DEDA	92	73	104	102	83	52	59	26	40	45	21	108	41
	oleuropein	25	70	72	12	11	234	8	264	257	163	191	14	147
	oleuroside	nd	5	8	nd	nd	21	1	28	26	23	14	7	12
	luteolin-4-glucoside	9	8	14	10	12	8	6	5	9	12	3	2	2
	luteolin-7-glucoside	35	27	41	23	18	57	26	76	59	48	42	40	41
	luteolin glucoside[b]	27	18	30	19	15	29	25	41	30	30	25	35	34
	caffeic acid	nd	1	6	6	2	4	10	2	6	7	1	4	5
	5-caffeoylquinic acid	nd	2	3	3	9	22	1	26	3	3	5	1	2
old season leaves	tyrosol	5	2	3	4	3	4	4	3	3	4	3	2	2
	hydroxytyrosol	39	15	52	55	55	67	42	111	84	96	89	68	61
	3,4-DHPEA-DEDA	21	3	4	10	13	21	7	nd	13	13	14	52	18
	oleuropein	20	6	31	94	64	156	18	196	186	160	138	20	67
	oleuroside	5	2	7	21	15	32	7	41	41	37	26	12	16
	luteolin-4-glucoside	11	6	21	14	14	18	10	13	14	16	6	4	4
	luteolin-7-glucoside	24	24	59	47	36	61	32	28	29	41	40	40	53
	luteolin glucoside[b]	32	19	40	35	22	45	36	46	37	42	30	31	36
	caffeic acid	nd	13	3	nd	18	24	6	4	25	26	2	16	17
	5-caffeoylquinic acid	17	3	4	4	40	9	18	13	52	25	19	2	2
seed	tyrosol	40	14	6	5	8	11	9	7	10	13	19	22	17
	hydroxytyrosol	8	10	9	3	1	2	2	1	1	2	9	4	3
	3,4-DHPEA-DEDA	2	1	4	3	4	5	4	6	4	3	3	2	2
	oleuropein	1	2	2	1	1	1	1	1	1	1	64	2	nd
	verbascoside	7	4	4	5	5	5	3	8	5	4	17	5	5
	nüzhenide	1	5	69	51	49	66	39	76	48	28	12	60	52
stone	tyrosol	8	7	5	2	3	2	4	3	2	1	6	4	2
	hydroxytyrosol	5	6	12	4	7	6	13	8	8	19	12	13	19
	3,4-DHPEA-DEDA	6	8	10	7	12	9	5	8	5	3	6	8	1
	oleuropein	nd	nd	1	1	1	2	1	nd	2	1	32	nd	1

[a] Numbers are expressed as mg/g tyrosol equivalents and are the average from four trees. [b] Unknown structure; nd, none detected.

sampling date, were averaged, since similar quantities of the various metabolites were observed, and metabolic trends were similar for each of the four trees.

Tissue Specificity. No major differences in phenolic composition were found between the various tissues analyzed (*9*). However, there were distinct differences between the tissues with respect to quantifiable phenols. Leaves, both old season and new season, displayed the most diverse range of phenols with 10 out of 12 quantified compounds represented (**Table 2**). The pulp had the next most diverse range of phenols (*7*), followed by seed (*6*) and stone (*4*). Leaves were unique in that they were the only tissues to contain quantifiable amounts of luteolin glycosides and oleuroside. Similarly, nüzhenide was confined to seeds, while pulp and seed were the only tissues to contain significant amounts of verbascoside. In contrast, four phenols, tyrosol, hydroxytyrosol, 3,4-DHPEA-DEDA, and oleuropein, were ubiquitous and quantifiable in all of the investigated olive tissues.

Data compilation (*11*) suggested that different tissues have a different phenolic composition supporting the notion that metabolism in each plant tissue is unique. However, our results presented earlier (*9*) and here indicate that metabolism of the individual plant parts is similar or that phenolic precursors are easily translocated between tissues for subsequent biosynthesis. A further interpretation of the data is that specific phenolic metabolism in different tissues is a real phenomenon; however, improved detection of a wide array of common phenols in all tissues may obscure the observation of this phenomenon. In this case, detection of a particular phenol in a plant tissue at low levels would have more to do with "leakage" from surrounding tissues rather than being directly related to the intrinsic metabolism of the particular tissue.

Metabolic Relationships Between Phenols. Metabolic relationships between the various phenols listed in **Table 2** have been inferred by observing concentration changes for structurally related phenols. The structural relationships between nine nonflavonoid phenols monitored in this study are shown in **Figure 1**. Single arrows indicate a progression from a simpler structure to a more complex one, usually by conjugation of two or more separate entities, e.g., formation of verbascoside from hydroxytyrosol and caffeic acid. Thus, a metabolic relationship between these species may be deduced if an increase in the concentration of verbascoside is accompanied by a decrease in the concentration of hydroxytyrosol and caffeic acid. It is reasonable to assume that structurally related compounds are also metabolically related, however a rigorous proof of this would require extensive radiolabeling studies, enzyme isolation, identification of regulatory genes, etc., beyond the scope of this investigation.

The double-headed arrow in **Figure 1** indicates a structural relationship that may be regarded as isomeric. Thus, oleuropein and oleuroside are related by a shift of the exocyclic double

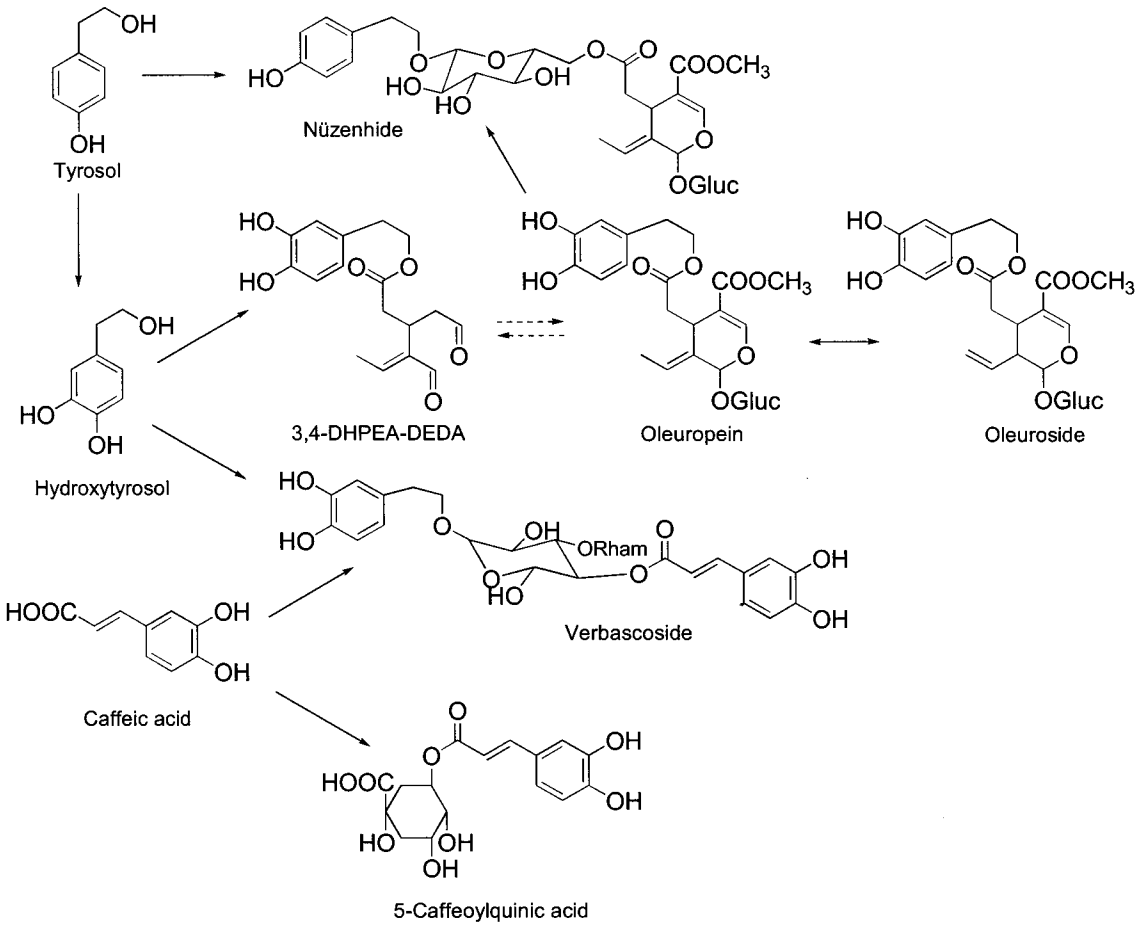

Figure 1. Structural relationships of important phenolic compounds in olive.

bond. This warrants investigation to determine whether the shift is photochemically induced or facilitated by an enzyme. On the other hand, there have been a number of reports of the conversion of oleuropein to 3,4-DHPEA-DEDA during the extraction of olive oil from the fruit (*12*−*15*). This is initiated by glucosidase activity during processing followed by ring opening to give the dialdehyde. As discussed below, we present evidence that in cv. Hardy's Mammoth, the reverse transformation can occur.

Although not shown in **Figure 1**, the flavonoid compounds are metabolically related to caffeic acid. They share a common precursor in 4-coumaric acid, and the metabolic pathway branches here to give caffeic acid on one arm, whereas on the other arm flavonoids are formed via a link with malonyl-CoA (*16*). Thus, concomitant changes in flavonoid and caffeic acid concentrations may indicate switching between the various pathways.

Metabolic Relationships within Leaves. There is an extensive amount of literature devoted to the phenomenon of alternate bearing in *O. europaea* (*17*), and it is believed (*18*) that the initial signal for alternance may be received by the leaves. Lavee and colleagues (*18*−*21*) have identified the central role of 5-caffeoylquinic acid (chlorogenic acid) in alternate bearing. Concentrations of 5-caffeoylquinic acid have been found to be 3−4 times greater in the mature full size leaves from the previous season of fruit-bearing trees as compared to the leaves of nonfruiting trees (*18*) with changes in the levels of 5-caffeoylquinic acid occurring during the flowering and fruit set period (*20*). By monitoring concentrations of both 5-caffeoylquinic acid and caffeic acid over two consecutive fruiting seasons, we were able to determine whether our sampling method was able to reliably track the known phenomenon of alternate bearing in olive metabolism.

The data for 5-caffeoylquinic acid (**Table 2**) are consistent with the findings of Lavee et al. (*19*−*21*) in that generally the levels were higher in both new season and old season leaves in season 1 as compared with season 2. This is also true for caffeic acid, and it would appear that in the high-yield season, regulatory mechanisms accumulate both acids rather than convert all caffeic acid to 5-caffeoylquinic acid. Thus, both compounds appear to be important in determining alternance. No quantitative correlation between caffeic acid and alternate bearing was reported in the previous studies (*18*), but it was shown that in contrast to 5-caffeoylquinic acid, caffeic acid had no effect on reducing fruit bud differentiation and on olive callus growth.

Some interesting trends are revealed when considering the fluctuation of levels of 5-caffeoylquinic acid and caffeic acid in leaves during a fruiting season. Beginning with new season leaves, caffeic acid was the first to build up and reach 6 mg/g, its first maximum, on 23 March (**Table 2**). 5-Caffeoylquinic acid accumulated more slowly and reached its first maximum, 22 mg/g, two sampling dates later (20 April) and was accompanied by a drop in caffeic acid concentration. This is consistent with caffeic acid being a metabolic precursor to 5-caffeoylquinic acid. However, it appears that rather rapid interconversion between the two compounds can occur. From 20 April to 3 May, there was a sharp drop in the 5-caffeoylquinic acid level from 22 to 1 mg/g with a corresponding rise in the amount of caffeic acid from 4 to 10 mg/g, which suggests that 5-caffeoylquinic acid has been converted back to caffeic acid. On the next sampling date, 18 May, 5-caffeoylquinic acid was

again dominant (26 mg/g) and caffeic acid once more was present in lower amounts (2 mg/g). These three sampling dates, 20 April, 3 May, and 18 May, correspond to a period approximately midway through the ripening phase of the fruit, between mature green fruit toward the end of the growing period of the fruit and mature black fruit. It is intriguing that large and rapid fluctuations of phenols occur at this time of the season, seemingly independent of the major developmental stages of the fruit. However, it is apparent that other phenols in new season leaves also experienced rapid and major fluctuations at this time.

Old season leaves showed a somewhat different pattern for levels of 5-caffeoylquinic acid and caffeic acid as compared to new season leaves (**Table 2**) during a fruiting season. However, the interplay between these two acids was also evident in old season leaves, occurring somewhat earlier in the season, the key dates being 6 April, 20 April, and 3 May. At the end of the sampling period in season 1, the levels of both 5-caffeoylquinic acid and caffeic acid in old season leaves were high relative to new ones. According to Lavee and co-workers (*19*−*21*), this is a signal for the low fruit yield in season 2. Thus, our method of periodically monitoring major phenolic metabolites in olive leaves was able to detect a signature for alternance and gives us confidence that other relationships between phenols in the tissues discussed below are genuinely related to metabolic processes occurring within the tree.

The flavonoids, all of which are luteolin glucosides, only occurred to any significant extent in the leaves. Levels of luteolin-4-glucoside did not vary much during season 1, with values ranging from 5 to 14 mg/g in new season leaves and from 6 to 21 mg/g in old season leaves (**Table 2**). In season 2, luteolin-4-glucoside amounts were noticeably lower and this may be related to alternate bearing. In contrast, luteolin-7-glucoside and an unknown luteolin glucoside isomer showed substantial fluctuations during season 1, which tended to change in parallel for both luteolin glucosides. Identification of the unknown isomer as a luteolin glucoside was based on ultraviolet and mass spectral data. From retention times of pure compounds, the isomer was not luteolin-6-glucoside nor luteolin-8-glucoside.

There were two periods where levels of these flavonoids fluctuated rapidly in both new and old season leaves. The first was early in season 1 (11 February and 8 March) where the amounts of luteolin-7-glucoside and unknown luteolin glucoside doubled (old season leaves) or nearly doubled (new season leaves) before steadily decreasing over the next three sampling dates. A further rapid rise in concentration of both flavonoids, again 2-fold, occurred on 20 April. This was followed by a drop in levels on the next sampling date (3 May) with a further rapid rise on 18 May in new season leaves. For new season leaves, the dates 20 April, 3 May, and 18 May were highlighted above because of rapid changes in levels of caffeic acid and 5-caffeoylquinic acid.

The five remaining phenols, tyrosol, hydroxytyrosol, 3,4-DHPEA-DEDA, oleuropein, and oleuroside, that were quantified in leaves are all structurally related to tyrosol (**Figure 1**). Tyrosol itself showed very little change in levels during both season 1 and from season 1 to season 2 (**Table 2**). This is consistent with tyrosol being in a steady state concentration whereby its rate of formation is approximately equal to its rate of catabolism. On the other hand, hydroxytyrosol and its glucoside showed a general rise across season 1 in both new and old season leaves. The most dramatic change in concentration of hydroxytyrosol species occurred in old season leaves on 18 May, a date already highlighted for other compounds. It is difficult to rationalize how such a rapid rise (from 42 to 111 mg/g) was sustained by leaf metabolism alone. This is because other hydroxytyrosol-containing species, such as oleuropein and oleuroside, also showed major increases in concentration on the same sampling date in both new and old season leaves. Thus, there was a large increase in the concentration of several hydroxytyrosol-containing species without a corresponding decrease in the concentration of precursors within the leaves, the exception being a small drop in concentration of 3,4-DHPEA-DEDA (**Table 2**).

Oleuropein has often been referred to as the dominant phenol in olives (*5*−*7*). In terms of peak concentrations during a sampling season, this was also true for the present study. The peak concentrations of oleuropein in season 1 occurred on 18 May for both new and old season leaves (264 and 196 mg/g, respectively) and were far in excess of any other phenol. However, the dominance of oleuropein was not sustained during the sampling periods of both season 1 and season 2, and at various times, 3,4-DHPEA-DEDA and hydroxytyrosol concentrations exceeded that of oleuropein. In new season leaves, it was 3,4-DHPEA-DEDA that was present in highest concentrations early in season 1, whereas hydroxytyrosol was more abundant in old season leaves at the beginning of season 1. The situation was reversed in season 2, with 3,4-DHPEA-DEDA reaching a peak midseason, whereas oleuropein concentrations went through a minimum at this time (**Table 2**). This highlights the complex interrelationship between these three structurally related metabolites, hydroxytyrosol, 3,4-DHPEA-DEDA, and oleuropein. Oleuroside, an isomer of oleuropein, is another phenol that is confined to the leaves in quantifiable amounts, with higher levels generally found in old season leaves. The change in amount of oleuroside parallels precisely that of oleuropein. This suggests that the two are not in equilibrium but rather that oleuroside synthesis is sustained from the conversion of oleuropein, which is always in higher concentrations than oleuroside.

We previously presented evidence (*9*) that in cv. Hardy's Mammoth, 3,4-DHPEA-DEDA is a genuine precursor for oleuropein rather than, as previously suggested (*12*−*15*), simply a breakdown product resulting from glucosidase activity during the oil extraction process. Examination of the data for new season leaves in **Table 2** is consistent with this proposition. In general terms, levels of 3,4-DHPEA-DEDA were high at the beginning of season 1 and then decreased until the end of the first sampling period. At the same time, oleuropein concentrations began at relatively low levels and increased as the sampling period progressed. For season 2, the interrelationship was even more closely followed with peaks in oleuropein concentrations coincident with troughs in concentration of 3,4-DHPEA-DEDA and vice versa. For old season leaves, the relationship between 3,4-DHPEA-DEDA and oleuropein was not well-correlated in season 1; however, the pattern for season 2 matched that observed for new season leaves. There is thus increasing evidence to suggest that the metabolism of oleuropein is far from well-established as suggested by Damtoft et al. (*22*−*25*) and that further studies of this compound are warranted in *O. europaea*.

Metabolic Relationships within Pulp. Olive pulp is characterized by the highest concentrations of verbascoside of any of the olive tissues. A previous paper by Amiot et al. (*5*) indicated an inverse relationship between verbascoside and oleuropein; however, our study has not found evidence to support this. The structurally related compounds caffeic acid and 5-caffeoylquinic acid occurred at very low levels in the pulp, and no clear trends emerged between the three species.

There is a trend in season 1 to increasing levels of verbascoside from 5 mg/g at the beginning of the sampling period to 16 mg/g at the final sampling date. This trend was not observed in season 2 and could be interpreted as an influence of alternate bearing on the metabolic processes occurring within the pulp. However, other related phenols offer no such indication. While oleuropein appears to follow different trends from season 1 to season 2, tyrosol, hydroxytyrosol, and 3,4-DHPEA-DEDA seem to follow similar patterns in both seasons.

Tyrosol was present in pulp at higher levels than in leaves and, unlike leaves, significant fluctuations in levels occurred particularly between 8 March and 23 March and between 3 May and 18 May. The first of these sharp falls was not accompanied by increases in structurally related compounds within the pulp. It is possible, therefore, that tyrosol may have been converted to a number of species that were not quantified in this study. The general trend was for tyrosol levels to decrease during the sampling period in both seasons accompanied by a simultaneous increase in hydroxytyrosol concentration. This contrasts with the steady state concentration of tyrosol observed in leaves.

Apart from a general inverse association with tyrosol, hydroxytyrosol levels appeared to be closely correlated with those of 3,4-DHPEA-DEDA. However, the correlation appeared to be direct early in season 1 but inverse from 20 April onward. A similar pattern emerged in season 2. In season 1, April 20 was identified as an important date coinciding with a peak in the level of 5-caffeoylquinic acid in new season leaves. Over the next 2−3 sampling dates, rapid fluctuations of some phenol concentrations were observed in both new and old season leaves. As found in the leaves, hydroxytyrosol levels in the pulp were higher at the end of each season than at the beginning. Liquid chromatography/mass spectrometry (LC-MS) with selected ion monitoring (*9*) allowed hydroxytyrosol and hydroxytyrosol glucoside to be identified independently. Only the hydroxytyrosol glucoside could be detected in pulp of black mature fruits sampled on the last sampling date in season 1.

Oleuropein levels in the pulp were found to increase fairly slowly during season 1 and were inversely correlated with concentrations of 3,4-DHPEA-DEDA, as was found for leaves. It should be noted that the sharp drop in oleuropein on 18 May at color change of the fruits was accompanied by a sharp rise in 3,4-DHPEA-DEDA. Other workers (*5*, *7*) have reported that oleuropein concentrations decrease as fruit ripens, in contrast to our findings in season 1. However, in season 2, we observed a decrease in oleuropein levels from a very high 444 to 14 mg/g, consistent with the literature.

Metabolic Relationships in Seed and Stone. Seed tissue is characterized by nüzhenide as the predominant phenol. As might be expected (**Figure 1**), there is a link between increasing nüzhenide levels and decreasing tyrosol levels early in season 1 (**Table 2**). Apart from nüzhenide, levels of phenols were well below those found in other tissues and no clear metabolic relationships can be established. Hydroxytyrosol accumulated in the stone as the glucoside at the end of season 1 as established for all other tissues, with the notable exception of the seed.

Of possible significance to the issue of alternate bearing was that at the beginning of season 2 ("off" year), a number of phenols were found in significantly higher concentrations than at the beginning of season 1 (the "on" year). This was the case for oleuropein, in both seed and stone; verbascoside and nüzhenide, in seed only; and hydroxytyrosol, in stone only.

Alternate Bearing and General Trends in Phenol Metabolism. As noted above, the accumulation of 5-caffeoylquinic acid in the leaves during a high-yield season has been proposed (*19*−*21*) to act as a signal to the olive tree to produce a low yield in the following season. Having confirmed this trend in the current study, we examined also gross changes in levels of other phenols possibly linked to alternate bearing. As leaves are the major metabolically active tissue of the tree, it might be expected that metabolic changes associated with alternate bearing would show up clearly in them. Hydroxytyrosol, oleuropein, and oleuroside all show different trends from season 1 to season 2 in both new and old season leaves. For each phenol, levels increased in season 1 but showed either little variation (hydroxytyrosol) or a decrease (oleuropein and oleuroside) in season 2. For new season leaves, the concentration of 3,4-DHPEA-DEDA decreased 2-fold over season 1 but increased 2-fold in season 2.

Evidence of the impact of alternate bearing on levels of phenol accumulation in fruit from one season to the next is provided by patterns of oleuropein and verbascoside concentration changes from season 1 to season 2 in pulp. Oleuropein increased in concentration over season 1 but decreased in season 2, whereas verbascoside levels increased during season 1 but remained reasonably constant in season 2. In seed and stone, oleuropein levels remained fairly constant in season 1 but fell during season 2. Further studies are required to establish if a causal inductive metabolic link occurs between alternate bearing and phenol accumulation in fruit or if the differences are a result of the metabolic pathways, specific for high and low fruiting years. Given the importance of the phenol fraction to olive oil quality (*26*), further studies in the relationship of phenol metabolism in old season leaves and fruit are needed.

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