RESEARCH PAPER

9-Lipoxygenase metabolism is involved in the almond/Aspergillus carbonarius interaction

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Abstract

Phyto-oxylipins are a group of biologically active molecules that play an important role in plant defence. Their production begins with the oxygenation of polyunsaturated fatty acids by lipoxygenases (LOX) to form 9- or 13-hydroperoxides that are substrates for several enzymes involved in the synthesis of final oxylipins, which can act as signal molecules and/or direct antimicrobials. In the present work, the response of the 9-LOX pathway in the almond/Aspergillus carbonarius (producer of ochratoxin A) interaction was studied. Both LOX gene expression and activity are up-regulated over the course of fungal infection in immature and mature almonds. The biochemical characterization of major LOX and hydroperoxide lyase (HPL) isoforms indicated that 9-LOX metabolism is specifically induced by A. carbonarius. Lipid peroxidation profiling showed that, in infected immature almonds, enzymatically produced 9-hydro(peroxy) fatty acids (HFAs) were the main HFAs and are further metabolized by HPL into C9-aldehydes. Both HPL gene expression and C9-aldehydes increased over the course of fungal infection. In mature almonds infected with A. carbonarius, levels of LOX expression and activity were lower than those found in immature seeds, and 9-HFA represented the minority of total HFA, which consisted of mostly 13- and non-enzymatically produced HFA. In these experimental conditions, no volatile aldehydes were recorded from these samples, even though HPL was up-regulated in infected mature almonds. The effects on the growth of A. carbonarius of the aldehydes produced by these enzymes were also tested in vitro. Results reported here led to the proposal that, in almond seed, the association of 9-LOX and HPL has an important role in seed defence mechanism against pathogen infection.

Key words: Almond, Aspergillus carbonarius, lipoxygenase, oxylipins, plant/microbe interaction, volatile aldehydes.

Introduction

Oxylipins are a group of biologically active molecules produced by the oxidative metabolism of polyunsaturated fatty acids (PUFAs). In plant cells, these compounds are believed to play an important role in several physiological events including plant defence, development, and senescence. Most of the phyto-oxylipins so far identified are synthesized via the lipoxygenase (LOX) pathway. In the first enzymatic step of this pathway, LOX catalyse the hydroperoxidation of PUFAs containing a cis,cis,1,4-pentadiene moiety, such as linoleic (C18:2) or linolenic acid (C18:3). Since oxygen insertion is stereo-specific, only 9S- or 13S- hydroperoxides can be produced from these substrates, and plant LOXs are commonly referred as 9- or 13-LOX (reviewed in Feussner and Wasternack, 2002; Porta and Rocha-Sosa, 2002). PUFA hydroperoxides are rapidly converted into other oxylipins by the action of other enzymes downstream in the pathway, including hydroperoxide lyase (HPL), allene oxide

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Abbreviations: LOX, lipoxygenase; HFAs, hydro(peroxy) fatty acids; HPL, hydroperoxide lyase; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; RP-HPLC, reverse-phase HPLC; SP-HPLC, straight-phase-HPLC; SPME, solid-phase micro-extraction.
synthase (AOS), divinyl ether synthase (DES), epoxy alcohol synthase (EAS), and peroxidase. The products of oxylipin metabolism include jasmonates, octadecanoids, 6- and 9-carbon aldehydes, o xoacid and divinyl ether fatty acids, bioactive compounds that act either in defence signalling or as direct antimicrobials (Blee, 2002; Feussner and Wasternack, 2002).

As far as seed LOXs are concerned, their physiological role is still largely unclear. In some species such as maize (L2-LOX; Jensen et al., 1997), peanut (PnLOX1; Burow et al., 2000), and almond (PdLOX1; Mita et al., 2001) lox genes are expressed in immature seed when the synthesis of storage proteins and lipids is not yet completed. In peanut (Burow et al., 2000) and maize (Wilson et al., 2001) these specific isoforms were also up-regulated during Aspergillus flavus infection. Recently, Tsitsigiannis et al. (2005) reported the sequence of two other lox genes (PnLOX2, PnLOX3) highly expressed in mature peanut seed, but which, differently from PnLOX1, PnLOX2, and PnLOX3s are both repressed during fungal infection (Tsitsigiannis et al., 2005a). Moreover, Calvo et al. (1999) reported that PUFA hydroperoxides produced by LOX can affect mycotoxin biosynthesis in Aspergillus spp., mimicking the effects of some sporulation factors and proposed that 9S- and 13S-hydroperoxides could have different effects on mycotoxin production since 9S-stimulated whereas 13S-hydroperoxides inhibited mycotoxin production. On the basis of these results, seed LOXs could have an important role in seed/fungi interaction and oxylipins produced by this pathway could be important molecular mediators in this interaction.

In previous studies (Mita et al., 2001; Santino et al., 2005), it was shown that in almond seed a 9-lox gene is specifically expressed in immature seed, but the physiological significance of this metabolism is still largely unknown. In the present work, the response of the 9-LOX pathway to Aspergillus carbonarius infestation of almond seeds at different developmental stages was studied. The biochemical characterization of major LOX isoforms confirms that 9-LOX metabolism is specifically induced by A. carbonarius. Hydro(peroxy) fatty acids (HFAs) were monitored in control and infected seeds over the course of fungal infection. Lipid peroxidation analyses suggested that, in infected almond seeds, 9-hydroperoxides could be converted into other antifungal oxylipins. To verify this hypothesis, the response of the hpl gene to fungal infection was studied and C9-aldehydes were monitored over the course of infection. The fungicidal properties of C9-aldehydes, together with the concomitant induction of both LOX and HPL responsible for their biosynthesis and the increased amount of C9-aldehydes during fungal infection, have prompted the suggestion that both enzymes play an important role in seed defence mechanism.

Materials and methods

Plant material

Immature (30 d and 45 d after flowering) and mature seeds of almond (Prunus dulcis, cultivar Scorza Verde) were harvested from the experimental field of the Istituto Sperimentale Frutticoltura, Caserta, Italy.

Microbial strains

Aspergillus carbonarius (strain ITEM 4722, producer of ochratoxin A) was used to infect mature almonds. Almonds were surface-sterilized (Burow et al., 2000) and immersed in 10 ml of 1×10⁶ spore suspension for 30 min. Control seeds were incubated with water. All seeds were incubated at 30 °C in Petri dishes lined with moist filter paper. Samples were collected at the time points indicated.

Protein extraction and LOX activity in almond seed

Microsomal and soluble protein samples were extracted from almond seeds as previously reported by Mita et al. (2001). LOX activity was assayed polarographically with a Clark O₂ electrode using linoleic acid as substrate, as previously reported by Santino et al. (2005). The reaction mixture (1.0 ml) consisted of 100 mM sodium phosphate buffer pH 6.0 containing 0.3 mM substrate and different amounts of protein samples. Decrease in O₂ concentration was monitored for 3 min and the enzymatic activity was calculated from the initial rate of O₂ uptake. One unit of LOX activity corresponded to the uptake of 1 nmol of O₂ min⁻¹. LOX activity was also assayed spectrophotometrically, monitoring the increase in absorbance at 234 nm for the conjugated-diene structures as previously reported by Santino et al. (2005).

HPLC analysis of hydro(peroxy) fatty acids in almond seed and specificity of almond LOX isoforms

PUFA hydroperoxides and other oxylipins were extracted from infected and control almond seeds after NaB₄H₄ reduction and saponification as previously reported by Montillet et al. (2004). The only exception was that fatty acids and oxylipins recovered after the saponification procedure were extracted with 2 vols of chloroform/methanol (2:1, v/v), dried, and resuspended in methanol:water:acetic acid (85:15:0.1 by vol.). An aliquot of the extracts was submitted to RP-HPLC with a C₁₈ Ultrasphere column (Beckman, 25 cm, 5 µ particle size) with a solvent system of methanol:water:acetic acid (85:15:0.1 by vol.). Detection of HFAs was carried out recording the absorbance at 234 nm (indicating the conjugated diene system). Analysis of HFA isomers was carried out as previously described (Mita et al., 2001) with a Silica Ultrasphere Column (Beckman, 0.46×25 cm) equilibrated with n-hexane:2-propanol:acetic acid (100:2:0.1 by vol). The enantiomer composition was carried out by chiral-phase HPLC on a Chiralcel OB column (Daicel Chem., Industries, 0.46×25 cm, 5 µ particle size) with a solvent system of hexane:2-propanol:acetic acid (100:5:0.1 by vol.) and a flow rate of 1 ml min⁻¹.

The analysis of product specificity of major LOX isoforms, detected in soluble protein samples and microsomal samples from control and infected almonds, was carried out as previously reported by Santino et al. (2003) by incubating protein samples for 30 min in 1 ml of 0.1 M sodium phosphate buffer pH 6.0 containing linoleic or linolenic acids (0.3 mM final concentration). The reaction products were reduced with sodium borohydride and separated by RP-HPLC and SP-HPLC.

Authentic standards of 9- and 13-hydroperoxides of linoleic and linolenic acids were purchased from Larodan (Malmö, Sweden).
The calibration curves (five measurements points) for these compounds were established.

HPLC analyses were carried out on an Agilent 1100 series HPLC system coupled to a diode array detector. All compounds were identified by their characteristic UV spectra and compared with those of authentic standards.

**Identification of HPL products**

The endogenous volatiles present in mature and immature almonds were characterized. A 2 g aliquot of seeds was ground to a fine powder in liquid nitrogen, sealed, and incubated first at 37 °C for 30 min and then at 55 °C for 10 min in a stirred 20 ml reaction tube closed with a septum. Headspace compounds were trapped by SPME (75 μm polydimethylsiloxane fibre, Supelco) and desorbed at 230 °C for 3 min in the injection port of a GC-MS (QP5050, Shimadzu; equipped with an AOC-5000 auto injector) column (Restek Stabilwax Crossbond Carbowax-PEG capillary column 30 m×0.32 mm). The temperature program used was as follows: 40 °C for 5 min; 40–200 °C, 8 °C min⁻¹; 200–250 °C, 10 °C min⁻¹; 250 °C, 2 min. Identification of products was by retention time compared with authentic standards and from mass spectra of the standards and the HPL products. Calibration curves (five measurement points) for C9-aldehydes and alcohols were established.

Authenticated, high-purity (>95%) standards of (2E)-nonenal and (2E,6Z)-nonadienal, nonanol; nonyl alcohol; (2E)-nonen-1-ol (Aldrich Chemical Co.) were used to establish calibration curves. (five measurement points). For each concentration, standard compounds were placed individually in a capillary tube. Capillary tubes containing the different standard compounds were placed in a 20 ml reaction tube closed with a septum to create a five-component mixture. The vials were held at 37 °C for 30 min before trapping volatile compounds by solid-phase micro-extraction (SPME).

**RNA extraction and real-time reverse transcription (RT)-PCR analysis**

Total RNA was isolated from 100 mg of almond seeds at different developmental stages using the RNeasy plant mini kit (Qiagen) following the manufacturer’s instructions. cDNA was prepared using 2 μg of total RNA, oligo(dT)₁₈ primer, and the Superscript II Polymerase (Invitrogen) according to the manufacturer's instructions. cDNA was used as template in real-time PCR experiments with oligonucleotides designed on the basis of the LOX and HPL gene sequences using the Beacon designer 2.0 software (Bio-Rad) according to the manufacturer’s instructions. cDNA was used as template in real-time PCR experiments with oligonucleotides designed on the basis of the LOX and HPL gene sequences using the Beacon designer 2.0 software (Bio-Rad) according to the manufacturer’s instructions. cDNA was used as template in real-time PCR experiments with oligonucleotides designed on the basis of the LOX and HPL gene sequences using the Beacon designer 2.0 software (Bio-Rad) according to the manufacturer’s instructions. cDNA was used as template in real-time PCR experiments with oligonucleotides designed on the basis of the LOX and HPL gene sequences using the Beacon designer 2.0 software (Bio-Rad). Primers and TaqMan probes are shown in Table 1. The oligonucleotides used for actin expression analysis were designed on the basis of a partial almond actin cDNA previously characterized (accession no. AM491134).

**Determination of minimum inhibitory concentration (MIC)**

The volatile compounds (2E)-nonenal, (2E,6Z)-nonadienal, hexanal, and (2E)-hexenal (purchased from Aldrich Chemical Co.) were screened in vitro for their ability to control the growth of A. carbonarius (strain ITEM 4722, producer of ochratoxin A). Agar plugs were collected from 3-d-old cultures and transferred onto the centre of freshly prepared PDA (potato dextrose agar) media in 9 cm plastic Petri dishes. The Petri dishes were then inverted and 6 cm Whatman No. 1 filter papers were attached to the inner surface of the lids. The filter paper was impregnated with different volumes (from 1 μl to 1 ml per dish) of the tested compounds, then the Petri dishes were immediately sealed and incubated at 25 °C in the dark.

Radial growth was measured daily for 7 d following inoculation. Each treatment was replicated three times. The minimum inhibitory concentration (MIC) for each fungus was determined.

**Results**

**Induction of lox during the almond/Aspergillus interaction**

It has previously been shown that 9-LOX metabolism accounts for most of the oxylipins produced during almond (P. dulcis) seed development (Mita et al., 2001; Santino et al., 2005). To verify a possible involvement of the almond lox gene (PdLOX1) in the almond/A. carbonarius interaction, immature (collected at 30 d and 45 d after flowering; stage I and II, respectively) and mature almonds (90 d after flowering; stage III) were inoculated with a spore suspension of A. carbonarius. Lox gene expression was studied by real-time RT-PCR using two specific oligonucleotides (Table 1) designed on the basis of the PdLOX1 gene previously reported (accession number AJ404331). Actin was used as an endogenous control, since its expression levels were almost constant across the control, since its expression levels were almost constant across the control, since its expression levels were almost constant across the control, since its expression levels were almost constant across the control, since its expression levels were almost constant across the control, since its expression levels were almost constant across the control, since its expression levels were almost constant across the control, since its expression levels were almost constant across the control, since its expression levels were almost constant across the control.

| Table 1. Oligonucleotides used in real-time RT-PCR to study LOX and HPL gene expression |
|--------------------------------------|--------------------------------------|
| LOX sense primer                     | 5’-CGT TAA CAA GAC ATT TCT CCC AAG T-3’ |
| LOX antisense primer                 | 5’-CTC CCT TCC CAT CTC TCA A-3’ |
| LOX TaqMan                           | 5’-FAM- ACA CGG TTG CCA CTG CGA AAA TAC AGA-3’TAM |
| HPL sense primer                     | 5’-CTA CCG CGT CTG CGC TTA TC-3’ |
| HPL antisense primer                 | 5’-GGG CTT CCA AGT TGA GAA ACA TG-3’ |
| HPL TaqMan                           | 5’-FAM- CCT TCG GAG CCC AAC CAC GCC AC-3’TAM |
| Actin sense primer                   | 5’-ACG CAG ATC ATG TTT GAG ACC TT-3’ |
| Actin antisense primer               | 5’-TAA CCT TCA TAG ATT GGC ACA GTG T-3’ |
| Actin TaqMan                         | 5’-FAM- ACC ATC ACC AGA GTC CAG CAC AAT ACC A-3’TAM |

As expected, expression of the almond PdLOX1 gene in control samples was only found in immature seeds (stages I and II; Fig. 1) and higher levels were recorded from stage I seeds (see also Mita et al., 2001; Santino et al., 2005). In infected samples, the levels of PdLOX1 transcripts were significantly higher than those recorded from control samples. Similar levels of PdLOX1 expression were recorded from stage I and II seeds (compare I stage 72 h and II stage 48 h); whereas lower levels (about seven times) were recorded from mature seeds (Fig. 1).

LOX activity was also monitored in control and infected seeds. LOX activity was assayed in the soluble and microsomal protein fractions from control and infected
seeds. A very low enzymatic activity was detected in the microsomal fraction purified from both control and infected seeds, with no significant differences in these samples (data not shown).

As reported in Fig. 1, LOX activity in the soluble protein fraction from infected seeds showed a similar trend in all samples and peaked at 48 h after inoculation. At these times, LOX activity was significantly higher than that calculated from control seeds. The product specificity of LOX isoforms induced in control and in infected almond seeds was studied since no hydroperoxide-hydrolysing activity was found in the soluble protein fraction from almond seeds (Mita et al., 2001, 2005). Figure 2 shows that a 13-LOX is clearly present in the soluble protein fraction from control seeds. 13-HFAs account for about 70% and 60% of total hydroperoxides produced by LOXs in control samples from mature and immature seeds, respectively (Fig. 2). In infected seeds at 24 h after inoculation a similar ratio of 13/9 hydroperoxides was found, but starting from 48 h, 9-HFA becomes the major hydroperoxide produced by almond LOXs from mature and immature seeds (Fig. 2), indicating that 9-LOX isoforms are up-regulated upon infection.

**Lipid peroxidation during fungal infection**

PUFA hydroperoxides, released after alkaline hydrolysis from total lipids of control and *A. carbonarius*-infected almonds, were reduced with sodium borohydride to the corresponding HFAs and analysed by a combination of RP, SP, and chiral-phase HPLC. As expected, HFAs derived from linoleic acid were more abundant than HFAs from linolenic acid. They accounted for about 60–70% of the total HFAs detected in immature almonds and about 80–90% in mature seeds. Together with 9- and 13-HFAs, other hydroperoxides were identified after SP-HPLC.

They showed retention times similar to those previously reported for 12- and 16-HFAs, known reactive oxygen species (ROS)-mediated peroxidation markers in *Arabidopsis thaliana* leaves (Montillet et al., 2004). Like these, 12- and 16-HFAs characterized from almond seeds...
showed a racemic composition (50/50 R/S ratio; data not shown) and, also in the current work, were collectively indicated as ROS-mediated HFAs.

The quantitative analysis of HFAs from stage II and III seeds is reported in Fig 3. In immature seeds, the highest levels of total HFAs were found in infected seeds at 24 h (Fig. 3). At that time, most of HFAs were represented by 9-HFA. After 24 h, both total and 9-HFAs diminished throughout infection. Interestingly, in control samples both total and 9-HFAs increased during treatment and peaked at 72 h (Fig. 3). An analysis of the composition of the enantiomers carried out on 9- and 13-HFAs indicated a prevalence of (S) enantiomers, as expected for hydroperoxides produced by specific LOXs (data not shown). A very low amount of ROS-mediated HFAs was recorded from control and infected seeds (Fig. 3).

In mature seeds, lower amounts of HFAs were found (Fig. 3). They were mostly represented by 13-HFA which showed a prevalence of the R enantiomer (data not shown). As far as 9-HFA is concerned, the amounts were very low.

Fig. 2. Analysis of hydro(peroxy) fatty acids (HFAs) produced by almond lipoxygenases from control and Aspergillus-infected almond seeds. Soluble protein fractions from stage II (45 DAF) and III (90 DAF) almonds were incubated in the presence of linoleic acid and the hydroperoxides produced separated by RP- and normal-phase HPLC as described in the text. Data represent the relative proportions (%) of each isomer. The data represent replicas of three experiments.

Fig. 3. HPLC analysis of hydro(peroxy) fatty acids (HFAs) in almond in response to A. carbonarius infection. Lipid peroxidation profiling at 24, 48, and 72 h in immature (stage II) and mature (stage III) control and infected almonds. The data represent means from three experiments.
and accumulated amounts similar to ROS-mediated HFAs. Anyway, they were the only HFAs showing a prevalence of S enantiomers (data not shown).

*Induction of hpl during the almond/Aspergillus interaction*

The results presented here have shown that, in almond seeds, the overexpression of the PdLOX1 gene and the increase of LOX activity did not parallel HFA accumulation during *A. carbonarius* infection. This was more evident in immature almonds where both total and 9-HFA diminished during infection. To verify the presence of volatile aldehydes and alcohols produced by the LOX pathway, volatile compounds from infected almonds were analysed using an SPME method combined with gas chromatography-mass spectrometry (GC-MS). No volatile C6/C9-aldehydes were detected in either control or infected mature almonds, probably because they were beneath the levels of detection (Fig. 4A). In contrast, both C9-aldehydes and alcohols produced by the LOX pathway were detected in immature almonds (Fig. 4B). Quantitative analysis carried out on C9-aldehydes and alcohols showed an increase of (2E)-nonenal and (2E,6Z)-nonadienal during fungal infections (Fig. 5).

To verify the responsiveness of the almond 9-HPL gene (PdHPL1; accession number AJ578748) to *A. carbonarius* infection, real-time RT-PCR experiments were carried out using two oligonucleotides (Table 1) specific for PdHPL1. As shown in Fig. 6, infected seeds always showed a significantly higher expression of the hpl gene than that recorded from control samples.

*Fungicidal activities of C9-aldehydes towards toxigenic fungi*

To verify the fungicidal properties of C9-aldehydes, the MICs of (2E)-nonenal and (2E,6Z)-nonadienal were evaluated, which have been characterized as major products of the almond LOX/HPL metabolism, were evaluated. The biological activity of these molecules was compared with that of hexanal and (2E)-hexenal, major compounds produced by 13-HPL and which have been reported to possess antifungal and antimicrobial activities (Croft *et al.*, 1993; Gardini *et al.*, 2001; Lanciotti *et al.*, 2003; Prost *et al.*, 2005). As reported in Table 2 and in

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**Fig. 4.** GC-MS analysis of volatile compounds detected in infected mature almonds (A) and in immature seeds (B). About 2 g of mature almonds 2 d after inoculation with *A. carbonarius* (A) and 2 g of immature almonds (B) were ground in liquid nitrogen, sealed and incubated first at 37 °C for 30 min and then at 55 °C for 10 min. Headspace compounds were trapped by SPME and analysed by GC-MS as described. Identification of products was by retention time of authentic standards and the comparison of the mass spectra of identified compounds with those of standards. The minor peak eluted at 23.4 min in (A) was identified as glutaraldehyde. Inset in (B) shows the chromatogram relative to a mixture of the following standards: 1, nonanol; 2, (2E)-nonenal; 3, (2E, 6Z)-nonadienal; 4, nonyl alcohol; 5, (2E)-nonen-1-ol.
Fig. 7A and B, both (2E)-nonenal and (2E,6Z)-nonadienal showed a marked inhibitory effect on the growth of Aspergillus carbonarius, with a MIC even lower than that calculated for hexanal and (2E)-hexenal [analysis of variance (ANOVA), $F_{4,25}=257.51, P=0.01$]. Similar values of MIC were recorded with another toxigenic fungus, Fusarium proliferatum (data not shown).

Finally, the resistance levels of immature seeds were verified. As shown in Fig. 7C and D, immature seeds were more resistant to $A. carbonarius$ than mature seeds. This is also confirmed by the observation that conidia were not able to germinate on immature almonds (Fig. 7E, F). However, $A. carbonarius$ showed normal growth if infection was carried out in not completely sealed conditions (Fig. 7G, H).

Discussion

These data indicate that 9-LOX metabolism is involved in almond seed defence against toxigenic fungi. In Aspergillus-infected almonds, OX and HPL appear to be concomitantly expressed. 9-LOX isoforms account for most of the LOX activity detected in both immature and mature seeds at 48 h and 72 h after infection. In control samples, a prevalence of 13-LOX, most probably related to the seed germination process (see also Santino et al., 2005), was observed. However, if compared with immature seeds (stages I and II), the levels of LOX expression and activity recorded from mature seeds were low. This is further confirmed by low levels of total and 9-HFAs detected in mature almonds. Taken together, these results could indicate that fungal colonization is promoted in those plant tissues, such as mature seed, whose physiological state does not permit a rapid activation of defence mechanisms mediated by LOXs. Alternatively, it is possible to hypothesize that toxigenic fungi can overcome such a mechanism by repressing gene expression and/or by inhibiting the enzymatic activity, thus blocking the flux of substrates to the other enzymes downstream in the pathway that are responsible for the synthesis of oxylipins with fungicidal activity. In peanut seeds, Aspergillus parasiticus colonization results in a strong induction of the PnLOX1 gene from 8 h through 48 h, but a decrease in

Table 2. Minimum inhibitory concentration (MIC) of volatiles of the lipoxygenase pathway against the toxigenic fungus Aspergillus carbonarius

<table>
<thead>
<tr>
<th>Compound</th>
<th>Aspergillus carbonarius MIC ($\mu$mol disc$^{-1}$)</th>
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<tbody>
<tr>
<td>(2E)-nonenal</td>
<td>27.75±2.62 (A)</td>
</tr>
<tr>
<td>(2E, 6Z)-nonadienal</td>
<td>31.35±2.18 (A)</td>
</tr>
<tr>
<td>Hexanal</td>
<td>80.5±3.56 (C)</td>
</tr>
<tr>
<td>(2E)-hexenal</td>
<td>39.7±4.67 (B)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>&gt;9000</td>
</tr>
</tbody>
</table>

Different letters designate significantly different means by a Tukey–Kramer multiple comparison test ($P<0.01$) of an ANOVA.
LOX activity in infected seeds (Burow et al., 2000). Recently, Tsitsigiannis et al. (2005b) reported the isolation of two other LOX genes (PnLOX2 and PnLOX3) highly expressed in mature peanut seed. Both these genes, encoding 13-LOXs, are repressed by A. flavus infection.

As far as the role of 9-hydroperoxides in the stimulation of mycotoxin production is concerned, our results relative to the almond/A. carbonarius interaction indicated a limited role for these oxylipins, since mature almonds (which are normally colonized by the fungus) showed low levels of 9-LOX expression and activity, and 9-HFA. On the other hand, in immature almonds (which are characterized by a high resistance level to A. carbonarius) higher levels of 9-LOX expression and activity, and 9-HFA were recorded. Moreover, these results indicated that HPL is strictly connected with LOX in the metabolism of 9-hydroperoxides which finally leads to the synthesis of the antifungal C9-aldehyde.

The results on the effect of C9-aldehydes on A. carbonarius and Fusarium proliferatum are in accordance with those reported by Cho et al. (2004) about the bactericidal effects of (2E)-nonenal and (2E,6Z)-nonadienal and those reported by Matsui et al. (2006) on the effects of these compounds on Botrytis cinerea and Fusarium oxysporum. The values of MIC here reported, together with those found in previous studies, do not reflect the concentration of volatile compounds found in nature. Matsui (2006) proposed that, being volatile, aldehydes heavier than air do not diffuse in every direction but stay near the area where they are synthesized, thus providing a microenvironment hostile to fungal growth. Our results on immature almonds confirm this hypothesis since, in these samples, the high resistance to A. carbonarius parallels C9-aldehyde synthesis, and fungal spores were able to germinate on wounded immature almonds only in incompletely sealed conditions (Fig. 7).

On the basis of their wide spectrum of activities, C9-aldehyde could be interesting volatile compounds to be used to extend the shelf-life of many plant products, as already proposed for their C6 counterparts (Lanciotti et al., 2003).

Our knowledge of plant oxylipins and their biological activities is expanding fast. A recent report by Prost et al. (2005) showed that the production of antimicrobial compounds is not separated from the production of signal molecules in the oxylipin pathway, therefore indicating that at least some phyto-oxylipins can display both functions. This is the case for volatile aldehydes produced by HPL, which not only can function as direct antimicrobial agents but also can induce the expression of defence genes (Bate and Rothstein, 1998; Prost et al., 2005).

In conclusion, these results indicate that the almond 9-LOX/HPL are involved in plant defence and can be considered an interesting biotechnological target in programmes aimed at improving plant resistance toward toxigenic fungi.

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