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# ANALYSIS OF VOLATILES FROM CALLUS CULTURES OF OLIVE *OLEA EUROPAEA*

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**Key Word Index**—*Olea europaea*; oleaceae; olive; callus; acyl composition; lipoxygenase pathway; volatiles.

**Abstract**—The lipoxygenase pathway in olive fruit gives rise to volatile products which influence the aroma of the harvested oil. In this study dynamic headspace gas chromatography was used in order to investigate volatile production by different olive cultivars. Olive callus cultures were used as a model for this purpose, because of the ease of standardising the growth conditions of the calli compared to working with field-grown material. The cultures contained substantial amounts of the polyunsaturated fatty acids, linoleate and α-linolenate, which are the normal substrates for lipoxygenases. Most of the major volatiles that characterise virgin olive oil were also produced by the cultures, especially the unsaturated and saturated $C_6$ aldehydes, alcohols and their acetyl esters. Moreover, by means of ANOVA test, a strong effect of cultivar on volatile production in olive cultures was clearly established. This agreed with data for olive oils from different cultivars, which are also easily distinguished by headspace gas liquid chromatography. We have also demonstrated that changing the culture conditions for temperature and pH produced qualitative and quantitative alterations in volatile production. 

## INTRODUCTION

Olive oil ranks sixth in the world's production of vegetable oils [1]. However, it is particularly appreciated and, hence, commands a high market price, because of its many culinary properties as well as its rather special acyl composition, being particularly enriched in the monoenoic fatty acid, oleic acid and low in saturated fatty acids, such as palmitic and stearic. Olive oil has high nutritional value in the human diet [2] and there is accumulating evidence to suggest that there is positive correlation between the consumption of this oil and a reduced risk of heart disease [3, 4].

There are many different types of olive oil ranging from extra virgin to lampante oil, the grades of the oil being based on the manufacturing extraction procedures which have now been standardised within the European Community [5]. Poor quality fruit produces an oil that requires refining, resulting in the loss of the sensory attributes. Therefore, virgin oil, which can actually be consumed without any refining treatment, is preferred by the gourmet cook as well as by average households, particularly in Mediterranean countries. In addition to the oil's organoleptic properties, it is also renowned for its delicious taste which makes it more palatable.

Sensory analysis of the volatiles has become increasingly important as the oils of different cultivars and geographical location have been analysed [6]. Indeed, the application of computer sensory wheel analysis has helped to elucidate the complexity of oil aroma, flavour and taste in relation both to cultivar and to the source of oil. These measurements have been standardised in compliance to European Community regulations [7]. The aromas of vegetable oils vary quite considerably and are derived mainly from the volatile constituents which are by-products of the lipoxygenase pathway. This pathway is widespread in the plant kingdom and has been reviewed well [8–11]. Some of the major volatiles of olive oil include the $C_6$ compounds hexanal and *cis*-3-hexenal [12, 13]. The latter undergoes isomerisation to the more stable form, *trans*-2-hexenal which is itself reduced to form *trans*-2-hexen-1-ol. These volatiles contribute to the fruity note of olive oil aroma.

The analysis of the volatile components by dynamic headspace gas chromatography has proved to be a very useful tool for the identification of the individual components [14–16]. Morales *et al.* [17] have, in fact, identified 56 of 100 volatiles detected in virgin olive oil

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using this technique. Of the large number of volatiles present, only a few have organoleptic properties which are considered to be of importance. Therefore, the emphasis of our analysis has been on the identifying only those components which possess organoleptic properties.

We have chosen to use olive cultures as a model system in order to investigate olive volatile production. Callus cultures have already been found to be reliable materials to study olive lipid metabolism [18] as well as possessing an acyl composition comparable with olive fruit [19]. Cultures are also useful because it is possible to strictly control growth conditions thus reducing the variability associated with samples of fruit obtained in the field. The model system can be used further to investigate the lipoxygenase pathway and the associated enzymes involved in volatile production. This could also include, for example, other products of the lipoxygenase pathway such as jasmonic acid, a growth regulator which, itself, may be involved in callus growth [20]. By understanding the detailed biochemistry of the lipoxygenase pathway in olives, we hope to be able to elucidate the origin as well as the regulation of key sensory components in olive oils. Such knowledge will be important commercially in helping to ensure the best quality, in terms of consumer preference, for olive oils in the future.

## RESULTS AND DISCUSSION

A major objective of this study was to verify the suitability of callus cultures as a model for the investigating volatile production in *Olea europaea*. Cultures were established from the major olive cultivars grown in Greece, Italy and Spain which were, respectively, Koroneiki, Coratina and Picual. All three cultivars produced considerable amounts of volatiles (Table 1). Picual was characterised by producing the most volatiles (i.e. $6.78 \pm 1.00$ ppm) during the experimental period, but possessed the least amount of total lipid ($3.95 \pm 1.31$ g total lipid/100 g dry wt) compared to the other two cultivars. The differences in total volatile production between the cultivars were statistically significant at the 5% level whereas there were no significant differences in the lipid content. This observation implies that the lipoxygenase pathway is more active in Picual compared to Koroneiki and Coratina varieties.

Werkoff and Bretschneider [14, 15] have suggested that the design of dynamic headspace sampling procedures can influence the volatile profile. Therefore, the calli were not blended prior to sampling in order to minimise acyl hydrolase activity. We have, in fact, found previously that homogenising the calli prior to sampling resulted in rapid lipid degradation due to acyl hydrolase activity (data not shown). Moreover, we varied the collecting period and showed that the quality of volatiles was not affected by the sampling period.

The lipid composition of olive callus cv Picual has been previously reported by the authors [19] and it was found in the present study that the lipid composition of this variety was comparable with those data (results not shown). There were some interesting differences in the acyl composition between the cultivars (Table 2). The most prominent fatty acids in the acyl fraction were palmitate, oleate, linoleate and α-linolenate, with linoleate being particularly prominent in Koroneiki. All three varieties were characterised by a relative high amount of the polyunsaturated fatty acid, α-linolenate (ranging from 24 to 33% of the total lipid fraction). Coratina was particularly enriched in this trienoic acid. In contrast, Picual was characterised by possessing the highest proportion of oleate (45% of total acyl composition). Non-esterified fatty acids (NEFAs) are minor components in healthy plant tissue and do not normally accumulate [21]. However, the acyl composition of this minor lipid group was most interesting because it is NEFAs and not complex lipids which are the preferred substrates of most lipoxygenase enzymes [10]. The NEFAs of each of the cultivars contained relatively high proportions of the saturated fatty acids, palmitate and stearate. Presumably, this reflected the fact that such fatty acids are catabolised slower than, say, polyunsaturated fatty acids which are substrates for the lipoxygenase pathway. Interestingly, Coratina which, as already mentioned, possessed the highest proportion of α-linolenate in the total lipid fraction still maintained this differential in the NEFA fraction.

Table 1. Analysis of variance (ANOVA) of the effect of cultivar on lipid content and volatile production in callus cultures of olive *Olea europaea*

| | Cultivar | | | | |
|---|---|---|---|---|---|
| | Picual (Spanish) | Koroneiki (Greek) | Coratina (Italian) | *F* ratio | *F* probability |
| Total lipid (g/100 g dry wt) | $3.95 \pm 1.31$ | $5.10 \pm 1.65$ | $7.09 \pm 1.64$ | 3.19 | 0.114 |
| Total volatiles (ppm) | $6.78 \pm 1.00$ | $4.05 \pm 0.69$ | $5.45 \pm 0.39$ | 10.30 | 0.011* |

Data represents mean ± S.D. ($n = 3$). The cultures were grown uniformly at 25 and sampled 21 days after subculturing.
* Statistically significant ($P < 0.05$). Lipids were quantified as reported previously [19]. Volatiles were quantified using isobutyl acetate as an internal standard, as reported previously [17].

Table 2. Acyl composition of callus cultures obtained from different cultivars of olive

| | | Acyl composition (g 100 $g^{-1}$) | | | | | |
|---|---|---|---|---|---|---|---|
| Cultivar | Lipid fraction | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 | Others |
| Picual | Total | 20±*tr* | 3±*tr* | 45±3 | 8±1 | 24±2 | nd |
| Koroneiki | Total | 23±*tr* | 3±2 | 28±2 | 21±*tr* | 25±2 | nd |
| Coratina | Total | 19±*tr* | 3±*tr* | 37±2 | 8±1 | 33±1 | nd |
| Picual | NEFA | 30±3 | 28±1 | 30±4 | *tr* | 7±1 | 5±*tr* |
| Koroneiki | NEFA | 42±7 | 17±3 | 34±3 | 1±1 | 6±*tr* | nd |
| Coratina | NEFA | 36±2 | 11±1 | 29±3 | *tr* | 19±3 | 4±2 |

Data represents means±S.D. ($n = 3$).

Abbreviations: NEFA = non-esterified fatty acids: nd = none detected; *tr* = 0.5%; Fatty acids are shown with the number before the colon giving the number of carbon atoms and the figure after indicating the number of double bonds. The fatty acids were fully identified and 16:0 = palmitate; 18:0 = stearate; 18:1 = oleate; 18:2 = linoleate; 18:3 = α-linolenate.

Analysis of volatile components in olive oil has been well reported [13, 17, 22] and, as a result, many of the individual volatile components have been identified in virgin olive oil. The volatile profiles of oils are believed to be influenced by a number of factors including cultivar [23], climate, soil, time of harvest and storage conditions of the extracted oil [24]. Indeed, it has been reported that stage of fruit ripeness also is an influencing factor [25]. In addition, Montedoro and his coworkers [13] have suggested that the milling and pressing processes particularly affected aldehyde production.

The volatile components of the olive callus cultures were identified by capillary GC–MS and data for major constituents are shown in Table 3. Of the 95 peaks detected in the samples, 30 of them were fully identified, all of which have been previously detected in olive and other vegetable oils [12, 17, 22, 26]. We did not attempt to identify all the individual volatile components, but concentrated on those constituents which are known to contribute to the aromatic properties of the tissue. The volatiles included hydrocarbons, alcohols, aldehydes, ketones and esters. Table 3 indicates that aldehydes were the most prominent of the volatiles (ranging from 27 to 37%) a result which compares well with previously published data for olive oil [13]. Hexanal, *trans*-2-hexenal, 3-methylbutan-1-ol, *trans*-2-hexen-1-ol, *cis*-3-hexen-1-ol, butyl acetate, 3-methylbutanal, hexyl acetate and methyl nonanoate are major volatile constituents of virgin olive oil [13, 27] and these were positively identified in the calli. Indeed, the presence of hexanal and *trans*-2-hexenal (and associated metabolites) is clear evidence of an active lipoxygenase pathway in the cultures. Moreover, we have conducted an initial characterisation of lipoxygenases in olive callus cultures recently and confirmed the presence of several isoforms (data not shown).

As shown in Table 3, ANOVA test highlighted that 13 peaks were statistically different (assuming that an $F$-probability of $< 0.05$ is significant). There was a significant difference in the amount of acetaldehyde produced by the calli ($F$ probability of 0.006). Coratina possessed the highest amount of this volatile which was at least double that of the other two varieties. This observation may well be related to the higher proportion of α-linolenate in the Italian variety. Hexanal and *trans*-2-hexenal were other prominent aldehydes and were typical major products of the lipoxygenase pathway [8] and major components of olive oil volatiles [12, 13]. The differences in the amount of hexanal production was very significant ($F$-probability $< 0.001$). Production of this aldehyde was 6-fold greater in the Spanish variety than the other two cultivars. *trans*-2-Hexenal was also more prominent in Picual. Octanal was noticeably higher in Picual ($F$-probability 0.002) than in the other cultivars which appears to agree well with Snyder *et al.* [26] who have suggested that olive oils with the highest amount of oleate are associated with a corresponding high octanal content than lower oleate-containing oils. *cis*-3-Hexenal was not detected either because it is unstable in an acidic environment (Murashige and Skoog medium is slightly acidic [28] (see Experimental) and, thus underwent isomerisation to the more stable *trans*- form, or it may have been a substrate for alcohol dehydrogenase producing *cis*-3-hexen-1-ol. $C_6$ alcohols were readily detectable in all cultures (see below), showing that alcohol dehydrogenase was appreciably active in each cultivar.

Other volatiles that showed significant differences between the cultivars were the $C_6$ alcohols. The alcohol derivatives of hexanal are recognised to be the leaf alcohols exhibiting a "green aromatic" note [29] and, as such, are important contributors to the aroma of an oil, although some of these may well be undesirable [27]. The differences in amounts of both hexan-1-ol and *cis*-2-hexen-1-ol were statistically significant and that for *cis*-3-hexen-1-ol only just failed to attain statistical significance (Table 3). For the esters also, there were significant differences in several components. Of these, ethyl propionate and hexyl acetate are sig-

Table 3. ANOVA of some important volatile compounds of callus cultures of olive

| | Peak-area ratio ( × 100) | | | | |
|---|---|---|---|---|---|
| | Picual | Koroneiki | Coratina | *F* ratio | *F* probability |
| Hydrocarbons | | | | | |
| *n*-Hexane | 0.045 | 0.092 | 0.097 | 0.53 | 0.612 |
| Octane | 0.149 | 0.027 | 0.119 | 4.81 | 0.057 |
| 3, 4-Dimethyl heptane | 0.256 | 0.412 | 0.694 | 5.03 | 0.052 |
| Hydrocarbon | 5.666 | 2.854 | 3.789 | 6.00 | 0.037* |
| | (21.9) | (23.0) | (28.8) | | |
| Alcohols | | | | | |
| 3-Methyl-butanol | 0.203 | 0.101 | 0.057 | 5.27 | 0.048* |
| Hexan-1-ol | 0.276 | 0.175 | 0.050 | 15.22 | 0.004* |
| *cis*-3-Hexen-1-ol | 0.068 | 0.072 | 0.023 | 4.54 | 0.063 |
| *trans*-3-Hexen-1-ol | 0.168 | 0.077 | 0.061 | 2.21 | 0.191 |
| *cis*-2-Hexen-1-ol | 0.072 | 0.315 | 0.075 | 8.39 | 0.018* |
| *trans*-2-Hexen-1-ol | 0.426 | 0.342 | 0.171 | 1.19 | 0.367 |
| Octan-2-ol | 0.084 | 0.033 | 0.059 | 1.71 | 0.258 |
| | (4.6) | (7.6) | (3.0) | | |
| Aldehydes | | | | | |
| 3-Methyl-butanal | 5.418 | 4.520 | 3.790 | 0.78 | 0.499 |
| Acetaldehyde | 0.262 | 0.388 | 0.861 | 13.76 | 0.006* |
| Hexanal | 0.687 | 0.104 | 0.104 | 36.66 | <0.001* |
| 3-Hexenal | 0.092 | 0.018 | 0.133 | 2.48 | 0.164 |
| *trans*-2-Hexenal | 0.607 | 0.141 | 0.181 | 33.52 | 0.001* |
| Octanal | 0.358 | 0.165 | 0.038 | 22.75 | 0.002* |
| Nonanal | 0.059 | 0.123 | 0.042 | 1.65 | 0.269 |
| | (26.7) | (37.2) | (31.5) | | |
| Ketones | | | | | |
| Acetone | 0.195 | 0.161 | 0.118 | 0.47 | 0.644 |
| Pentan-2-one | 2.257 | 0.056 | 0.683 | 142.02 | <0.001* |
| | (8.8) | (1.5) | (4.9) | | |
| Esters | | | | | |
| Ethyl acetate | 0.702 | 0.137 | 0.220 | 3.91 | 0.820 |
| Ethyl propionate | 0.216 | 0.062 | 0.117 | 13.96 | 0.006* |
| Butyl acetate | 2.555 | 1.059 | 1.378 | 2.55 | 0.158 |
| Hexyl acetate | 0.683 | 0.081 | 0.078 | 34.91 | <0.001* |
| *cis*-3-Hexenyl acetate | 0.858 | 0.880 | 0.272 | 2.95 | 0.128 |
| Methyl nonanoate | 0.629 | 0.233 | 0.057 | 11.46 | 0.009* |
| Other | 1.742 | 0.335 | 0.427 | 35.16 | <0.001* |
| | (26.4) | (18.9) | (20.5) | | |
| Acid | | | | | |
| Acetic acid | 0.094 | 0.166 | 0.038 | 2.35 | 0.176 |
| Others | 3.155 | 1.561 | 1.792 | | |
| | (11.1) | (10.6) | (10.9) | | |

There were three replicates for each sample.
Numbers in parenthesis represent the group percentages of the total volatiles.
* Statistically significant ($P < 0.05$).

nificant components of olive oils where they contribute to the sweet fruity notes [17].

Temperature is an important environmental factor which particularly influences plant lipid metabolism [30]. Therefore, we investigated the effects of this parameter on volatile production by the calli. They were cultured at temperatures compatible with the Mediterranean climate i.e. 25–35°C. Table 4 shows clearly that total volatile accumulation was inversely proportional to increased growth temperature. This observation may well be related to the relative effects of temperature on enzyme activities. Further work on this aspect of the research is necessary in order to clarify this point.

Table 4. The effect of growth temperature and medium pH on volatile production in olive callus cv Picual

| | Total volatiles (ppm) | | |
|---|---|---|---|
| pH | 25°C | 30°C | 35°C |
| Control (5.8) | 6.78 ± 1.00 (33 : 27 : 33 : 7) | 3.91 ± 0.57 (43 : 36 : 14 : 7) | 3.07 ± 0.30 (41 : 41 : 1 : 6) |
| 5.0 | 4.95 ± 1.00 (46 : 31 : 15 : 8) | nm | nm |
| 6.5 | 4.17 ± 0.88 (55 : 20 : 20 : 5) | nm | nm |
| 7.0 | 3.58 ± 0.18 (35 : 41 : 18 : 6) | nm | nm |

Data represents means ± S.D. (where $n = 3$).
Abbreviations: nm = not measured.
Numbers in parenthesis represent the percentages of identified aldehydes: hydrocarbons: esters: alcohols (for list see Table 3).

Moreover, apart from the quantitative effects on volatile production, Table 4 also indicates that the aroma of the cultures is influenced by alterations to the growth temperature. There were, in fact, changes in the proportions of the volatile constituents particularly of the hydrocarbon and aldehyde groups. When one considers that, in vegetable oils, the alcohol, aldehyde and ester constituents contribute particularly to the desired aroma of an oil, it could be assumed that, in olive cultures, elevated temperatures may well have significant effects on their characteristic fragrance. A relative reduction in alcohol production could well have been associated with a reduction in the activity of alcohol dehydrogenases or because of the results of endogenous oxidation reactions. In the latter connection, Snyder *et al.* [26] have reported that saturated aldehydes such as hexanal, pentanal, octanal and nonanal are readily detectable in oxidised olive oil.

Olive cultures are grown optimally in medium that has a pH of 5.8 [19]. We were also interested to determine whether manipulation of the growth medium pH would affect volatile production since this could have relevance to agricultural practice (including soil quality) as well as to manufacturing conditions. Once again both quantitative and qualitative changes to the volatiles produced by the cultures were observed (Table 4), especially to the changes in the proportions of aldehydes and hydrocarbons. How these changes reflect alterations in amounts or activity of individual enzymes of the olive lipoxygenase pathway, some of which have been partly characterised [31, 32], needs further research.

The results reported here have shown that it is possible to use olive callus cultures as model systems for studies of the production of volatiles by the lipoxygenase pathway. The calli produced all the volatiles found within the fruit and oils and, moreover, show cultivar specificity, just as olive oils do. In addition, clear effects have been shown which illustrate the changing environmental conditions (temperature, pH) on volatile production by calli in agreement with sensory data for oils from different harvesting regions [6]. These observations, together with greater knowledge of the lipoxygenase pathway in different calli, should help with predictions about how the quality and quantity of olive volatiles can be controlled.

## EXPERIMENTAL

### *Tissue cultures*

Callus cultures were established with three olive (*O. europaea* L.) varieties. Each cultivar was from a different Mediterranean country; Spain (cv Picual), Italy (cv Coratina) and Greece (cv Koroneiki). The olive pericarp was removed from the fruit and surface-sterilised with 4% sodium hypochlorite (w/v) for 30 min and imbibed for 8 h in running water at 20°. The kernels were aseptically germinated at 25°.

Etiolated embryos emerged from the kernels after 4–5 days. These were aseptically excised and bisected so that the cotyledons were cut transversely into two segments. The tissue slices were transferred to Murashige and Skoog medium (pH 5.8) [28] which was supplemented with an auxin–cytokinin combination consisting of 2,4-dichlorophenoxyacetic acid (12 $\mu$M) and benzylaminopurine riboside (0.56 $\mu$M). Activated charcoal (0.025%, w/v) was added to the medium in order to counteract any toxins produced by the cultures in the growth medium. The embryos were incubated at 25° with a 12 h light/dark cycle. Callus was established within 28 days and then transferred to a medium containing one half the concentration of the above-mentioned plant growth regulators. The calli were subcultured at four-weekly intervals. The light was provided by cool white fluorescent tubes (20 $\mu$mol $m^{-2} s^{-1}$). In order to investigate the effects of growth

temperature (25, 30 and 35°) as well as the pH of the medium (5.0, 5.8, 6.5 and 7.0), Picual callus was transferred from the above Murashige and Skoog medium at 25° to new media with adjusted pH and appropiate incubation temperatures. Moreover, the cultures were only sampled after two successive subcultures covering a period of at least 8 weeks, so that the cultures had fully adjusted to their new growth conditions. Samples were then analysed uniformly 21 days after subculturing.

*Volatile analysis*

Olive callus (20 g) was heated at 40° and a stream of nitrogen gas was flushed over the surface of the tissue at a rate of 200 ml $min^{-1}$. The carrier gas was passed through a condenser in order to remove any water vapour which might hinder the chromatographic analysis. The volatiles were then trapped in a sample tube packed with Tenax TA (Perkin–Elmer). The sampling time was 30 min. Isobutyl acetate was used an internal standard.

Thermal desorption of the trapped volatiles was achieved using an Automatic Thermal Desorption System (Perkin–Elmer ATD 400 model) set at 250°. The volatiles were then flushed from the sample tube again using nitrogen carrier gas (18–22 psi) and concentrated on a second trap containing Tenax TA but at a temperature of −30° for 10 min. The cold trap was flash heated to 250° and the volatiles were automatically injected onto a GC capillary column. A Perkin–Elmer (PE) Autosystem GC was fitted with a fused-silica Supelcowax 10 capillary column (60 m × 0.32 mm ID, 0.5 μm film thickness), using helium as carrier gas and nitrogen as make-up gas. The oven temperature was set initially at 40° for 4 min and was programmed to rise at 4° $min^{-1}$ until 240° was reached. The injection temperature was 175° and the detector temperature was 275°. Both the Autosystem GC and the ATD400 were controlled automatically by a PE Nelson Model Integrator Programmer. Tentative identification were made by comparing retention times of the GC peaks with authentic standards. Peaks were identified by mass spectral analyses by using a Fisons MD800 Mass Selective Detector. The software used was MassLab VI.3. Sample components were verified by comparison of the mass spectral data with those of authentic reference compounds. When standards were not available, the components were identified by mass spectra obtained from the mass spectral library collection.

*Statistical analysis*

ANOVA was used to study the reproducibility of the samples, which were analysed in triplicate. All calli types were treated with the same growth conditions so that the only variable was that of cultivar. Hence, the experimental stucture consisted of a $2^1$ factorial set.

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