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Lipoxygenase Distribution in Coffee (*Coffea arabica* L.) Berries

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In this paper lipoxygenase (LOX) presence was investigated in coffee berries to determine its involvement in lipid degradative metabolism of plants grown in organic and conventional cultivations. An immunochemical analysis has evidenced a ca. 80 kDa protein, cross-reacting with an anti-LOX antibody, only in the pulp fraction of berries obtained from plants of both cultivations. LOX activity in this fraction could be monitored either as conjugated diene formation or reaction products (determined by HPLC) and was mainly associated with a heavy membrane fraction (HMF, enriched in tonoplast, endoplasmic reticulum, plasma membrane, and mitochondria) and a light membrane fraction (LMF, enriched in plasma membrane and endoplasmic reticulum, with low levels of tonoplast and mitochondria). The LOX activity of LMF from berries of both cultivations showed an optimum at pH 8.0. The HMF exhibited a different activity peak in samples from conventional (pH 8.0) and organic (pH 5.5) cultures, suggesting the presence of different isoenzymes. These findings were also confirmed by variation of the ratio of 9- and 13-hydroperoxides in organic (1:1) and conventional cultivations (1:10), indicating that the organic one was subjected to an oxidative stress in the coffee pulp fraction leading to the expression of an acidic LOX. Such de novo synthesized LOX activity could be responsible for the production of secondary metabolites, which may interfere with the organoleptic profile of coffee.

KEYWORDS: Lipoxygenase; coffee; 9/13-hydroperoxyoctadecadienoic acid (9/13-HPODE) production; organic cultivation; oxidative stress

INTRODUCTION

Lipoxygenases (LOXs) are multifunctional enzymes that play an active role in plant lipid degradative metabolism. By their versatile activity, LOXs catalyze both the dioxygenation of lipid substrates (oxygenase reaction) and the secondary conversion of hydroperoxy lipids (hydroperoxidase reaction) (*1*), leading to the production of short-chain alcohols and aldehydes (*2, 3*). These enzymes belong to the complex metabolic pathway commonly known as the "oxylipin pathway".

Even if LOXs have been shown to be present in several plant species (*4*), their involvement has been scarcely investigated in coffee plants (*Coffea* sp.); hitherto only reports regarding their participation in the response to biotic and abiotic stress can be found (*5, 6*). Technical difficulties in evidencing and characterizing lipoxygenase activity in lipid-enriched fruit tissues could partially explain data shortage on such a topic.

Similarly to other fruits (e.g., olive, hazelnut, almond), coffee berries are rich in lipids. In particular, fatty acids inside cotyledons (green coffee) represent 9–15% of the dry weight (*7*): the most representative one (about 50%) is linoleic acid (*8, 9*), which is together with linolenic acid a well-known substrate for LOXs.

Furthermore, it has been shown that also cultivar (*10*), drying procedures (*11*), storage conditions (*12*), and roasting processes (*13*) are all sources of variability for both the lipid profile and fatty acid pool composition, causing a higher or lower susceptibility to further degradation by lipoperoxidative reactions. With regard to the agronomical techniques, it has been shown that in the organic cultivation practices, plants under stress conditions produce a significant amount of secondary metabolites through the so-called oxylipin pathway, such as jasmonic acid, traumatic acid, and alkenals, suggesting that LOXs are involved in the activation of a defense response (*14*). In addition, these

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secondary metabolites can lead to the production of volatile compounds, which play an important role in food flavor and quality (*15*).

As a consequence of the features above described, it is rational to hypothesize that also in coffee berries LOXs initiate the oxylipin cascade producing fatty acid hydroperoxides, leading then to the production of a large amount of secondary products along seven potential degradation pathways, which are well-summarized in Feussner and Wasternack (*16*).

The worldwide importance of coffee in human life is universally accepted, so it appears necessary to elucidate the lipoxygenase pathway in coffee plants, taking into account the pivotal role that volatile compounds, derived from fatty acid degradation, play in aroma and organoleptic perception (*17*). It has been shown that this enzyme can affect the shelf life of plant products. In particular, a correlation between quality and LOXs (*18*) in several food products has been established [e.g., beer (*19*), tomato (*20*), olive (*21*)]. Similarly, it has been noted that specific off-flavors present in brewed coffee (e.g., "woodiness") are more evident in coffee beans that have suffered different forms of stress during cultivation (E. Illy, personal communication). Therefore, oxidative stress, involving lipoxygenase activity in coffee fruits, could be associated with the generation of these off-flavors.

For these reasons, this work aimed to highlight the presence of LOXs in different coffee berry tissues. Furthermore, possible differences in lipoxygenase activity and their hydroperoxide product profile were determined in fruits harvested from plants grown in both organic and conventional cultivations.

MATERIALS AND METHODS

Chemicals. All chemicals, unless otherwise specified, were obtained from Sigma-Aldrich (St. Louis, MO). Authentic standards of 13-*Z*,*E*- and 9-*E*,*Z*-hydroperoxides of linoleic acid were purchased from Cayman Chemicals (Ann Arbor, MI).

Plant Material. Mature berries and green coffee beans of *Coffea arabica* L., grown in organic (OC) or conventional (CC) cultivations, were harvested in two consecutive years (2005 and 2006) from plantations located at 900 m above sea level in Campinas, Brazil. Plants in CC were supplied with 300 kg ha^{-1} of nitrogen and potassium mineral fertilizers, whereas plants in OC were supplied with liquid organic fertilization (4000 kg ha^{-1}), a procedure characterized by high denitrification phenomena. Moreover, CC received complete protection against pests and weeds by means of three treatments, whereas OC was not subjected to any treatment. In mature berries, pulp was separated from parchment seed; the two fractions were frozen in liquid nitrogen and stored at −20 °C. Green coffee beans obtained separately were subjected to the same treatment.

Antioxidant Capacity Assay. The antioxidant capacity of the different coffee fruit portions was evaluated by crocin kinetic competition test as described by Tubaro et al. (*22*). This test was performed on the hydrophilic compounds extracted from 1 g of ground material obtained from green coffee beans, pulps, and parchment seeds and extracted in phosphate buffer, pH 7.5, for 30 min at 25 °C. Peroxy radicals were generated in situ by diazo compound decomposition. The bleaching of crocin was directly correlated with radical production and occurred with a constant speed. When part of the peroxyl radicals was quenched by other antioxidants, the bleaching rate was lower, and it was correlated to the concentration of the antioxidants present in the sample. The ratio between the bleaching speed of crocin without or with other antioxidants yielded the whole antioxidant capacity of the sample.

The slope of a known antioxidant, such as Trolox, was calculated. Other substances, even complex mixtures, such as coffee extracts, could be measured and their values related to that of Trolox. The results were expressed as millimolar equivalents of Trolox.

Lipid Hydroperoxide Extraction. Ten grams of each coffee fruit portion was ground in a blender and extracted twice with hexane and isopropanol (95:5, v/v) and finally acidified at pH 2 with 1 N HCl. The organic phase was dried with sodium sulfate, evaporated under a nitrogen flow, and then resuspended in the mobile phase (acetonitrile/water/acetic acid, 80:20:0.1, v/v/v). Samples were then subjected to RP-HPLC measurement as hereafter described.

Acetone Powder Preparation. Pulps, parchment seeds, and green coffee beans were ground in a blender to obtain a fine powder. Five grams of material was extracted in 50 mL of acetone (chilled to −20 °C) and kept under stirring for 4 h at 4 °C. The samples were centrifuged at 1900*g* for 15 min, and the pellet was collected. The precipitate was rinsed with an equal volume of diethyl ether (chilled at −20 °C) to wash away residual lipids and subjected to another centrifugation at 1900*g* for 15 min. The pellet obtained was dried under nitrogen and then resuspended in 20 mM Tris-HCl, pH 7.5/10 mM histidine, and recentrifuged at 2700*g* for 15 min. The supernatant was lyophilized and resuspended in 1 mL of 20 mM Tris-HCl, pH 7.5/10 mM histidine.

Membrane Fraction Preparation. Pulp tissues (30 g) were homogenized with an Ultra-turrax (Ika-werk Sweden) in 150 mL of grinding buffer composed of 50 mM HEPES–KOH, pH 7.5, 0.25 M sucrose, 20 mM KCl, 2 mM $MgCl_2$, 5 mM EDTA, 7 mM mercaptoethanol, 3 mM DTE, 0.1% (w/v) ascorbate, 10 mM histidine, and 10% (v/v) glycerol, in the presence of 5% (w/v) PVPP, as described by Salas et al. (*23*). The homogenized pulp was filtered through 200 μm nylon mesh and submitted to differential centrifugations at 1000*g* for 5 min by a Sorvall RC-5B centrifuge (SS-34 rotor), then to 28000*g* for 12 min, and finally to 100000*g* for 1 h by a Beckman LE80K ultracentrifuge (70ti rotor). The 1000*g* precipitate was discarded; the 28000*g* (heavy membrane fraction, HMF) and 100000*g* (light membrane fraction, LMF) pellets were resuspended in 20 mM Tris-HCl, pH 7.5/10 mM histidine and further utilized for LOX assays. A soluble fraction was also collected and tested for both LOX activity and Western blotting assay.

Membrane Marker Enzyme Activities. Vanadate-sensitive (plasma membrane marker enzyme) and bafilomycin A_1-sensitive (tonoplast marker enzyme) ATPases were assayed as previously described (*24*). Antimycin A-insensitive cytochrome *c* reductase (marker enzyme for endoplasmic reticulum) and cytochrome *c* oxidase (marker enzyme for mitochondria) were detected as described by Lord et al. and Hodges et al., respectively, (*25, 26*) Glucose-6-phosphate dehydrogenase (marker enzyme for plastids) was assayed as described by Bergmeyer et al. (*27*)

SDS-PAGE and Western Blotting. Proteins from acetone powder preparation (15 μg) or from HMF and LMF (25 μg) were separated by SDS-PAGE in a 10% (w/v) polyacrylamide gel, under reducing conditions, stained with Coomassie Brilliant Blue R-250; immunoblotting was performed according to standard techniques (*28*). The antipea LOX B antibody (Ab) was used at a 1:2000 dilution. The immune reaction was detected by means of an antirabbit IgG alkaline phosphatase-conjugated secondary Ab (used at 1:1000 dilution), followed by addition of the substrate (Sigma-Fast; 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) buffered substrate tablets).

Preparation of Linoleic Acid. Linoleic acid was dissolved under nitrogen flux in 5 mM of borate buffer (pH 9.0), 0.1 mM EDTA, and 9.6% ethanol, with the addition of a few drops of Tween 20 to help the solubilization of the fatty acid. The solution was then divided into aliquots, which were flushed with nitrogen and stored at −20 °C.

Lipoxygenase Activity Assays. LOX activity was measured in different membrane fractions as conjugated diene formation, followed as an increase of absorbance at 234 nm by a Perkin-Elmer λ15 spectrophotometer. The activity was screened over a wide pH interval, ranging from 4.5 to 9.5 values, according to experimental procedures used in Braidot et al. (*29*), but the data presented are referred to the pH characterized by the maximal activity in each fraction. The incubation mixture consisted of 0.1 M sodium acetate, pH 5.5 (for HMF from organic samples), 0.1 M EPPS-KOH, pH 8.0 (for HMF from conventional samples and for LMF from both samples), and 20 μL of sample (about 20 μg of protein) in a final volume of 2 mL. The reaction was initiated by the addition of 250 μM linoleic or linolenic acid and proceeded at 25 °C. An extinction coefficient (ε_M) of 25000 M^{-1} cm^{-1} was used to estimate the amount of conjugated dienes.

RP-HPLC Analysis of Octadecadienoic Hydroperoxides (HPODE). These compounds were initially extracted and assayed on different fruit portions as previously described. Subsequently, RP-HPLC was applied to the reaction products from both HMF and LMF, isolated from pulp fruits and buffered in experimental mixtures at the same pH values that exhibited the maximal LOX activity. The reaction was started by the addition of 250 μM linoleic acid to 0.2 mL (containing about 200 μg of protein) of the crude extract dissolved in 10 mL of the incubation buffers mentioned above; the solution was stirred at 25 °C for 10 min. The reaction mixture was acidified to pH 2 with 1 N HCl and then extracted twice with 10 mL of hexane and isopropanol (95:5, v/v). The solvent used for the extraction contained 0.22 mM BHT as an internal standard. The extract was dried with sodium sulfate, evaporated under a nitrogen flow, and redissolved in 200 μL of the HPLC mobile phase. Reaction products were identified by RP-HPLC on a C18 Ultrasphere ODS column (5 μm, 4.6 mm × 250 mm, Beckman Instruments, Fullerton, CA) using an isocratic elution with acetonitrile/water/acetic acid (80:20:0.1, v/v/v) at a flow rate of 1 mL min^{-1}. Conjugated dienes were measured at 234 nm by means of an UV–vis detector (Beckman Instruments). The amount of HPODE was determined by integration of the chromatogram peaks, using the Star chromatography workstation version 5.5 (Beckman Instruments). The peaks observed were referred to that of a standard of 13-*Z*,*E*-HPODE.

Regioselectivity of HPODE Production by Pulp HMF. Extracts from both cultivations were additionally analyzed by LC-MS/MS to separate, clearly identify, and quantify the 9/13-HPODE isomers. Separation was obtained using LC-MS grade acetonitrile/water (55:45, v/v) under addition of 0.1% formic acid at a flow rate of 0.3 mL min^{-1} on a C18 Gemini column (3 μm, 3 mm × 150 mm, Phenomenex, Aschaffenburg, Germany). The isomers were detected in ESI negative MRM mode using a Q-TRAP triple-quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Darmstadt, Germany). Maximum response was tuned using a HPLC mix of the HPODE (9/13) and 13-HPOTE (Cayman Chemical) resulting in a spray voltage of −4500 V and a declustering potential of −20 V. Nitrogen was used for solvent drying (250 °C, 45 PSI) and as nebulizer gas at 25 PSI. Collision energy was set to −20 V, and collision gas pressure was 7 mPa (nitrogen). For 9-HPODE MRM of the ion *m*/*z* 311 → 185 was used for quantification and ion *m*/*z* 293 → 185 as qualifier at dwell times of 150 ms. For 13-HPODE ion *m*/*z* 311 → 113 and *m*/*z* 293 → 113 were used, respectively. Analyst software version 1.4.1 was used for data acquisition and processing.

Protein Determination. The protein content was determined according to the Bradford method with the Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions and using crystalline bovine serum albumin (BSA) as a standard.

RESULTS

Results were obtained in two consecutive years, and the data displayed the same experimental pattern. For this reason they are presented in each figure as a unique data set.

The antioxidant capacity of different fruit portions, evaluated as equivalent millimolar concentration of Trolox, clearly indicates that the parchment seed fraction of coffee berries showed the highest antioxidant activity in aqueous extractions, if compared with the pulp and green coffee (**Figure 1**). In addition, the results did not indicate a remarkable difference in the level of antioxidative compounds between OC and CC in each fruit fraction. Nevertheless, **Figure 2** shows that HPODE, determined by RP-HPLC in different coffee berry portions, was highest in the parchment seed when compared to the pulp and the green coffee. On the other hand, in the parchment seed and in the green coffee there was not a significant difference in the hydroperoxide level between OC and CC, whereas in the pulp the two treatments were different. In particular, the amount of hydroperoxides in the coffee pulp from OC was about 30% higher than that in CC.

It is well-known that fatty acid hydroperoxides can be produced by either enzymatic or nonenzymatic pathways. An

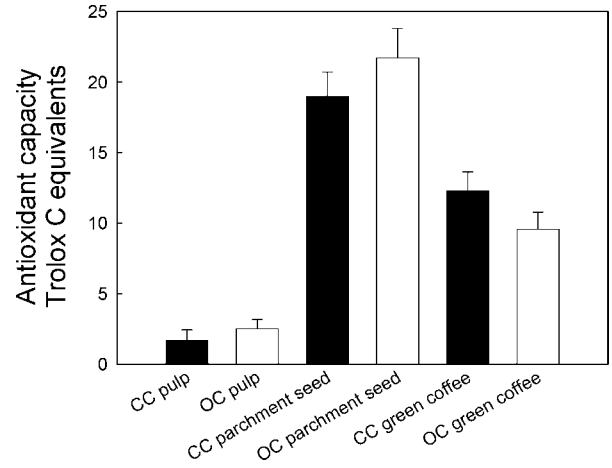

Figure 1. Antioxidant activity of aqueous extracts (phosphate buffer, pH 7.5), determined by crocin kinetic competition, in different portions of coffee berries from plants cultivated by organic (OC) and conventional (CC) techniques: (black bars) CC plants; (open bars) = OC plants. The results are expressed as concentration of Trolox C millimolar equivalents. Data are means of at least three replicates ± SD.

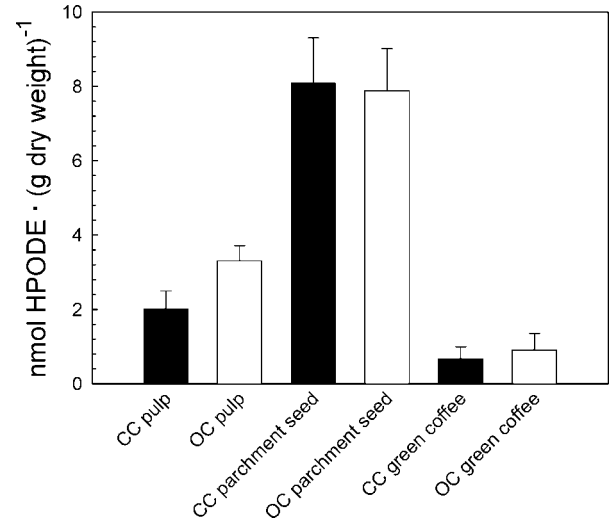

Figure 2. HPODE content evaluated by RP-HPLC in different coffee berry portions from plants cultivated by organic (OC) and conventional (CC) techniques: (black bars) CC plants; (open bars) OC plants. Data are means of at least three replicates ± SD.

immunochemical method was used to elucidate the possible presence of LOX in different coffee berry portions. **Figure 3** shows the SDS-PAGE of proteins derived from cold acetone extraction (panel **A**) and the corresponding Western blot (panel **B**). An Ab raised against pea LOX B cross-reacted with a protein of about 80 kDa, detectable only in the pulp fraction from both OC and CC. In addition, the presence of LOX was examined in different membrane fractions extracted from pulp berries (**Figure 4**). The results obtained from SDS-PAGE of proteins and the corresponding Western blot again showed cross-reactivity in either HMF (28000*g*) or LMF (100000*g*). Although HMF exhibited a stronger reaction, in comparison with LMF, a slight difference was detectable between the two cultivation techniques. No cross-reactivity was detected using soluble fractions obtained from all of the samples (results not shown).

These membrane fractions could be a mixture of several types of membranes (e.g., tonoplast, endoplasmic reticulum, plasma membrane) and organelles (e.g., mitochondria). Therefore, they were further characterized by marker enzyme activities to better

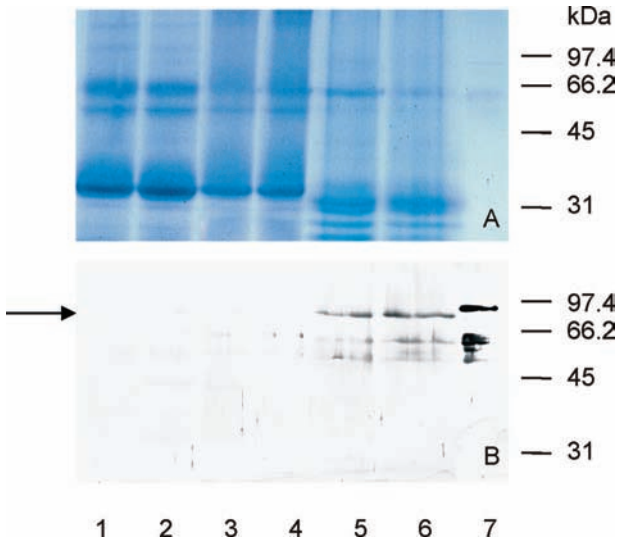

Figure 3. SDS-PAGE (**A**) and Western blot (**B**) of cold acetone extracts of coffee proteins obtained from different parts of the fruit. In panel **B** the arrow indicates cross-reactivity with pea anti-LOX B Ab. Figures below the plot represent (1) CC seeds, (2) OC seeds, (3) CC parchment seeds, (4) OC parchment seeds, (5) CC pulp, (6) OC pulp, (7) soybean LOX (Sigma).

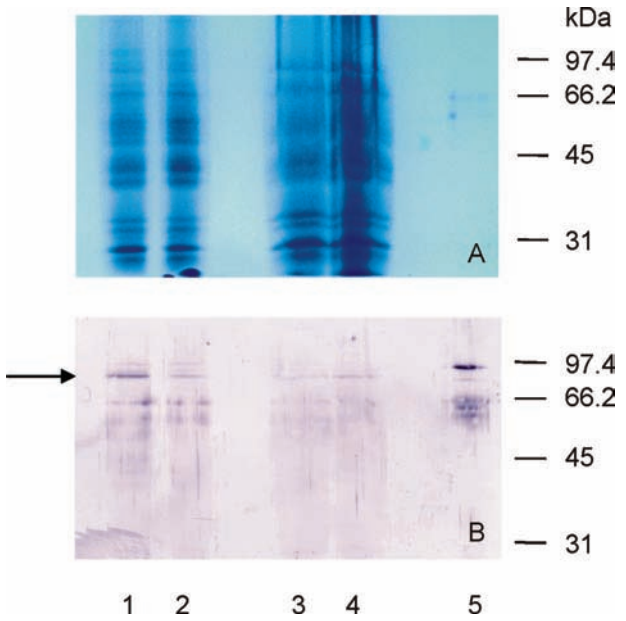

Figure 4. SDS-PAGE (**A**) and Western blot (**B**) of coffee proteins obtained from pulp HMF and LMF. The arrow in panel **B** indicates cross-reactivity with pea anti-LOX B Ab. Figures below the plot represent (1) conventional cultivation HMF, (2) organic cultivation HMF, (3) conventional cultivation LMF, (4) organic cultivation LMF, and (5) soybean LOX (Sigma).

clarify their purity and origin (**Table 1**). HMF showed a 49% level of vanadate-sensitive (plasma membrane marker enzyme) and a higher level (85%) of bafilomycin A_1-sensitive (tonoplast marker enzyme) ATPase activity, in comparison to LMF. Such a fraction thus contained plasma membrane vesicles (36%) and negligible levels of tonoplast membranes (2%). The activities of antimycin A-insensitive cytochrome *c* reductase (endoplasmic reticulum marker enzyme) were the same in both types of membranes; in contrast, cytochrome *c* oxidase (mitochondrial marker enzyme) was significantly higher in HMF than in LMF. Finally, glucose-6-phosphate dehydrogenase (plastid marker enzyme) activity was not detected in either HMF or LMF.

LOX activity was evaluated, in a pH range from 4.5 to 9.5, by linoleic acid-dependent conjugated diene formation, in pulp HMF and LMF, obtained from coffee plants grown in OC and CC (**Figure 5**). Such preliminary assays indicated that pulp HMF exhibited a different activity optimum: pH 8.0 and 5.5 for conventional and organic samples, respectively. On the contrary, the same activity peak (pH 8.0) for the pulp LMF extracted from both cultivations was detected.

The hydroperoxide formation in pulp HMF, determined by RP-HPLC, showed higher values in CC than in OC (**Figure 6A**). However, the addition of nordihydroguaiaretic acid (NDGA), a typical LOX inhibitor, caused a decrease in hydroperoxide production, and a comparable HPODE amount in both situations was left. This NDGA-insensitive amount could represent constitutive hydroperoxides, already present in the samples. Instead, the hydroperoxide production, due to enzymatic activity, was 31 and 41 nmol of HPODE · (min · mg of protein)$^{-1}$ for organic and conventional HMF, respectively. The presence of LOX activity in coffee pulp HMF was further confirmed by measuring hydroperoxide formation by linoleic acid-dependent conjugated diene formation assay (**Figure 6B**). LOX activity in HMF from OC and CC coffee pulps exhibited an initial rate of 268 and 352 nmol of HPODE · (min · mg of protein)$^{-1}$, respectively. The enzymatic activity associated with different pH values suggests the presence of at least two LOX isoforms in HMF. LOX activity was then shown in pulp LMF, both as HPODE formation (**Figure 6C**) and linoleic acid-dependent conjugated diene formation (**Figure 6D**). Although LMF was characterized by a lower activity with respect to pulp HMF, a substantial similarity was found between the samples from the two agronomical cultivations. First of all, the pH optimum for LOX activity was 8.0 in both cases; furthermore, hydroperoxide determination by RP-HPLC was 13 and 14 nmol of HPODE · (min · mg of protein ·mL)$^{-1}$ in LMF obtained from OC and CC plants, respectively. Such results were confirmed by linoleic acid-dependent conjugated diene formation, which showed an initial rate of 138 nmol of HPODE · (min · mg of protein)$^{-1}$ for OC and 150 nmol of HPODE · (min · mg of protein)$^{-1}$ for CC. A linolenic acid-dependent activity was also detected in all membrane fractions, but its rate was markedly lower than the linoleic acid-induced one (results not shown).

LOX activity products yielded in pulp HMF and obtained from CC and OC plants were further characterized by LC-MS analysis (**Figure 7**). This method allowed the determination of the different positional specificities of HPODE formation in CC and OC plants: both samples displayed a mixture of 9- and 13-HPODE with retention times of 26 and 25 min, respectively. The two isomers were identified by mass spectra analysis, and the relative amount was determined as summarized in **Table 2**. The amount of HPODE due to LOX activity, indicated as NDGA-sensitive HPODE production, was calculated by the difference between the whole amount of HPODE detected in the assay mixture and the quantity of HPODE evaluated in the presence of NDGA, indicated as NDGA-insensitive HPODE production. LOX activity present in OC pulp was characterized by a 9/13-HPODE ratio near 1:1, whereas 13-HPODE appeared to be the most represented in CC (1:10 ratio). Such clearly different patterns are in agreement with the presence of different isoenzymes. Instead, the regiospecificity measured in samples treated with NDGA showed the same ratio (1:3) in both CC and OC coffee pulp membranes.

DISCUSSION

LOXs are enzymes widespread in several plant organisms, recognized to be expressed through different isoenzymes, which

Table 1. Marker Enzyme Activities in HMF and LMF Extracted from Coffee Berry Pulps[a]

	HMF		LMF	
	nmol · (mg of protein · min)$^{-1}$	% of control	nmol · (mg of protein · min)$^{-1}$	% of control
ATPase				
1 mM ATP (control)	208 ± 32	100	58 ± 15	100
+ 200 μM vanadate	106 ± 21	51	37 ± 12	64
+ 1.5 μM bafilomycin A_1	32 ± 9	15	57 ± 19	98
antimycin A-insensitive cytochrome *c* reductase	143 ± 27		143 ± 31	
cytochrome *c* oxidase	1381 ± 95		524 ± 74	
glucose-6-phosphate dehydrogenase	ND		ND	

[a] Data are means of at least three replicates ± SD. ND, not detected.

perform specific and peculiar functions during various developmental stages. In particular, it is well-known that the up-regulation of some LOXs in correspondence to seed and fruit maturation is counterbalanced by the down-regulation of other isoenzymes (*30*, *31*).

In some cases ex novo synthesized LOX isoenzymes could be ascribed to "vegetative storage proteins", which constitute a nitrogen storage especially in *Fabaceae* plants (*32*). Moreover, some of these isoenzymes are responsible for the degradation of lipid membranes during senescence and the biosynthesis of flavor compounds and defense metabolites in ripening fruits (*33*).

In this paper we show evidence for the presence of LOXs in coffee pulp obtained from mature fruits of plants grown in conventional and organic cultivations. These two different agronomical techniques did not influence the whole antioxidant capacity of coffee berries, measured as Trolox millimolar equivalents, but by analyzing different fruit portions a significant distinction could be evidenced. Among these fractions, parchment seed showed the highest oxidative protection; nevertheless, it also exhibited the highest HPODE content, if compared to pulp and green coffee portion. These apparently controversial results could be explained, however, by taking into account the huge amount of polyphenolic substances (chlorogenic acid, in particular) present in parchment seed (*34*). Such a high concentration of antioxidant compounds could exert an opposite effect from that generally expected, increasing the alteration of the lipid fraction stored in the seed (*35*). Furthermore, it could be stressed that HPODE production, evaluated in green coffee and parchment seed, was not caused by lipoxygenase activity,

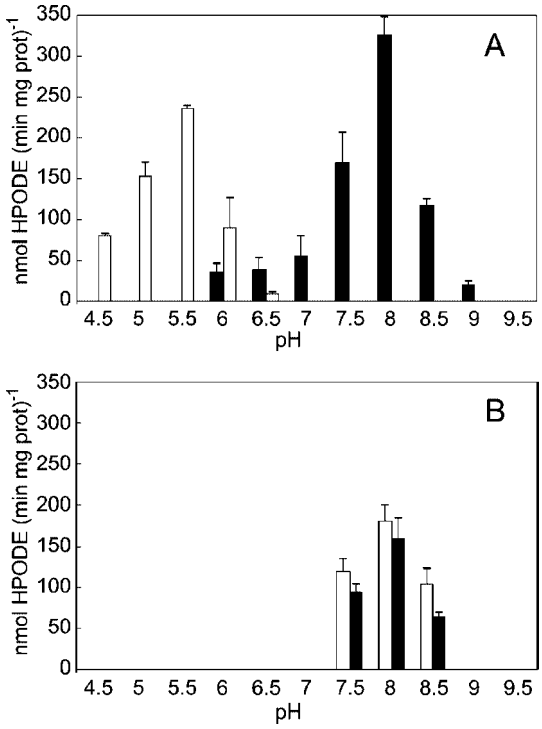

Figure 5. Effect of pH on linoleic acid-dependent LOX activity, measured by conjugated diene formation in HMF (**A**) and LMF (**B**). Black bars are referred to CC samples plus 250 μM linoleic acid, whereas open bars are referred to OC samples plus 250 μM linoleic acid. Data are means of at least three replicates ± SD.

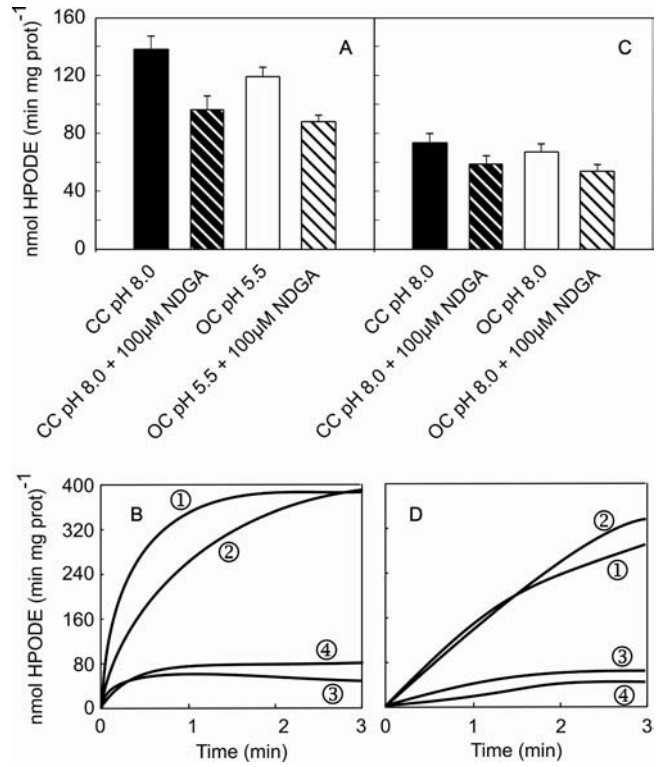

Figure 6. Lipoxygenase activity measured by RP-HPLC evaluation of HPODE produced (**A**, **C**) and by conjugated diene formation (**B**, **D**). Panels **A** and **B** are related to coffee pulp HMF, whereas panels **C** and **D** refer to coffee pulp LMF. (**A**, **C**) black bars, CC samples; open bars, OC samples; black slashed bars, 250 μM linoleic acid; open slashed bars, 250 μM linoleic acid plus 100 μM NDGA. Data are means of at least three replicates ± SD. (**B**) trace 1, CC samples plus 250 μM linoleic acid, pH 8.0; trace 2, OC samples plus 250 μM linoleic acid, pH 5.5; trace 3, CC samples plus 250 μM linoleic acid and 100 μM NDGA, pH 8.0; trace 4, OC samples plus 250 μM linoleic acid and 100 μM NDGA, pH 5.5. (**D**) trace 1, CC samples plus 250 μM linoleic acid, pH 8.0; trace 2, OC samples plus 250 μM linoleic acid, pH 8.0; trace 3, CC samples plus 250 μM linoleic acid and 100 μM NDGA, pH 8.0; trace 4, OC samples plus 250 μM linoleic acid and 100 μM NDGA, pH 8.0. Traces are representative of at least three replicates.

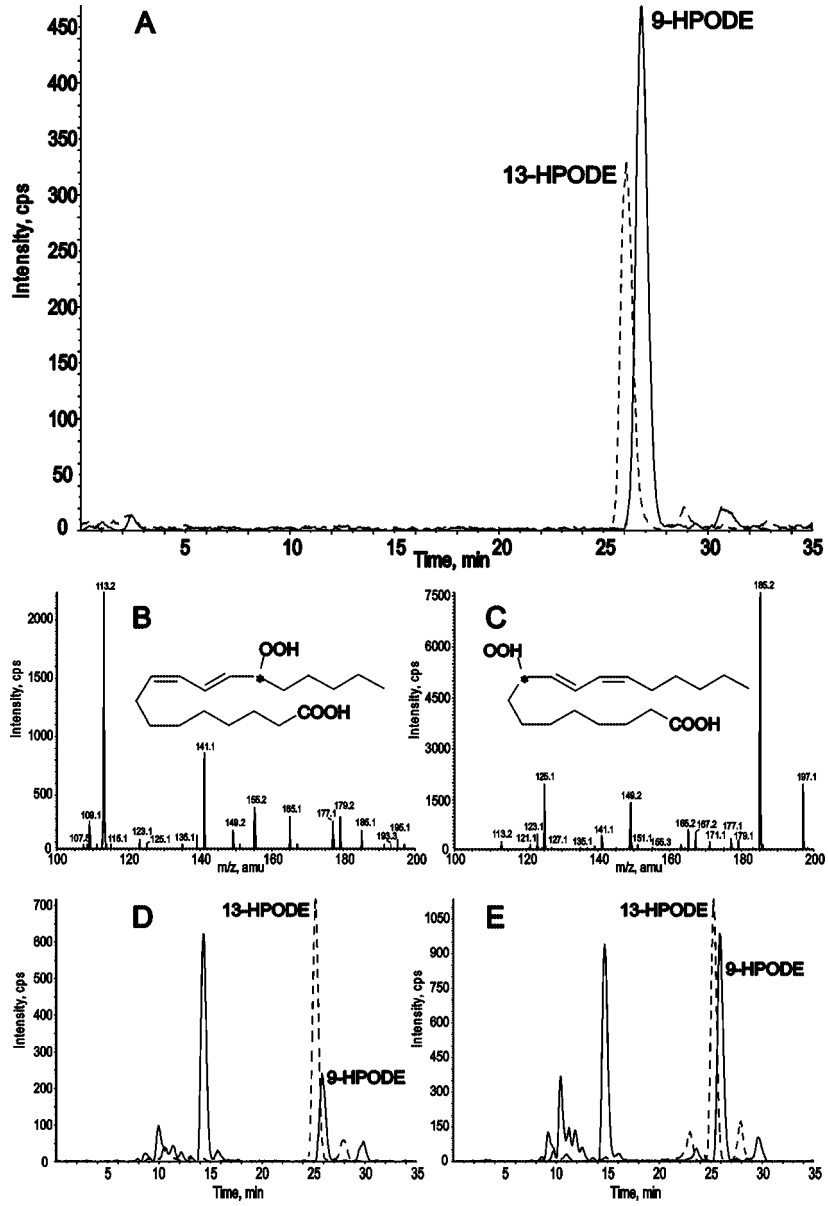

Figure 7. LC-MS determination of regiospecificity in HPODEs produced by lipoxygenase activity in pulp HMF extracted both from CC and OC plants: (**A**) RP LC-MS/MS (ESI^-) chromatogram of regioisomers 9/13-HPODE standard mass transition *m/z* 311 → 185 for 9-HPODE and *m/z* 311 → 113 for 13-HPODE. (**B**) MS^3 (311/293) spectrum of 13-HPODE. (**C**) MS^3 (311/293) spectrum of 9-HPODE. Panels **C** and **D** show LC-MS/MS MRM chromatograms for HMF LOX products from CC and OC coffee pulp, respectively.

Table 2. HPODE Isomer Distribution in Coffee Pulp HMF Obtained from OC or CC Coffee Plants[a]

	CC coffee		OC coffee	
regiospecificity	13-HPODE (%)	9-HPODE (%)	13-HPODE (%)	9-HPODE (%)
NDGA-sensitive HPODE production	91.9	8.1	55.1	44.9
NDGA-insensitive HPODE production	74.2	25.8	74.3	25.7

[a] Results are expressed as percentage of total HPODE amount determined either in NDGA-sensitive aliquot and in NDGA-insensitive fraction.

due to the lack of cross-reactivity in such portions with an Ab raised against pea LOX B. It is, therefore, reasonable to suggest the presence of previously formed HPODE, derived from the activity of no longer present LOXs or built up in the seed together with lipid stores. Given the protective role of the parchment coat, which increases the germinating power of seed with respect to the green coffee, it could be suggested that the presence of high HPODE content may be a further defense factor useful in preventing biotic stresses. On the other hand, no significant difference could be detected in HPODE content in the fruit fractions, when the two agronomical techniques were compared, except for the pulp one, which exhibited a higher concentration of HPODE in the OC fruits.

This observation is consistent with immunochemical and biochemical data, showing the presence of a LOX enzyme, with an apparent molecular mass of about 80 kDa, the protein of which reacted against the pea anti-LOX B Ab. Such cross-reactivity with this type of antibody is not surprising (*29*, *36*), due to the fact that LOXs have a highly conserved sequence. The enzyme was associated with low and heavy membrane

fractions from both CC and OC pulps. The amount of LOX was higher in HMF than in LMF, and this observation was confirmed by measurement of the catalytic activity. In agreement with recent findings of Leone et al. (*33*), the presence of LOX activity in HMF of senescing fruits could be explained with LOX association with lipid–protein aggregates. On the other hand, it is already known that LOX could be linked to plasma membranes, lipid bodies, chloroplasts, tonoplast, and mitochondria (*37*). The presence of LOX in these organelles could be related to membrane alterations occurring during biotic stress, senescence, and wounding, as well as in membrane remodeling during germination. Therefore, LOX evidenced in coffee pulp membranes could be correlated with these physiological functions.

As shown in **Table 1**, HMF contained plasma membranes and endoplasmic reticulum, but was mainly enriched in tonoplast vesicles and mitochondria. LMF also contained plasma membranes and endoplasmic reticulum, whereas it was devoid of tonoplast vesicles and possessed a low contamination of mitochondria. Therefore, it is possible to speculate that LOX activity at pH 8.0 may be associated with plasma membranes and/or endoplasmic reticulum, being present in both fractions (HMF and LMF) from CC plants. LOXs from OC plants with activity maximum at pH 5.5 (the same value present in vacuolar compartment) and 8.0 could be present in tonoplast and in a still unknown membrane/compartment, respectively. In any case, more work is needed to better characterize the presence of this LOX in such types of membranes.

Interestingly, LOX activity was characterized by different pH optima in HMF obtained from CC or OC, indicating the presence of different isoenzymes. Such an assumption was corroborated by the kinetic behavior of the alkaline LOX (pH 8.0) in CC pulps, which showed an immediate increase of hydroperoxide production followed by a quick achievement of steady-state conditions, in this resembling the behavior of the well-known alkaline soybean LOX1 (*28*). On the contrary, acidic LOX activity (pH 5.5) in OC pulps displayed a more constant catalytic rate over time.

The isoenzymes were characterized by HPLC analysis, showing that acidic LOX was a 13/9-LOX, because of the 1:1 ratio of its products (9-HPODE and 13-HPODE). On the contrary, the alkaline one was mainly a 13-LOX, because the 13-HPODE represented about 90% of the HPODE production due to enzyme activity. The presence of a LOX activity was further confirmed by the comparison between the NDGA-sensitive and NDGA-insensitive HPODE production. The latter amount of lipid peroxidation could be considered not directly linked to LOX activity; in this case the 9/13-HPODE ratio was 1:3 and it was the same in both, being significantly different from those detected following enzymatic activity.

The effect of the cultivation method on LOX expression was not detectable in LMF, where both CC and OC pulp fractions displayed similar LOX activities, characterized by the same pH optimum around 8.0. The same pattern of LOX expression was instead different in HMF, because in CC only alkaline LOXs, linked to plasma membranes and/or endoplasmic reticulum, seemed to be expressed; on the contrary, in OC an acidic LOX, associated with tonoplast, and another LOX of unknown origin appeared to be expressed.

On the other hand, it is widely accepted that biotic and abiotic stresses could induce acidic LOX isoenzyme expression (*5*, *38*, *39*). Organic cultivation has a lower environmental impact, but offers a weaker protection, due to the absence of pesticides and chemical auxiliary products. Owing to those modified conditions, plants, in response to pathogen attack, are able to induce a de novo synthesis of specific acidic LOXs, which are responsible for oxylipin production, a large family of LOX metabolites involved in signaling defense and antimicrobial activity (*40*).

These results are in agreement with others showing a role for the acidic LOX in response to stress and, in particular, with the induction of 13/9-LOX in several physiological responses caused by plant–pathogen interactions (*41–43*). We, hence, hypothesize that such acidic LOX represents a natural biochemical defense, particularly in organic cultivated coffee plants, which are more susceptible to pathogen infection lacking protection supplied by chemical auxiliary products. Similarly to other species, also in coffee has been demonstrated the existence of a relationship between stress occurrence and the induction of an acidic LOX in the fruit. Moreover, the products of the oxylipin pathway synthesized during coffee fruit maturation could also have great interest in relation to the shelf life of green coffee and to the final coffee aroma. In particular, LOXs could represent the starting point for additional enzymatic and nonenzymatic mechanism, still largely unknown. Further studies are needed to elucidate better the metabolic pathway as well as practical implications in quality, more specifically on the coffee organoleptic profile.

ABBREVIATIONS USED

Ab, antibody; BSA, bovine serum albumin; BHT, butyl hydroxytoluene; CC, conventional cultivation; DTE, 1,4-dithioerythritol; (ESI^-) electron spray negative ionization, liquid chromatography; HMF, heavy membrane fraction; HPODE, hydroperoxyoctadecadienoic acid; HPOTE, hydroperoxyoctadecatrienoic acid; LMF, light membrane fraction; MRM, multiple reaction monitoring; NDGA, nordihydroguaiaretic acid; OC, organic cultivation; ODS, octadecylsilane; PSI, pounds per square inch; PVPP, polyvinylpolypyrrolidone; RP LC-MS/MS, reverse phase liquid chromatography–tandem mass spectrometry; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UV–vis, ultraviolet–visible spectrophotometry.

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