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Role of Olive Seed in the Biogenesis of Virgin Olive Oil Aroma

PILAR LUACES, ANA G. PÉREZ, AND CARLOS SANZ*

Department of Physiology and Technology of Plant Products, Instituto de la Grasa, C.S.I.C., Padre García Tejero 4, 41012 Seville, Spain

Results obtained in a set of experiments point to an effective participation of olive seeds in the biosynthesis of olive oil aroma through the lipoxygenase pathway during the extraction process to produce virgin olive oil. Data showed that olive seeds should contain enzymatic activities metabolizing 13-hydroperoxides other than hydroperoxide lyase, giving rise to a net decrease in the content of C6 unsaturated aldehydes during the olive oil extraction process. Olive seeds seem also to supply this process with alcohol dehydrogenase activity, being more specific for saturated C6 aldehydes and not acting on C5 alcohols. Moreover, olive seeds would be responsible for the biosynthesis of 30−50% esters during the olive oil extraction process of intact fruits. Thus, olive seeds would afford a load of alcohol acyltransferase activity that might be quite unspecific in terms of substrate, producing any kind of esters.

KEYWORDS: Olive oil; seed; aroma; LOX pathway, *Olea europaea* L.

INTRODUCTION

A large increase in demand for high-quality virgin olive oil during the past few years can be attributed not only to its potential health benefits, but also to its particular organoleptic properties. The aim of increasing the quality standards for virgin olive oil is continuously stimulating the search for new technologies. In this sense, a new technological procedure is being developed that includes stone removal before the olive oil extraction process. Several authors suggested that stone removal gives rise to an increase in oxidative stability in the oils (*1*). This effect would be due to the absence of the olive seed during the crushing−malaxation process occurring in the olive oil production. Moreover, this stone removal seems also to improve the organoleptic quality of olive oil. In this sense, our group established a decade ago the participation of the lipoxygenase (LOX) pathway in the biosynthesis of C6 compounds in olive oil aroma (*2*). Aldehydes and alcohols of six straight-chain carbons (C6) and the corresponding esters are the most important compounds in the aroma of virgin olive oil, either from a qualitative or a quantitative point of view (*3*, *4*). These compounds are synthesized from polyunsaturated fatty acids containing a (*Z*,*Z*)-1,4-pentadiene structure such as linoleic (LA) and linolenic (LNA) acids. In a first step of this pathway LOX produces the corresponding 13-hydroperoxide derivatives that are subsequently cleaved by hydroperoxide lyase (HPL) to C6 aldehydes (*2*, *5*, *6*). C6 aldehydes can then undergo reduction by alcohol dehydrogenases (ADH) to form C6 alcohols. Finally, by means of an alcohol acyltransferase activity C6 alcohols can be transformed into the corresponding esters.

Work by Angerosa et al. (*7*) has demonstrated also the relevance of C5 compounds in the aroma of olive oil. C5 compounds would be generated through an additional branch of the LOX pathway from LNA that would involve the production of a 13-alcoxyl radical by LOX as demonstrated in soybean seeds by Gardner's group (*8*, *9*). This radical would undergo subsequent nonenzymatic β-scission to form a 1,3-pentene allylic radical that could be chemically dimerized to form pentene dimers or react with a hydroxyl radical to form pentenols. These pentenols would be the origin of C5 carbonyl compounds present in the aroma of olive oil through an enzymatic oxidation by ADH, as suggested to occur in soybean leaves (*10*).

Different studies have dealt with the role of olive pulp in the biosynthesis of olive oil aroma. These studies have been carried out either from an analytical (*11*) or a biochemical point of view (*2*, *5*, *6*, *12*, *13*). However, no study has been conducted to identify the contribution of olive seed in the synthesis of olive oil aroma. The aim of the present work was to establish the level of participation of olive seed in the biosynthesis of olive oil aroma.

MATERIALS AND METHODS

Plant Material. Olive fruits (*Olea europaea* L.) cultivar Verdial were harvested in Opracol Huelva orchards (Villarrasa, Huelva, Spain) at two maturity stages, color index 1 (green stage) and color index 4 (ripe stage), according to García et al. (*14*). Fruits were immediately transported to the Instituto de la Grasa, randomly distributed in 2-kg batches for every treatment in triplicate, and conditioned to 4 °C.

Fruit stoning was carried out by means of an Ulrich stoner at 4 °C. Pulp was immediately processed for olive oil extraction and "olive pulp" oils were obtained. "Wounded olives" oil samples were produced by adding the stones (resulting from the stoning process) to olive pulp just before the crushing−malaxation step for olive oil extraction.

For the experiments involving addition of stones to intact fruits, stones were collected using an Ulrich stoner and cleaned of remaining

* Corresponding author. Phone: +34 95 611550. Fax: +34 95 616790. E-mail: sanzca@cica.es.

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Table 1. Volatile Contents (ng/g olive oil) in the Aroma of Verdial Olive Oils Obtained from Control Fruits, Olive Pulp (Stoned Fruits), and Wounded Olives (Stoned Fruits Plus Stones Added Before the Crushing–Malaxation Process to Obtain Virgin Olive Oil) at Two Different Ripeness Stages (Green Fruits and Ripe Fruits)[a]

	green fruits			ripe fruits		
	control	olive pulp	wounded olives	control	olive pulp	wounded olives
(*E*)-hex-3-enal[b]	911.1	1105.9	835.3	1139.5	1174.6	883.2
(*Z*)-hex-3-enal	8753.6	14182.7	13993.9	9962.4	12312.2	10208.2
(*Z*)-hex-2-enal[b]	1224.3	1727.3	1418.3	1958.9	2224.2	1875.3
(*E*)-hex-2-enal	10087.2	9399.8	7006.8	15951.9	12382.2	11842.5
(*E*)-hex-3-enol	0.0	25.0	12.2	14.4	23.1	14.5
(*Z*)-hex-3-enol	368.2	4524.7	3580.7	1040.1	1848.7	1677.1
(*E*)-hex-2-enol	0.0	41.4	41.6	19.4	63.6	13.7
ΣC6/LNA	21344.4	31006.6	26888.8	30086.6	30028.6	26514.5
pentene dimers[b]	4975.5	2110.5	2679.3	7344.5	6091.3	4296.7
pent-1-en-3-one	833.2	297.6	339.7	497.0	332.0	269.9
(*Z*)-pent-2-enal[b]	104.5	58.5	54.4	49.1	62.7	50.8
(*E*)-pent-2-enal	81.9	40.6	42.8	57.5	53.0	41.9
pent-1-en-3-ol	929.5	232.4	397.8	396.7	286.8	275.7
(*E*)-pent-2-en-1-ol	69.2	24.7	42.4	68.9	63.1	56.2
(*Z*)-pent-2-en-1-ol	662.8	341.6	403.3	319.4	266.5	197.2
ΣC5/LNA	7656.5	3105.8	3959.8	8733.2	7155.4	5188.4
hexanal	1227.9	2479.9	2568.3	3290.3	5158.7	4542.2
hexan-1-ol	57.6	1300.6	493.4	376.3	536.3	659.1
ΣC6/LA	1285.5	3780.4	3061.8	3666.6	5695.0	5201.3
hexyl acetate	156.7	97.5	170.3	625.2	491.2	765.7
(*Z*)-hex-3-en-1-yl acetate	666.4	494.7	1218.2	1413.9	1351.0	1958.9
ΣLOX esters	823.1	592.2	1388.5	2039.1	1842.2	2724.7
methyl acetate	222.6	224.9	294.6	300.0	329.0	717.0
ethyl acetate	46.8	20.9	50.6	49.1	52.0	46.2
3-methylbut-1-yl acetate	10.8	3.8	5.8	64.2	31.1	60.9
methyl hexanoate	2.5	24.9	12.9	200.4	154.1	124.2
ethyl hexanoate	0.0	0.0	0.0	3479.3	2172.9	3770.4
Σnon-LOX esters	282.7	274.5	364.0	4093.1	2739.2	4718.6

[a] Average coefficient of variance was 5.1%. [b] Compounds tentatively identified on the basis of mass spectra.

pulp in tap water using a Waring blender at low speed. Stones were rubbed and dried with filter paper and immediately added to intact fruits for olive oil extraction so that fruit batches reached 200% and 300% in the number of stones in relation to control fruits (100%).

Olive Oil Extraction. The oil from every treatment was extracted separately using an Abencor analyzer (Comercial Abengoa, S. A., Seville, Spain) that simulates at lab scale the industrial process of virgin olive oil production (*15*). Malaxation was carried out for 30 min with the Abencor thermobeater operated at 30 °C. After the oils were centrifuged, they were decanted and filtered. Samples for volatile analysis (0.5 g) were taken in 10-mL vials, which were sealed and stored at −18 °C until analysis.

Analysis of Volatile Compounds. Olive oil samples were conditioned to room temperature and then placed in a vial heater at 40 °C. After 10 min equilibrium time, volatile compounds from the headspace (HS) were adsorbed on a SPME fiber DVB/Carboxen/PDMS 50/30 μm (Supelco Co., Bellefonte, PA). Sampling time was 50 min at 40 °C and carried out in duplicate. Desorption of volatile compounds trapped in the SPME fiber was done directly into the GC injector. Volatiles were analyzed using a HP-5890 gas chromatograph equipped with fused silica capillary column DB-Wax (30 m × 0.25 mm; J&W Scientific, Folsom, CA). Operating conditions were as follows: N_2 as carrier gas; injector and detector at 250 °C; column was held for 6 min at 40 °C and then programmed at 2 °C/min to 120 °C. Quantification was performed by using individual calibration curves for each identified compound which had been prepared by adding known amounts of different compounds to a re-deodorized high oleic sunflower oil. Compound identification was carried out on a HRGC-MS Fisons series 8000 equipped with a similar stationary phase column and two different lengths, 30 and 60 m, matching against the Wiley/NBS Library and by GC retention time against standards.

Chemicals and Reagents. Reference compounds used for volatile identidication were supplied by Sigma-Aldrich (St Louis, MO) except for (*Z*)-hex-3-enyl acetate which was purchased from Givaudan Co. (Clifton, NJ) and (*Z*)-hex-3-enal which was generously supplied by S. A. Perlarom (Louvaine-La-Neuve, Belgium).

RESULTS AND DISCUSSION

Volatile analysis using the HS-SPME technique proved to be a very suitable tool for the quantitative and qualitative analysis of virgin olive oil aroma. Our preliminary studies showed that, compared to other fiber coatings, the DVB-Carboxen-PDMS presented the highest detector responses, as well as a greater linearity within the compound concentration intervals under study (data not shown). These results are in good agreement with those by Vichi et al. (*16*) except for the fact that 50 min of fiber exposure to headspace was needed in our studies to reach equilibrium for most compounds of olive oil aroma. Using this HS-SPME technique, as described in Material and Methods, the volatile compound profile of oils from fruits with different ripeness degrees was studied first. Verdial olive oils displayed different volatile compositions based on the fruit ripeness degree (**Table 1**, Controls column). Olive oil aroma from ripe fruits presented a higher content of every class of compounds derived from the LOX pathway, total C6 compounds from the HPL cleavage of LNA and LA 13-hydroperoxides, and total C5 compounds derived from the homolytic cleavage of LNA 13-hydroperoxides. Angerosa and Basti (*17*) reported quite similar results in three Italian cultivars for the same ripeness degrees.

The first approach to determine the contribution of olive seed in the synthesis of olive oil aroma was to study the effect of stone removal on the composition of olive oil aroma. Aroma volatile composition of olive oils obtained from olive pulp (stoned fruits) showed an increase in C6 compounds with a

strong dependency on ripeness degree (**Table 1**). On the other hand, esters derived either from LOX or other metabolic pathways showed a decrease as a result of stone removal in green and ripe fruits. Following this pattern, C5 compounds content also showed a reduction as a consequence of stone removal, being higher in green fruits (41%) than in ripe fruits (18%). Angerosa et al. (*11*) also reported an increase in the content of C6 compounds in olive oils extracted from Coratina stoned olives, but contents in C5 compounds showed almost no variation as a consequence of stone removal. Our studies in other olive cultivars (Picual and Manzanilla) indicate that C5 compound contents always decreased in the olive oil aroma from stoned fruits (published elsewhere), and that the level of this decrease is related to cultivar. The organoleptic importance of C5 compounds in olive oil was studied by Angerosa et al. (*7*) and showed that these compounds, especially pent-1-en-3-one, strongly affect most of the taste and odor attributes.

Wounding caused during the stone removal process could affect the aroma composition of the resulting olive oils. **Table 1** displays the comparative aroma composition of olive oils obtained from control fruits and wounded fruits, that is, stoned fruits added with their stones before the crushing−malaxation process. Data show that wounding gave rise to a modification in the aroma composition of olive oils characterized also by a dependency on the degree of fruit ripeness. Thus, wounding promoted an increase in C6 compounds derived from LNA in green fruits (26%) but a decrease in ripe fruits (12%). C6 compounds derived form LA, as well as esters, increased irrespectively of fruit maturity. On the other hand, wounding produced a decrease in the content of C5 compounds in both green and ripe fruits in the range 40−50%. This reduction in C5 compounds by wounding was at the same level in pentene dimers content that it was in C5 alcohols and carbonyls. This fact could be evidence for a common biochemical origin of C5 compounds in olive oil, as suggested by Angerosa et al. (*18*), through an additional branch of the LOX pathway from LNA that would involve the production of a 13-alcoxyl radical by LOX as described in the Introduction.

A comparison of data on aroma composition of olive oils from fruit pulp and wounded fruit (olive pulp plus stones) would give an approximate idea of the participation of olive seed in the synthesis of olive oil aroma. The same degree of wounding is caused to the fruits before oil extraction in both cases so that seed participation in the biogenesis of olive oil aroma can be discussed. Data show that seed presence during the olive oil extraction process produced a decrease in the content of C6 compounds in both green and ripe fruits. These results and the higher concentration of C5 compounds, derived from LNA, found when seed is present during the olive oil extraction process of green fruits could well account for a HPL inhibitor in the seed. This inhibitor would procure an increase in the content of LNA 13-hydroperoxide so that homolytic cleavage is favored yielding C5 compounds. However, this pattern is different in ripe fruits. Also, no differences were found in C5 compounds when considering alcohols and carbonyl derivatives separately. This fact would indicate that no ADH activity, acting on C5 alcohols, seems to be present in olive seed in both green and ripe olive fruits.

A high increase in the content of esters derived from the LOX pathway was observed in green (134%) and ripe fruits (48%) as a consequence of the presence of seed during the olive oil extraction process. Thus, it seems that an active form of AAT could be present in the olive seed contributing to the formation of esters derived not only from the LOX pathway but also from other metabolic pathways.

Georgalaki et al. (*19*) reported the isolation and partial characterization of an active form of LOX located in the membranes of oil bodies of olive endosperm. This endospermal LOX acting during the crushing-malaxation process, and the fact that a decrease in the content of C6 compounds in olive oil aroma was observed when seed was present during the olive oil extraction process, points to the presence in the seed of other enzymes metabolizing 13-hydroperoxides. In this sense, in the plant kingdom jointly to HPL action, hydroperoxide dehydratase and peroxigenase activities were shown to metabolize 13-hydroperoxide into compounds with a possible regulatory role or involved in plant defense mechanisms (*20*, *21*).

To overcome the wounding effect on the biosynthesis of olive oil aroma, which could distort results aimed to elucidate the role of olive seed in this biosynthesis, a different approach was carried out. For this purpose ripe Verdial olives were chosen because of the higher content of volatile compounds observed in their oils (**Table 1**). Whole intact olive fruits were supplemented with pulp-free olive stones, reaching 200% and 300% stones, for the olive oil extraction process as described in Material and Methods and compared to control fruits (100% stones). Olive oils obtained in these conditions were analyzed as before and results are shown in **Figure 1**. Plots of the content of the different classes of compounds against stone percentage used for the olive oil extraction process were drawn. In absence of a point showing oil aroma composition from "intact seedless olive fruits", an estimated point representing 0% stones was calculated by subtracting the effect of wounding from volatile contents of olive pulp oil. This wounding effect factor was previously calculated from data (**Table 1**) by comparing olive oil aroma composition of control and wounded fruits.

Figure 1 shows that correlation coefficients (R^2) higher than 0.9400 were found for all classes of compounds in the range 0−200% stones, except for esters from the LOX pathway which displayed $R^2 = 0.9046$. Despite the increasing enzymatic load provided by supplementing with extra amounts of stones during the olive oil extraction process, no significant modification of olive oil aroma was observed with 300% stones. This fact seems to indicate that substrate concentration is a limiting factor in the biosynthesis of olive oil aroma when more than 200% stones were present in the olive oil extraction process. Results are in good agreement with the conclusions raised by Schwab (*22*) in a recent review on plant metabolomics on the main role of substrate availability in the formation of secondary plant metabolites.

The linearity observed in the range 0−200% stones for the production of volatile compounds of olive oil aroma allows the discussion about the role of olive seed in the biosynthesis of olive oil aroma. As shown in **Figure 1A**, the main compounds in olive oil aroma, C6 from LNA, seem to follow a different formation pattern for aldehydes and alcohols in relation to percentage of stones. Data derived from the calculated line equation for aldehydes would indicate that C6 aldehydes from LNA have their metabolic origin in the olive pulp. Thus, olive seeds would promote a reduction (7%) in the content of this class of compounds during olive oil extraction of intact fruits (100% stones). On the other hand, formation of C6 alcohols from LNA are also synthesized mainly in the olive pulp. In this sense, three ADH activities have been identified in olive pulp (*12*). Olive seeds would be responsible for just 11% of the C6/LNA alcohol production in olive oil from intact fruits.

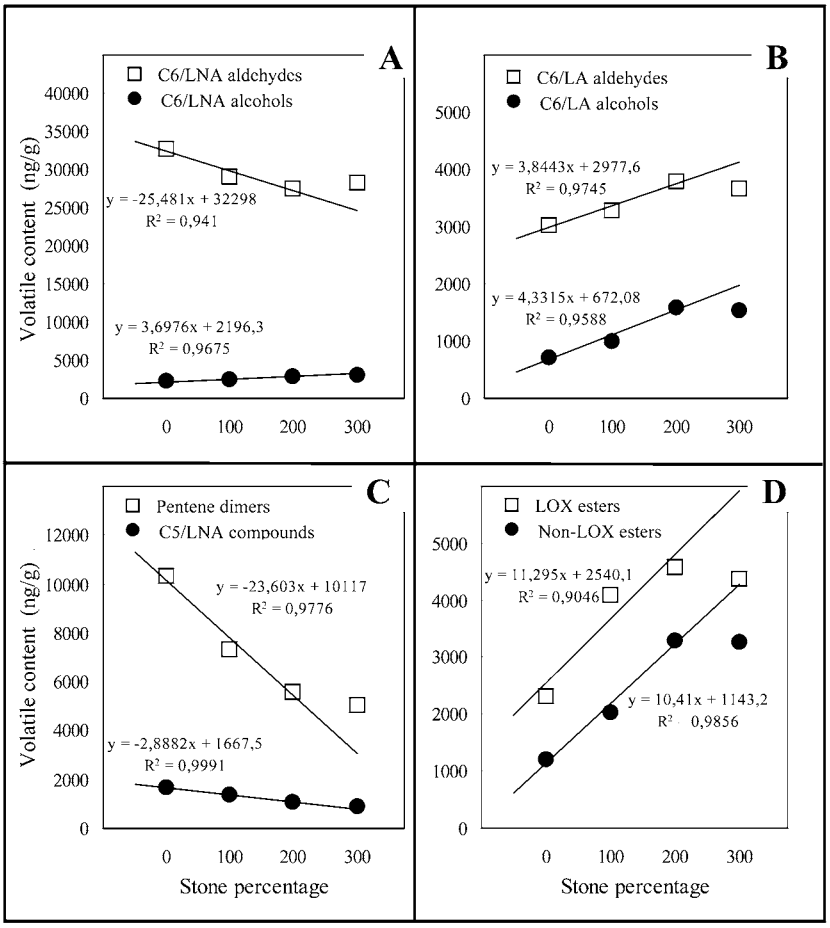

Figure 1. Contents of the main classes of volatile compounds in olive oil aroma as a function of stone amount during the virgin olive oil extraction process. Line equations and regression coefficients (R^2) were calculated in the range 0–200% stone percentage.

Equations obtained for C6 compounds from LA (**Figure 1B**) indicate that olive pulp is mainly responsible for aldehyde synthesis as well, but, contrary to what was observed in C6 aldehydes from LNA, seeds would be responsible for 9% production of C6 aldehydes from LA. This contribution of seeds to the production of this class of compounds could be explained by the presence of a LOX activity in the seed, probably the endospermal LOX found by Georgalaki et al. (*19*), highly specific for LA. It could be explained, additionally, by the much higher content of LA than LNA that has been reported in olive seeds (*23*). Similarly to the pattern observed for C6 alcohols derived from LNA, C6 alcohols derived from LA seem to be produced at 39% by olive seed. Different rates of production by olive seeds for alcohols derived from LNA and LA suggest that an ADH activity is located in the olive seed acting during the crushing−malaxation process with a higher affinity against saturated aldehydes.

As expected after the results described for C5 compounds in olive oils from ripe fruits (**Table 1**), content of C5 compounds in the aroma of olive oil is clearly influenced by the presence of olive seed during the olive oil extraction process (**Figure 1C**). C5 alcohols and carbonyl derivatives followed a similar production pattern, and they are formed by the olive pulp. Olive seeds contribute to olive aroma by reducing the amount of these C5 compounds, either as alcohol and carbonyl derivatives or as pentene dimers. This content reduction was quite similar for both classes of compounds (average 17%). These data point out the presence in olive seed of enzymatic activities metabolizing LNA 13-hydroperoxides different from HPL activity. This is supported by the observed decrease in the content of C6 aldehydes from LNA caused by oil seeds during the olive oil extraction of intact fruits that is not totally explained by the increase in C6 alcohols from LNA or esters derived from these alcohols.

Figure 1D shows ester content as a function of stone amount in the olive oil extraction process. Both esters derived from the LOX pathway and esters from other metabolic pathways showed a dependency on the amount of stone present during this extracting process. An olive seed contribution to ester formation has been calculated in the range of 30−50% in olive oil from intact fruits, which would mean that AAT activity could be found in olive seed. This activity should be quite unspecific if considering the curve slopes for both kinds of esters. On the contrary, AAT activity identified in olive pulp seemed to be more specific for C6 alcohols (*2*, *24*).

The results obtained in this set of experiments point to an effective participation of olive seeds in the biosynthesis of olive oil aroma through the LOX pathway during the extraction process to produce virgin olive oil. Although from this work no conclusions can be made about the presence of LOX activity in olive seeds, the literature seems to demonstrate that this activity is in the seed endosperm of olive fruits. The participation of olive seeds would be by supplying enzymatic activities metabolizing 13-hydroperoxides other than HPL giving rise to a net decrease in the content of C6 unsaturated aldehydes. Olive seeds seem also to be a good source of ADH activity. This ADH activity would be more specific for saturated than unsaturated C6 aldehydes, and, apparently, it would not act on C5 alcohols. Moreover, olive seeds may contain an important level of AAT activity that would explain the observed increment in the content

of esters when seeds are present during the crushing−malaxation process to obtain virgin olive oil.

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