PERGAMON

Phytochemistry 53 (2000) 13–19

PHYTOCHEMISTRY

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Lipoxygenase pathway in olive callus cultures (*Olea europaea*)

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Received 1 June 1999; accepted 21 August 1999

Abstract

Stimulation of the lipoxygenase pathway in olive fruit initiates a cascade of reactions that begins with the regio- and stereo-specific di-oxygenation of polyunsaturated fatty acids containing a *cis*, *cis*-1,4 pentadiene moiety. Later products of the pathway include volatiles that influence the organoleptic properties of harvested olive oil. In this study, we have investigated lipoxygenase activity in olive callus cultures, and found that there is evidence of several isoforms of the enzyme with different pH optima and substrate specificities. Endogenous lipoxygenase activity was detected throughout the growth cycle of olive callus, particularly during the log phase of growth, suggesting that olive lipoxygenases are intimately involved in growth. The most prominent lipoxygenase activity in tissue cultures was found to be soluble but significant activities were detected in the plastid fraction. In addition, hydroperoxide lyase (HPL) activity was measured in the calli; both 13- and 9-HPL activities were found which were particulate.

Keywords: *Olea europaea*; Oleaceae; Callus cultures; Lipoxygenase pathway; Lipoxygenase isoforms

1. Introduction

There is an increasing demand for olive oil in the world market. It ranks sixth in vegetable oil production (Gunstone, Harwood & Padley, 1994), but it is regarded as being superior in quality to other vegetable oils because of its rather special acyl composition as well as its delicious taste and distinctive aromatic qualities. Virgin olive oil is unique in that it is extracted without any refining treatment. It is also appreciated for its health properties (Kaftos & Comas, 1990). The organoleptic properties of virgin olive oil are derived from by-products of the lipoxygenase pathway. Many of the volatile constituents of virgin olive oil (Morales, Aparicio & Rios, 1994) have also been identified in olive tissue cultures (Williams, Morales, Aparcio & Harwood, 1998) which serve as a convenient year-round model system. Therefore, we have studied the lipoxygenase pathway in both systems.

Lipoxygenases (LOX: EC 1.13.11.12) are found widely in the plant and animal kingdoms and catalyse the regio- and stereo-specific dioxygenation of polyunsaturated fatty acids containing a *cis*, *cis*-1,4-pentadiene moiety (Gardner, 1991). In plants, the immediate products of the LOX reaction are conjugated (*Z*, *E*)-diene hydroperoxy (HPO) fatty acids (Blée, 1998). These compounds are highly reactive as well as being toxic to cells. They are rapidly degraded into metabolites that lead to the production of jasmonates, conjugated dienoic acids and volatile aldehydes (Wasternack et al., 1998). The latter are particularly important to the olive industry because they contribute to the organoleptic properties of the harvested oils.

Hydroperoxy lyase cleaves hydroperoxy C_{18}-fatty acids into C_6-aldehydes and 12-oxo-acids. The C_6-aldehydes include hexanal and *Z*-3-hexenal. The latter is an unstable compound and undergoes rapid isomerisa-

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0031-9422/00/$ - see front matter
PII: S0031-9422(99)00468-9

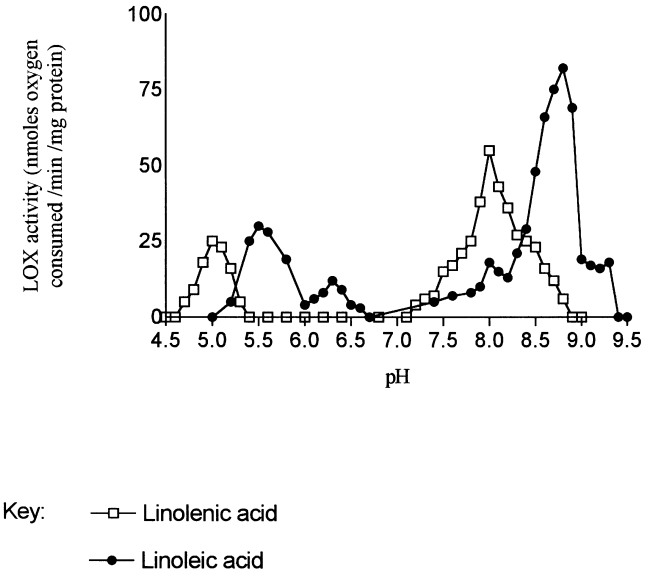


Fig. 1. pH profile of lipoxygenase activity in resuspension of acetone powders of olive callus.

tion to the more stable compund, *E*-2-hexenal. Both hexanal and its unsaturated homologues undergo reduction, forming alcohols (Siedow, 1991). The latter are recognised as being responsible for the fruity notes of an oil (Morales et al., 1994). Sensory evaluation of olive oil, incorporating the application of computer sensory wheel analysis, has been instrumental in demonstrating the importance of cultivars and the geographical location of individual olive groves (Morales et al., 1994) and is used in line with EC regulations (Aparicio, Alonso, Morales & Calvente, 1994). Of the large numbers of volatile constituents in vegetable oils such as aldehydes, alcohols, hydrocarbons, ketones, furans, acids and esters, only a comparative few have organoleptic properties that are of any importance (Snyder, Frankel & Selke, 1985).

In contrast to the considerable data available in the literature about other plant LOXs, very little has been published concerning olive LOXs (e.g. Georgalaki, Sotiroudis & Xenakis, 1998). However, a clear understanding of the LOX reaction in harvested fruit and during the extraction process is necessary to optimise olive oil manufacturing processes. Hence, we have been studying the LOX pathway in olive callus cultures. Olive callus has an acyl composition that is comparable to developing fruit (Williams, Sanchez, Hann & Harwood, 1993) and has proved to be a reliable material for the study of olive lipid metabolism (Williams & Harwood, 1994; Rutter, Sanchez & Harwood, 1997). Most significant has been the finding that olive cultures produce volatiles that are qualitatively similar with those detected in virgin oil (Williams et al., 1998), thus demonstrating that results obtained from olive cultures can also be applied to the parent olive plant.

2. Results and discussion

2.1. Olive calli contain LOX isoforms

The use of plant tissue technology has greatly facilitated the study of lipid metabolism in olive fruit and was first reported by Williams et al. (1993). Olive cultures were also instrumental in demonstrating that the olive cultivar is a major factor that influences volatile production (Williams et al., 1998) as it is with olive fruit (Aparicio, Morales & Alonso, 1997). There is also considerable interest in LOX activities in oleaginous plants within the food industry because LOXs are responsible for the degradation of fatty acids with subsequent loss of food quality and colour (Eskin, Grossman & Pinsky, 1977).

LOXs exist in multiple isoforms in some plants such as tomato (Smith, Linforth & Tucker, 1997) and cucumber (Feussner & Kindl, 1994). It is likely that different isoforms have distinctive physiological roles (Hildebrand, 1989). Fig. 1 shows the pH profiles for LOXs from acetone powders of olive callus. Similar pH profiles for olive fruit (data not shown) emphasise the suitability of using tissue culture technology for LOX investigations. Thus, three distinct peaks of activity were found with linoleic acid and two with linolenic acid. The results suggest that there are several LOX isoforms in olive which differ in their substrate specificity and pH optima. The most significant LOX activity for oil quality is probably in the acidic region which is within the malaxation pH range for oil extraction. The maximum LOX activity, however, was associated noticeably with the alkaline range (for both substrates), a finding that has also been observed in soybean cotyledons (Axelrod, Cheesebrough & Laakso, 1981) and in the lipid-bodies of cucumber (Feussner & Kindl, 1994).

2.2. Effect of tissue development on LOX activity

We extended our studies in order to investigate the influence of the stage of callus development on LOX activity. Therefore, we measured LOX activity throughout the growth cycle. Olive calli were routinely subcultured at 35 day intervals. Fig. 2 shows that, following subculturing of callus, there was an initial lag phase of about six days in growth. Thereafter, there was a gradual increase in fresh weight, with a doubling in mass after a further 14 days (21 days after subculturing). We assayed LOX activity during the total subculture period, using the optimal pHs for each fatty acid substrate (see Fig. 1). The lag phase of growth was characterised by a sudden increase in LOX activity but, this declined until the log phase of growth was established. During the log growth phase, LOX activity increased significantly

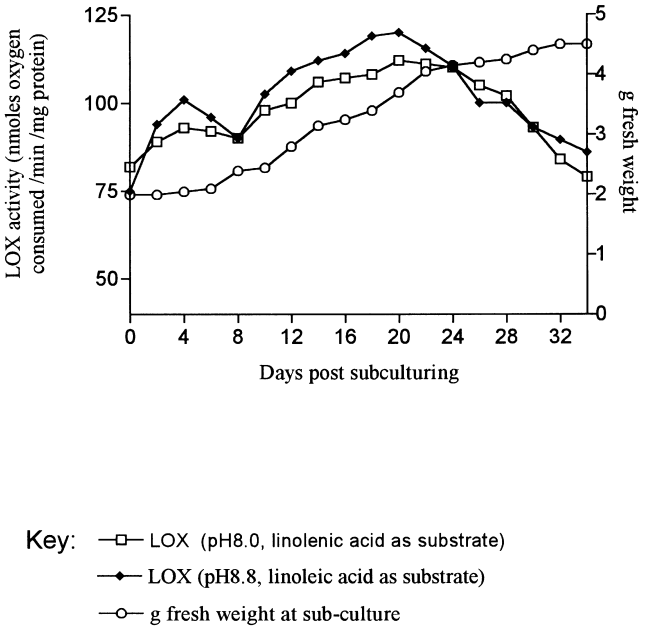


Fig. 2. Time-course of growth and lipoxygenase activity in acetone powders of olive callus cultures. Data represents means (n = 3).

(after day 8) to peak on day 20. Thereafter, LOX activity began to decline, returning more or less to its original level (on a protein basis) by the 34th day after subculturing. The data indicate, therefore, that LOX activity changes significantly during different growth phases, an observation which has also been reported for other species and suggests that LOXs are intimately involved in biological regulation during growth (see Hildebrand, 1989; Siedow, 1991). Of course, the calli are maintained in the presence of exogenous plant growth regulators which, undoubtedly, interact with products of the LOX pathway (Parthier, 1989) by keeping the cultures in a relatively undifferentiated state.

Although LOX activity with linoleate showed the same pattern of changes as that for α-linolenate, the relative changes in activity were different with the activity towards linoleate altering more. This was further evidence for different isoforms in olive tissue.

LOX activity is well recognised to be associated with growing plant tissue (Siedow, 1991), but the initial peak of activity on the 4th day may have been induced by a "transfer effect", a phenomenon which is observed with several enzymes. We noted that, although the overall pattern of changes in LOX activity were consistent, the duration of the lag phase depended on the size of the calli subcultured (the larger the tissue sample, the shorter the lag phase). This suggested that the initial peak of activity was regulated by the stage of development and not induced by mechanical stress during subculturing.

2.3. LOX responses to wounding in olive callus

Tissue wounding ellicits a jasmonic acid signal response which varies in length and is known to amplify the LOX reaction (Blée, 1998; Wasternack et al., 1998; Siedow, 1991). Elevated LOX activity associated with wounding has been widely reported in soybean leaves (Saravitz & Siedow, 1995), potato tubers (Geerts, Feltkamp & Rosahl, 1994) and tomato leaves (Conconi, Miquel, Browse & Ryan, 1996). Moreover, because volatile products from the LOX pathway are so important for sensory properties, we were also interested in possible alterations of the LOX pathway following fruit damage or, later, during malaxation. Homogenisation of the callus was associated with a rapid decrease in total lipid and polar lipid content (Fig. 3), no doubt as a result of the release of previously compartmentalised lipase and acyl hydrolase activities (Galliard & Chan, 1980). Even though non-esterified fatty acids (NEFA) do not normally accumulate in Nature, there was an initial increase in the amount of NEFAs for up to 10 min. Thereafter, the NEFA level decreased back to its initial value (Fig. 3). We included inhibitors of β-oxidation in our incubations to prevent such catabolism of liberated fatty acids (data not shown) so the decline in NEFA levels after 10 min was probably due mainly to LOX activity (see below).

The acyl lipid composition of some of the important lipid classes were analysed during the time-course study and data for total lipids and NEFAs are shown in Fig. 4. Whereas the acyl composition of the total

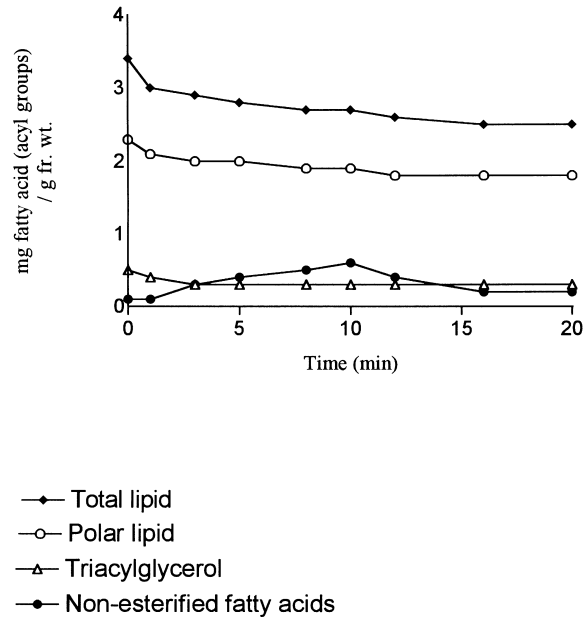
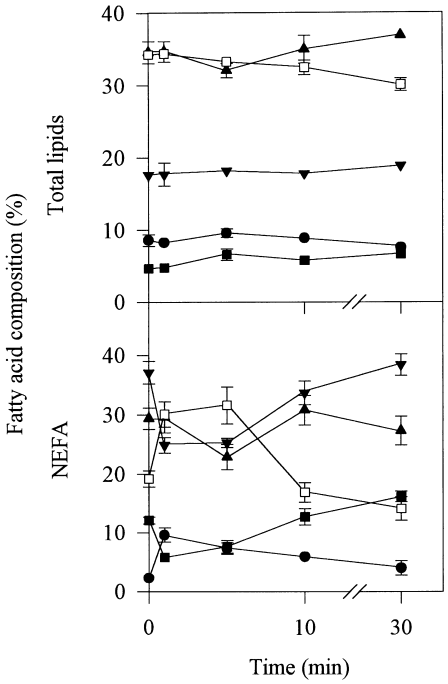


Fig. 3. Time-course of lipid alterations in homogenates of olive callus. Data represents means (n = 3).



KEY:

- ▼ Palmitic acid
- ■ Stearic acid
- ▲ Oleic acid
- ● Linoleic acid
- □ Linolenic acid

Fig. 4. Time-course of fatty acid metabolism in homogenised callus. Data represent means ±S.D. (n = 3). Abbreviation: NEFA = non-esterified fatty acids. Fatty acids were shown with the number before the colon indicating the number of carbon atoms and the figure after showing the number of double bonds. The unsaturated fatty acids were virtually exclusively oleic (18:1), linoleic (18:2) and α-linolenic acids.

lipid remained relatively unchanged during the time-course, there was a dramatic change in the ratio of saturated to unsaturated fatty acids of NEFAs. There was an initial increase in polyunsaturated fatty acids (PUFAs) after 1 min of the time-course, probably as a result of the hydrolytic attack on phospho- and glycosylglycerides and release of NEFAs. The rise in the proportion of PUFAs in the NEFA fraction was maintained for 5 min after which it declined back to around the initial levels. These data, together with the temporary increase in NEFA levels (Fig. 3), show that wounding by homogenisation caused a preferential liberation of PUFAs which were then catabolised. Since the LOX pathway uses such acids efficiently in comparison to other oxidative pathways, then the results indicate the importance of LOX activity during wounding of olive callus.

2.4. Subcellular localisation of LOX and hydroperoxide lyase activity

LOXs are widely distributed in plant organs (Siedow, 1991). Plant LOXs are primarily soluble, but there is increasing evidence that some isoforms are associated with particulate fractions e.g. lipid bodies (Feussner, Hause, Nellen, Wasternack & Kindl, 1996), vacuoles (Wardale & Lambert, 1980) and chloroplasts (see Gardner, 1991). LOX activity in olive fruit has shown by Salas, Williams, Sanchez & Harwood (1999) to be associated primarily with chloroplasts. All five putative LOX activities were detected in the soluble fraction. Of these, it is possible that the acidic activity (pH 5.5) against linoleate had originated from the chloroplast stroma. However, the alkaline (pH 8.8)

Table 1
Localisation of lipoxygenase activity in subcellular fractions from olive callus cultures. Abbreviation nd = none detected. Cultures were 21 days old. LOX activity was measured using a Clarke-type oxygen electrode at 25°C

Fraction	pH	Substrate	Specific activity (nmoles oxygen/min/mg)
Soluble	8.8	18:2	184.3
	6.3	18:2	23.2
	5.5	18:2	17.1
	8.0	18:3	87.2
	5.0	18:3	34.7
Plastid	8.8	18:2	26.4
	6.3	18:2	nd
	5.5	18:2	24.7
	8.0	18:3	27.7
	5.0	18:3	nd
Thylakoid membranes	8.8	18:2	12.9
	6.3	18:2	nd
	5.5	18:2	nd
	8.0	18:3	17.4
	5.0	18:3	nd

Table 2
Localisation of hydroperoxide lyase activity in olive callus. Data represent means ±S.D. (where $n = 3$). Products were quantified by head-space gas chromatography using isobutyl acetate as an internal standard

Fraction	Substrate	Total volatile aldehydes (ppm/mg protein)	
		Control	Sample control
Microsomal	13-HPO-18:2	0.9 ± 0.2	9.3 ± 0.8
Plastid	13-HPO-18:2	2.1 ± 0.4	13.2 ± 1.0
Soluble	13-HPO-18:2	0.3 ± 0.2	nd
Microsomal	9-HPO-18:2	1.3 ± 0.3	11.7 ± 0.8
Plastid	9-HPO-18:2	1.7 ± 0.5	15.6 ± 1.4
Soluble	9-HPO-18:2	1.1 ± 0.4	nd

activities against linoleate and (pH 8.0) against α-linolenate are unlikely to have arisen solely from the chloroplast stroma because their specific activities were significantly higher in the soluble fraction (Table 1). These two activities were also shown to be appreciable in a thylakoid membrane fraction. Thus, we can conclude that, in olive callus there are both membrane-associated and soluble activities with significant, but not exclusive, localisation with a plastid fraction. Some activity was found in the microsomal fraction, but we conclude that these membranes were contaminated by thylakoid fragments because of the presence of *trans*Δ3-hexadecenoic acid (see Harwood, 1980) and pigments in this particulate fraction (data not shown).

The HPO fatty acid products of LOX are themselves substrates for hydroperoxide lyases. Soluble and particulate fractions were incubated with either 13- or 9-HPO fatty acids, although it should be noted that 13-HPOs are the main products of LOX in olive tissue cultures (Williams & Harwood, 1998) as well as in olive fruit (Salas & Sanchez, 1998a). The volatile products of the reaction were collected by headspace analyses and analysed by capillary GC. Table 2 shows that the total aldehyde volatile production was associated with the particulate (microsomal and plastid) fractions, but not with the soluble fraction. These data indicate that hydroperoxy lyase in olive calli is membrane-bound and probably derives from the chloroplasts. Because of the small amounts of material available, we could not carry out further experiments to determine whether all the microsomal activity was due to chloroplast membranes. However, the importance of chloroplasts as a location for hydroperoxide lyase (Table 2) agrees with data for olive fruits (Salas & Sanchez, 1998b).

Two isoforms of hydroperoxide lyase have been partly purified from olive fruits at a middle maturation stage. Neither had activity with 9-HPO (n − 10) substrates (Salas & Sanchez, 1998b) and this clearly differs from the preparations assayed in the present work (Table 2). However, as discussed above (see also Salas & Sanchez, 1998a; Salas & Sanchez, 1998b) olive lipoxygenases generate mainly the 13-HPO, and it is cleavage of this product which is important for the generation of volatile components in olive oil.

3. Conclusion

The present studies indicate that there are several LOX isoforms in olive callus. These results have important implications for the olive oil industry. Whereas there is much concern in the food industry concerning spoilage of produce and the subsequent need for blanching prior to storage (e.g. vegetables and wheat dough), the LOX pathway in olive fruit is responsible for the production of the highly desirable organoleptic properties that help to differentiate virgin olive oil from other vegetable oils. LOXs are activated by the mechanical crushing of the fruit and their activity continues during the malaxation of the olive paste. The flavour and aroma of olive oil is primarily derived from C_6 aldehydes and alcohols that enhance the green and fruity notes of the oil. Any factor that can influence the production of these volatiles is, therefore, worthy of investigation.

Olive LOXs are distributed between the soluble and particulate fractions of callus tissue. Soluble LOXs have been detected in many other plant tissues e.g. tomato (Smith et al., 1997) and in so much that olive callus is highly vaculated (Williams et al., 1993), this finding was not unexpected. In contrast, olive fruit LOXs with acidic pHs have been found to be associated with plastid membranes (Salas et al., 1999). This apparent discrepancy can be rationalised when one considers that olive callus is primarily heterotrophic and, as such, plastid numbers are greatly reduced compared to photosynthetic olive fruits.

The results reported here add further evidence to the proposal that olive callus cultures can be used as a convenient experimental system for the study of lipid biochemistry. In this study, we have reported on the activity of enzymes associated with the lipoxygenase pathway. Further research on this important catabolic process will be useful for the elucidation of volatile generation in olive oil and for the optimisation of the extraction procedure.

4. Experimental

4.1. Plant materials

Photomixotrophic olive calli (*Olea europaea* cv. Coratina) were established using etiolated embryos as

reported previously (Williams et al., 1998). Callus was maintained thereafter on Murashige and Skoog medium (pH 5.8) which was supplemented with an auxin-cytokinin combination consisting of 2,4-dichlorophenoxyacetic acid (12 μM) and benzylaminopurine riboside (0.56 μM). Activated charcoal (0.025%) was also added to the medium in order to minimize the effects of toxin production by the callus. The cultures were grown in a Gallenkamp incubator at 25°C with a 12 h light/dark cycle. The light intensity was 20 μmol m^{-2} s^{-1}. Calli were subcultured every 35 days.

4.2. LOX extraction and assay

Olive callus (50 g) was ground and extracted three times in a total volume of 500 ml acetone at −20°C using a polytron blender. The resultant acetone powders were filtered under vacuum, rinsed with diethyl ether and then air-dried. The acetone powders were stored desiccated at −70°C until required.

Acetone powders (1 g) were resuspended using a Potter homogeniser in 20 ml of Hepes buffer (50 mM, pH 7.2) containing 4 mM DTE and 10 mM MgCl. The crude homogenate was then mixed gently for 1 h at 4°C before centrifuging at 10,000 g for 10 min. The supernatant was desalted by passing it through a column of Sephadex G-25M (PD-10, Pharmacia) and then concentrated by ultrafiltration in a Centricon C-30K concentrator. All of the above procedures were carried out either on ice or at 4°C. The enzyme preparation was then stored at −70°C in the presence of glycerol (30%, v/v).

LOX activity was measured at 25°C using a Clark-type oxygen electrode (Rank Bros.). The electrode was calibrated using an oxygen saturation curve. The reaction mixture, which had a total volume of 3 ml, consisted of 20 mM borate buffer pH 8.8 (2.45 ml), 10 mM linoleic acid/α-linolenate(50 μl) and 500 μl of enzyme preparation (approximately 150 μg protein). When α-linolenate was the substrate, the buffer was adjusted to pH 8.0. A sodium phosphate buffer system (pH 4.5–8.2) and a sodium borate buffer system (pH 8.0–9.5) were used for establishing pH profiles of both olive fruit and callus.

4.3. Extraction and analysis

Lipids were extracted by a high salt extraction method (Garbus, de Luca, Loomans & Strong, 1963) which has been modified for plant lipids (Smith, Douce & Harwood, 1982). The total lipid fraction was further purified by thin-layer chromatography using pre-coated (E. Merck, Darmstadt, Germany) silica gel 60 plates which were activated at 70°C for 1 h, prior to use. Lipids were separated using a non-polar solvent system consisting of petrol (60/80°fr.)-Et_2O-HOAc (80:20:1, by vol.). Lipid bands were revealed by spraying with 8-anilino-1-naphthalene sulphonic acid in methanol (0.2%, w/v) and viewed under UV light. Individual lipids were identified by co-chromatography with authentic lipid standards and their identity confirmed by the use of specific spray reagents (Kates, 1986).

Fatty acid methyl esters (FAMEs) of the total lipid fraction and the individual complex lipid classes were produced by acid-catalysed transmethylation as previously described (Williams, Francis, Hann & Harwood, 1991). These were analysed by gas chromatography using a Perkin–Elmer F33 model using a glass column (4.0 mm × 2 m) packed with EGSS-X (15% w/w) on Chromosorb WAW (120 mesh), at 175°C. Heneicosanoic acid was used as an internal standard.

4.4. Callus homogenate incubations

For the time-course degradation studies, callus (21 days after subculturing) was gently homogenised with a pestle and mortar for 30 s at room temp. The homogenates were then left for various incubation time periods. At the end of each incubation time, homogenate samples were immediately immersed in hot propan-2-ol (at 70°C) for 30 min in order to prevent further lipid catabolism. Lipid analysis was performed as reported previously (Williams et al., 1993).

4.5. Subcellular fractionation of olive callus

Callus cultures were destarched by placing them in the dark for three days. Callus (100 g) was then homogenised using a pestle and mortar in a buffer consisting of Hepes (25 mM), sorbital (330 mM), magnesium chloride (2 mM), potassium chloride (20 mM), EDTA (2 mM), sodium sulphate (10 mM), DTE (4 mM), β-mercaptoethanol (12 mM), BSA (0.1%) and ascorbic acid (0.2%), pH 7.2 at 4°C. The homogenate was filtered through two layers of Miracloth (Calbiochem) and then centrifuged at 3000 g for 10 min at 4° in order to isolate the crude plastid fraction. A putative thylakoid fraction (as judged by the enrichment of the appropiate markers) was isolated at 15,000 g for 15 min. The supernatant was eventually subjected to ultracentrifugation at 100,000 g for 1 h. The microsomal pellet (10 mg/ml) was also resuspended in the above mentioned Hepes buffer. The supernatant was concentrated by ultrafiltration as for acetone powders (above). The resuspension buffer for the particulate fractions consisted of Hepes (50 mM), sorbitol (330 mM), DTE (4 mM) and Triton X-100 (0.1%), pH 7.2 at 4°C.

4.6. Localisation of hydroperoxide lyase activity

Microsomal (125 μg protein/100 μl), plastid (90 μg protein/100 μl) and soluble (250 μg protein/100 μl) fractions (10 ml) were individually subjected to a steady stream of air through the incubation mixtures (flow rate of 10 ml/min) in an enclosed glass container for 30 min at room temperature. The carrier gas was passed through a water-cooled condenser in order to remove any water vapour which might hinder the chromatographic analysis. Volatiles from the above incubations were trapped in a Tenax TA sample tube (Perkin–Elmer). Thermal desorption of the sample volatiles was achieved using an Automatic Thermal Desorption System (Perkin–Elmer ATD 400 model) set at 250°C. The volatiles were automatically injected onto a GC capillary column (Perkin–Elmer Autosystem GC) as described previously (Williams et al., 1998). Both the Autosystem GC and the ATD 400 were controlled by a Perkin–Elmer Nelson Model Integrator Programmer.

After the initial 30 min sample period, 20 μl of HPO linoleic acid (9- or 13-isomers) was added to the incubation and the volatiles were collected for a further 30 min. Hydroperoxide lyase activity was assayed by difference measurements from two chromatograms and by reference to an internal standard of isobutyl acetate.

Acknowledgements

We are grateful to the EC (AIR 3-93-67) for financial support and to Dr. Franca Angerosa (Istituto Sperimentale per la Elaiotechnica, Pescara, Italy) for kindly providing olive fruits at different stages of ripening.

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