European Journal of Plant Pathology **106:** 857–865, 2000.

Lipoxygenase metabolites of α-linolenic acid in the development of resistance in pigeonpea, *Cajanus cajan* (L.) Millsp, seedlings against *Fusarium udum* infection

P. Uma Maheswari Devi[1], P. Srinivas Reddy[1], N.R. Usha Rani[2], K.J. Reddy[2], M. Narsa Reddy[1] and P. Reddanna[1,*]
[1]*School of Life Sciences, University of Hyderabad, Hyderabad – 500 046, India;* [2]*Department of Botany, Osmania University, Hyderabad – 500 007, India;* [*]*Author for correspondence: (Fax: +91 40 3010120/3010145; E-mail: prsl@uohyd.ernet.in)*

Accepted 17 September 2000

Key words: 13-hydroperoxyoctadecadienoic acid, 13-hydroperoxyoctadecatrienoic acid

Abstract

Lipoxygenase (LOX) activity was measured in germinating pigeonpea *Cajanus cajan* seedlings, resistant (ICP-8863) and susceptible (ICP-2376) to wilt fungus, before and after infection with *Fusarium udum*. LOX activity was significantly higher in the resistant than in the susceptible cultivars of pigeonpea and was enhanced further in response to infection with *Fusarium udum*. This increase in LOX activity in the resistant cultivars of pigeonpea appears to be due to the induction of lipoxygenase isozymes in response to infection. Analysis of the endogenous LOX metabolites in pigeonpea seedlings revealed the predominant formation of 13-hydroperoxyoctadecadienoic acid (13-HPODE) in healthy seedlings and 13-hydroperoxyoctadecatrienoic acid (13-HPOTrE) in infected seedlings. Further studies on the effects of LOX metabolites on the growth and multiplication of *Fusarium udum* showed that HPOTrEs, LOX metabolites of α-linolenic acid, are more anti-fungal compared to HPODEs, LOX metabolites of linoleic acid.

Introduction

Lipoxygenase (LOX – linoleate : Oxygen oxidoreductase, EC 1.13.11.12) catalyzes the addition of molecular oxygen to polyunsaturated fatty acids (PUFAs), which present a cis, cis-1, 4-pentadiene site, as in linoleic acid (LA-18 : 2), α-linolenic acid (ALA-18 : 3) and arachidonic acid (AA-20 : 4). These non-heme iron containing dioxygenases are widely distributed among plants and animals (Gardner, 1991; Siedow, 1991).

Lipoxygenase (LOX) products have been implicated in various regulatory functions, such as growth and development, senescence and defense responses. When plant tissues are injured by insects or pathogens or mechanical wounding, lipid degrading enzymes are activated (Narvaez-Vasquez et al., 1999). This provides the necessary polyunsaturated fatty acid substrates for LOX and the fatty acid peroxides thus generated can be further metabolized to biologically active compounds. These include jasmonic acid and traumatin, which evoke a variety of cellular responses (Farmer and Ryan, 1990; Farmer et al., 1992; Rosahl, 1996; Vijayan et al., 1998; Staswick et al., 1998), and highly reactive aldehydes with anti-microbial activity (Hamberg and Gardner, 1992; Hildebrand et al., 1988).

Increased lipoxygenase activity has been reported in dicots and monocots after infection with pathogenic microorganisms and viruses e.g., in tobacco infected with tobacco mosaic virus (Ruzicska et al., 1983), *Phytophthora parasitica* (Fournier et al., 1993; Rance et al., 1998), *Phytophthora cryptogea* (Suty et al., 1996; Rusterucci et al., 1999), in potato infected with *Rhizoctonia soloni* (Reddy et al., 1992), in tomato infected with powdery mildew (Kato et al., 1992), in *Pseudomonas syringae* – inoculated bean (Croft et al., 1990), tomato (Koch et al., 1992) and *Arabidopsis thaliana* (Melan et al., 1993), in wheat infected with *Puccinia graminis* (Ocampo et al., 1986) and in rice

infected with *Manaporthe griesea* (Ohta et al., 1991). In most cases, a correlation between induction of LOX activity and resistance of the plant has been shown. In the present study, an attempt was made to examine the role of LOX in resistant and susceptible cultivars of pigeonpea seedlings infected with *Fusarium udum*. The LOX metabolites formed *in situ*, in response to infection, were also isolated, identified and evaluated for their role in defense responses.

Materials and methods

Seeds and microbial culture

Pigeonpea (*Cajanus cajan*) seeds, resistant (ICP-8863) and susceptible (ICP-2376) to wilt fungus and strains of *Fusarium udum* were obtained from the International Crops Research Institute for Semi Arid Tropics (ICRISAT), Hyderabad, India. LOX products of linoleic acid (13-HPODE, 9-HPODE, 13-HODE and 9-HODE) and α-linolenic acid (13-HPOTrE, 9-HPOTrE, 13-HOTrE and 9-HOTrE) were prepared using soybean and potato LOX enzymes (Reddanna et al., 1990).

Growth of pigeonpea seedlings

Seeds of pigeonpea cultivars ICP-8863 and ICP-2376 (Nene et al., 1981), resistant and susceptible to wilt fungus respectively, were surface sterilized and grown in autoclaved river bed sand placed in 15 cm earthenware pots aseptically in an incubator under constant light at 25 °C for 7 days until the formation of first leaf. The seedlings were inoculated with *Fusarium udum* and the level of lipoxygenase activity monitored daily for a period of 7 days.

Preparation of Fusarium udum spore suspension

The spore suspension of *Fusarium udum* diluted to contain approximately 6.5×10^5 spores/ml was prepared from 7-day-old culture grown on potato dextrose broth. About 20 ml aliquots of the spore suspension was taken into sterilized glass tubes (150 × 15 mm).

Root dip inoculation

Seven-day-old pigeonpea seedlings with first leaf grown under aseptic conditions were removed and the root system was washed repeatedly by rinsing in sterilized distilled water. One seedling with the root system dipped in the 20 ml of spore suspension in each of the tubes was held in position by a cotton plug and incubated at 25 °C. After every 24 h sterilized distilled water was added aseptically to each of the tubes to make up the loss. Seedlings dipped in sterilized distilled water served as healthy controls. The seedlings were harvested at 24 h intervals for 7 days after inoculation and used for lipoxygenase extraction.

Preparation of LOX crude extract

The LOX enzyme was prepared from pigeonpea seedlings after inoculation with *Fusarium udum*. Pigeonpea seedlings were homogenized (20% homogenate w/v) in 100 mM potassium phosphate buffer, pH 7.0. The homogenate was passed through four layers of cheese cloth and centrifuged at 10,000*g* for 30 min. The resulting supernatant was used as the enzyme source.

Measurement of LOX activity

The LOX activity was assayed polarographically in the seedlings at 24 h intervals for 7 days (Reddanna et al., 1990). The reaction mixture consisted of 2.9 ml of 0.1 M potassium phosphate buffer pH 6.5 and 100 µl of enzyme. Reaction was initiated by the addition of 10 µl of substrate (linoleic or linolenic acid), to give a final concentration of 250 µM. The enzyme activity was expressed as Units/mg protein, wherein one Unit is defined as 1 µ mole of oxygen consumed/min. Protein content was estimated according to the method of Lowry et al. (1951).

Analysis of LOX isozymes on native-PAGE

Healthy and inoculated pigeonpea seedlings of the cultivars ICP-8863 were harvested 4 days of infection. The crude extracts of harvested seedlings were separated on 10% native-PAGE under cold conditions. After electrophoresis, the gel was washed in 0.1 M potassium phosphate buffer, pH 6.5 for 15 min at 4 °C. The gel was incubated with substrate solution, prepared by mixing one volume of 0.53 M substrate (linoleic acid or linolenic acid) with equal volumes of methanol and 0.53 M potassium hydroxide to give potassium linoleate or linolenate. The potassium linoleate or linolenate was mixed with 100 volumes of fresh 0.1 M KH_2PO_4 pH 6.5 to give a final concentration of 2.5 mM

substrate in the solution along with 0.1% sodium cholate. The gel was incubated for 5 min at 25°C temperature. After incubation, the gel was washed with 0.1 M KH_2PO_4 of pH 6.5 or with double distilled water to remove the excess of the substrate attached to the gel. The washed gel was stained with O-dianisidine hydrochloride in 0.1 M potassium phosphate buffer for color development (Heydeck and Schewe, 1985).

Analysis of endogenous lipoxygenase products

Healthy and inoculated seedlings were harvested 4 days of infection. The harvested seedlings were homogenized and centrifuged at 10,000*g* for 30 min. The resulting supernatant was extracted with hexane : ether (50 : 50) twice and the organic extracts were pooled, evaporated to dryness under vacuum and re-dissolved in HPLC mobile phase (hexane : propanol : acetic acid, 1000 : 15 : 1 v/v/v).

The LOX products from healthy and infected seedlings were separated on straight phase HPLC (Shimadzu LC 6A equipped with Rheodyne injector using Shimadzu CLC–SIL column 4.0 mm×25 cm). The products were monitored at 235 nm and the individual peaks detected were identified based on UV-visible spectral characterization, co-chromtography with authentic standards and GC-MS analysis. GC-MS analysis of the endogenous LOX products was done at The Pennsylvania State University, USA with the facilities of Dr. C. Channa Reddy, using Hewlett Packard 5890 series II gas chromatograph coupled to a Hewlett Packard 5971 mass spectrometer (Reddy et al., 1992). The LOX products were reduced with sodium borohydride, methylated and silylated using BSTFA of Supleco, Bellefonte, PA, before GC-MS analysis. The separation conditions were: 15 m fused silica column, 0.20 mm internal diameter with 0.20 μm film thickness, temperature program 3 min/70 °C, then 10 °C/min to 240 °C. The gas carrier was helium, 2 ml min. The quantification of individual LOX products was done based on peak areas and UV/VIS spectral data of peaks collected from HPLC.

LOX metabolites – filter paper disc bioassay

Fusarium udum Butler was maintained on potato dextrose broth on a reciprocal shaker for 4 days at 25 °C and conidial suspensions were obtained. Mycelial fragments were removed by filtering through sterile glass wool. After washing in sterile water, the conidial suspension was adjusted to the desired concentration (2×10^4 spores/ml). Approximately 1 ml of spore suspension was pipetted onto a fresh and dry Malachite green agar plates and evenly distributed with a glass spreader. To test the effect of hydroperoxides and hydroxides on the growth of *Fusarium udum*, sterile 14 mm×2 mm filter discs (Whatman) were placed centrally on the agar surface of previously seeded Malachite green agar plates. Different concentrations of hydroperoxides (13-HPODE, 9-HPODE, 13-HPOTrE and 9-HPOTrE) and hydroxides (13-HODE, 9-HODE, 13-HOTrE and 9-HOTrE) dissolved in 10 μl of 10% ethanol were pipetted on to filter discs in petriplates. Control plates were prepared by pipetting 10 μl of 10% ethanol on to filter discs. Plates were then sealed with parafilm and incubated at 25 °C for 24 h. Plates were examined for zones of growth inhibition around each disc. The lowest concentration of hydroperoxide or hydroxide that produced a detectable zone of inhibition was considered as minimum inhibitory concentration (MIC) and is expressed as micrograms of hydroperoxide or hydroxide per disc. The experiment was repeated 3 times.

LOX metabolites – microtitre plate bioassay

Anti-fungal activity of hydroperoxides and hydroxides on the growth of *Fusarium udum* was assessed in microtitre plates. Tests were performed with 20 μl of test solution (LA/ALA hydroperoxides/hydroxides with varying concentrations i.e., 5, 10, 20 and 40 μg/ml in 10% ethanol) and 40 μl of spore suspension of *Fusarium udum* (approximately 2×10^4 spores/ml) in half strength potato dextrose. Change in absorbance was monitored at 490 nm on an Enzyme-Linked Immunosorbant Assay (ELISA) reader after 12 h of incubation at 30 °C. Control sample was maintained by adding 10 μl of 10% ethanol to 40 μl of spore suspension. For each treatment, three replicates were examined.

Percent inhibition in the growth was calculated using the following formula:

$$\text{Percent inhibition} = \frac{\text{Absorbance of corrected control}}{\text{Absorbance of control} - \text{Absorbance of corrected test}} \times 100$$

where absorbance of corrected control = Abs of control at 12 h – Abs of control at 30 min and absorbance

of corrected test = Abs of test at 12 h – Abs of test at 30 min.

Results

Lipoxygenase activity in healthy and inoculated pigeonpea seedlings

Lipoxygenase activity in healthy and inoculated pigeonpea seedlings of resistant (ICP-8863) and susceptible (ICP-2376) cultivars, studied at different time intervals is shown in Figure 1. LOX activity, in the pathogen-inoculated seedlings of the resistant cultivar (ICP-8863), increased progressively starting from day 1 of infection, reached maximum by day 4 (approx. 10-fold) and declined gradually thereafter (Figure 1). LOX activity in this cultivar remained higher in comparison to the healthy seedlings throughout the period of infection. In the susceptible seedlings (ICP-2376) LOX activity was lower than that of the healthy seedlings throughout the period of infection (Figure 1). Also, the levels of LOX activity in the healthy and infected seedlings of resistant cultivar were higher than those in the susceptible cultivar at all the periods of study.

Analysis of LOX isozymes on native-PAGE

In order to analyse the expression of LOX isozymes in healthy and infected seedlings of the resistant cultivar of pigeonpea (ICP-8863), the proteins were separated on native-PAGE and stained with O-dianisidine hydrochloride. As shown in Figure 2, L_1, L_2, L_3 and L_4 isozymes were expressed in healthy seedlings. However, in the inoculated seedlings five LOX isozymes L_2, L_3, L_4, L_5 and L_6 were expressed with a decrease of L_1 isozyme. The highest LOX activity observed in inoculated seedlings may be due to the expression of new LOX isozymes (L_5 and L_6), in addition to induction in other isoforms.

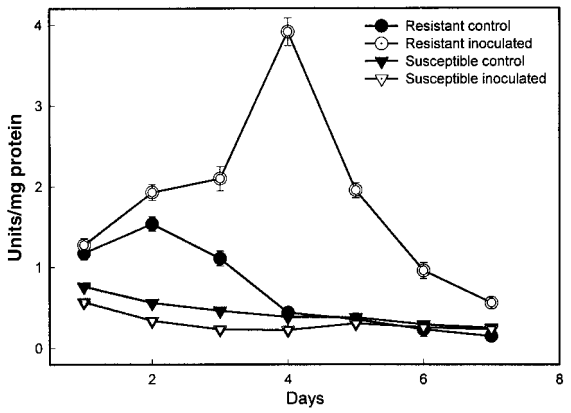

Figure 1. LOX activity in pigeonpea (resistant-ICP8863 and susceptible-ICP2376) seedlings in response to *Fusarium udum* infection. Data presented as mean ± standard error from three independent experiments. One unit is defined as one μmole of O_2 consumed per minute.

Endogenous LOX products

LOX metabolites formed in healthy and inoculated seedlings of the resistant cultivar of pigeonpea (ICP-8863) were extracted and separated on straight phase HPLC (SP-HPLC). HPLC analysis of the endogenous

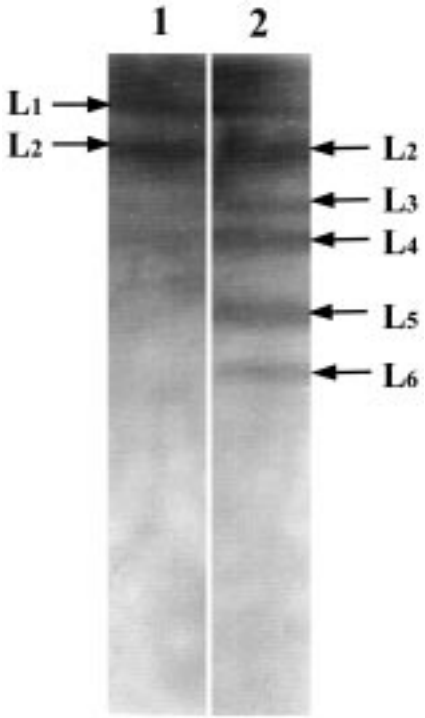

Figure 2. Activity staining of LOX isoenzymes in the control and infected (*Fusarium udum*) pigeonpea (ICP-8863) seedlings. Proteins separated on 10% native PAGE were incubated with substrate (LA) solution and the peroxides generated were stained with O-dianisidine hydrochloride. Lane 1 represents the LOX isozymes from 11 day old seedlings (control). Lane 2 represents LOX isozymes from 7 day old pigeonpea seedlings infected with *Fusarium udum* and harvested after 4 days.

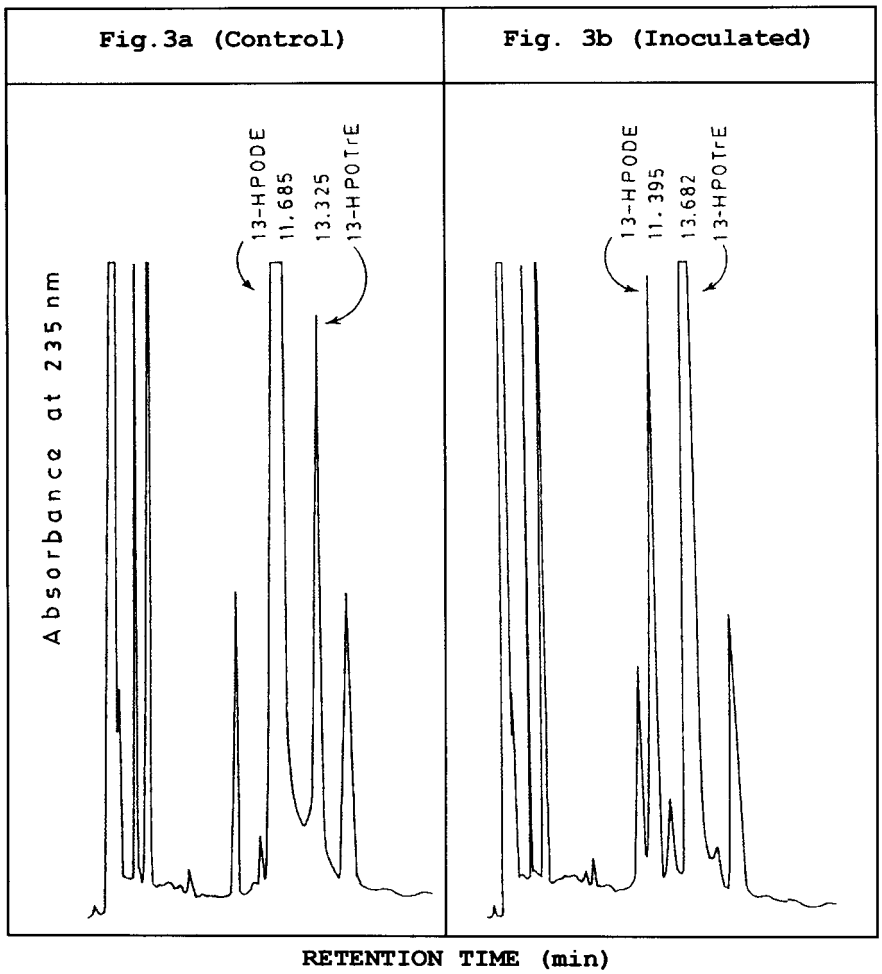

Figure 3. (a) HPLC separation of endogenous LOX products extracted from 11 days old pigeonpea control seedlings on a straight phase silica CLC–SIL column. The LOX products present in the homogenate supernatant (10,000*g* for 30 min) were extracted with hexane : ether (50 : 50) twice and the organic extracts pooled, evaporated to dryness, redissolved in HPLC mobile phase (hexane : propanol : acetic acid, 1000 : 15 : 1) and then applied to the column (straight phase silica column). The products were eluted with the mobile phase at a flow rate of 2 ml/min with detection at 235 nm. (b) HPLC separation of endogenous LOX products extracted from 7 days old pigeonpea seedlings infected with *Fusarium udum*. After 4 days of infection, the LOX products present in the homogenate supernatant were extracted and then separated on straight phase silica column as per the methods described in Figure 3a.

products from healthy seedlings showed a major peak with retention time 11.685 min and a minor peak with retention time of 13.325 min (Figure 3a). Both the peaks showed typical conjugated diene spectra with absorption maximum at 235 nm. The peak with a retention time of 11.685 min was identified as 13-hydroperoxyoctadecadienoic acid (13-HPODE) after co-chromatography of the compound with authentic 13-HPODE and GC-MS analysis of the reduced product after methylation and trimethylsilyl derivatization (data not shown). Similarly, the peak with RT 13.325 min was identified as 13-hydroperoxyoctadecatrienoic acid (13-HPOTrE) after co-chromatography and GC-MS analysis. The relative ratio of HPODE to HPOTrE, calculated basing on peaks areas on HPLC and UV/VIS spectral data, was 7 : 3.

Similarly, SP-HPLC analysis of the LOX products formed in the infected pigeonpea seedlings showed a minor peak with RT 11.395 min corresponding to 13-HPODE and a major peak with RT 13.682 min corresponding to 13-HPOTrE (Figure 3b). This pattern is

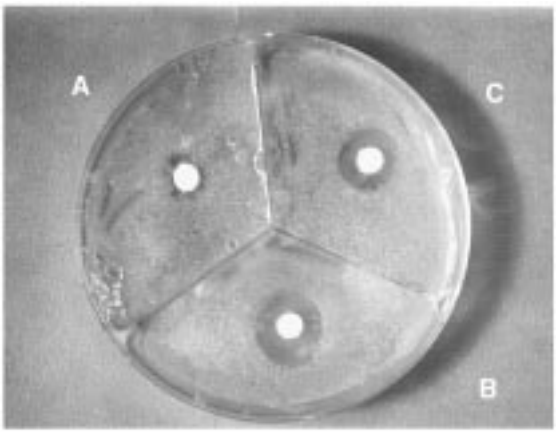

Figure 4a. Photomicrograph showing the effects of LOX products on the growth of *Fusarium udum* (Filter disc assay). Sterile filter discs (Whatman) were placed centrally on agar plates and different LOX products dissoved in 10 ml of 10% ethanol were added onto the filter discs. Plates were then sealed with parafilm, incubated at 25 °C for 24 h and later the photomicrographs were taken. A. 13-HODE (1 μg/ml); B. 13-HPODE (5 μg/ml) and C. 9-HPODE (5 μg/ml).

exactly opposite to that observed in healthy pigeonpea seedlings. Also, the relative ratio of HPODE to HPOTrE changed to 2 : 9 from 7 : 3 observed in healthy pigeonpea seedlings.

Antifungal activity of LOX metabolites filter paper disc bioassay

Hydroperoxides and hydroxy metabolites of LA and ALA spotted on filter paper discs inhibited the growth of *Fusarium udum* on Malachite green agar plates (Figure 4a). Of all the compounds tested maximum inhibition of fungal growth was observed with HPOTrEs compared to HPODEs (Table 1). In control filter discs, however, no inhibition of fungal growth was observed (Figure 4b).

Antifungal activity of LOX metabolites microtitre plate bioassay

As shown in Table 2, ALA hydroperoxides (13-HPOTrE and 9-HPOTrE), exhibited maximum percent inhibition (81% and 84% respectively) even at 5 μg/ml, when compared with LA hydroperoxides (13-HPODE 71% and 9-HPODE 80%). Further, 13-HOTrE and 9-HOTrE were found to be more potent compared to

Figure 4b. Photomicrograph showing the effects of LOX products on the growth of *Fusarium udum* (Filter disc assay). Sterile filter discs (Whatman) were placed centrally on agar plates and different LOX products dissoved in 10 ml of 10% ethanol were added onto the filter discs. Plates were then sealed with parafilm, incubated at 25 °C for 24 h and later the photomicrographs were taken. Control plates were prepared by adding 10 μl of 10% ethanol onto filter discs. A. 13-HPOTrE (5 μg/ml); B. 9-HPOTrE (5 μg/ml) and D. Control.

Table 1. Minimum inhibitory concentration (MIC) of hydroperoxyoctadecadienoic acids (HPODEs) and hydroperoxyoctadecatrienoic acids (HPOTrEs) on the growth of *Fusarium udum* on filter disc assay. MICs were calculated based on the concentration of hydroperoxide required to show a detectable zone of inhibition on the growth of *Fusarium udum* on filter disc assay

Treatment	Minimum inhibitory concentration	
	μg/disc	μM
13-HPODE	5.0	15.80
9-HPODE	5.0	15.80
13-HPOTrE	2.5	7.80
9-HPOTrE	2.5	7.80

13-HPOTrE and 9-HPOTrE as well as to those of LA hydroperoxides (HPODEs).

Discussion

In the present study, a higher level of LOX activity was observed in the resistant pigeonpea cultivar than in the susceptible cultivar. Lipoxygenase activity increased upto 10-fold in the resistant cultivar over a period of 4 days following infection with *Fusarium udum*. Similar results have been reported in other plant-microbe

Table 2. Percent inhibition in the growth of the fungus, *Fusarium udum* in the presence of different LOX metabolites, as shown by the microtitre plate method. Percent inhibition was calculated as per the equation given in the methodology section. Data preseted as mean ± SD of two trials with three replicates. All the values are significant at $P < 0.05$ compared to control value

Treatment	Percent inhibition			
	5 μg/ml	10 μg/ml	25 μg/ml	40 μg/ml
13-HPODE	70.6 ± 3.06	82.9 ± 1.47	89.8 ± 2.48	98.9 ± 2.77
9-HPODE	79.6 ± 2.22	86.2 ± 2.63	92.4 ± 2.72	96.3 ± 2.99
13-HPOTrE	81.3 ± 2.80	93.3 ± 2.22	97.3 ± 0.89	100 ± 0.49
9-HPOTrE	83.7 ± 2.45	90.2 ± 1.58	95.9 ± 0.89	100 ± 0.73
13-HOTrE	85.7 ± 1.50	95.2 ± 2.77	99.5 ± 0.89	100 ± 1.43
9-HOTrE	85.7 ± 1.50	93.9 ± 2.85	98.0 ± 1.34	100 ± 1.23

Values are mean ± SD of two trials with three replicates.

systems (Koch et al., 1992; Melan et al., 1993; Peng et al., 1994). The induction of lipoxygenase activity upto 7-fold was reported in tobacco leaves over a period of 11 days following infection with a powdery mildew, *Erysiphe cichracearum* (Lupu et al., 1980). Similar induction of LOX activity was recorded in tobacco leaves treated with cryptogein, a protein of the fungus *Phytophthora cryptogea* (Rusterucci et al., 1999). In potato tubers also, LOX activity increased upto 4 weeks following fungal infection with *Phoma exigua* (Galliard, 1978). In the susceptible pigeonpea seedlings, on the other hand, the LOX activity decreased immediately after inoculation and remained at lower levels compared to control seedlings. Thus, resistant and susceptible pigeonpea cultivars showed differential pattern in the levels of lipoxygenase activity in response to pathogen infection.

The endogenous product profiles of infected pigeonpea seedlings, however, were quite different from that of healthy pigeonpea seedlings. The relative ratio of LA to ALA products formed endogenously in healthy seedlings was 7 : 3 (LA : ALA) while it was 2 : 9 in infected seedlings, indicating a shift in LOX specificity towards ALA. The most important finding of the present study is the formation of ALA hydroperoxides (HPOTrEs) during infection in preference to LA hydroperoxides (HPODEs). Similar shifts towards ALA products was reported in the tobacco leaves treated with a fungal protein (Rusterucci et al., 1999). This shift is probably aimed towards the formation of traumatin and jasmonic acids, which are formed only from ALA. This may be possible with the specific release of ALA from membrane phospholipids by octadecanoid signal transduction pathway in plants in response to pathogen attack (Farmar and Ryan, 1992; Creelman and Mullet, 1995). Similar enhanced production of ALA metabolites in response to injury/infection was observed in potato tubers (Reddy et al., 2000).

LOX may also have a direct role in the protection of plant tissues. LOX metabolites might be acting directly on the fungal pathogen leading to the development of resistance. In order to test such a possibility, different LOX products of both LA and ALA were screened for their anti-fungal activity by filter disc assay and microtitre plate method. Agar plate assays showed that both hydroperoxides and hydroxides were effective anti-fungal agents, while the latter being more effective than the former. The minimum inhibitory concentration calculated for 13-HPOTrE was 7.8 μM compared to that of 15.8 μM for 13-HPODE/9-HPODE. These studies suggest that LOX metabolites exert their effects directly on the fungal pathogen.

The quantitative assessment of hydroperoxides and hydroxides on the growth of *Fusarium udum* by the microtitre plate method showed that HPOTrEs were more effective in inhibiting the growth of pigeonpea wilt fungus than HPODEs. Incidentally 13-HPOTrE, the LOX metabolite of ALA, is the major endogenous LOX product formed in pigeonpea seedlings infected with *Fusarium udum*. From these observations, it appears that ALA metabolites of LOX pathway might be involved in mediating the defense responses of the plant against wilt fungus. Similar inhibitions in the germination of conidia of rice blast, *Pyricularia oryzae* by 13-HOTrE and 9-HOTrE, (Shimura et al., 1983) and *Aspergellus niger* by 13-HPODE and 13-HPOTrE (Sailaja et al., 1997) and inhibition of cystospore germination of *Phytophthora capsici* by 9- and 13-HPOTrEs as well as by the hydroxy derivatives of arachidonic acid (Ricker and Bostock, 1994) were reported .

In general, it can be concluded that LOX activity is higher in the resistant cultivar of pigeonpea which is further enhanced in response to infection by *Fusarium udum*. The LOX metabolites formed endogenously in the tissue showed potent anti-fungal activity towards *Fusarium udum*, suggesting their possible involvement in the development of resistance. The resistance developed in pigeonpea seedlings against pathogen attack appears to be mediated through hydroperoxy/hydroxy metabolites of ALA.

Acknowledgements

This work was supported by the grants from Department of Biotechnology, New Delhi (Grants: BT/18/06/96-PID). We thank Dr. V.B. Reddy Senior Scientist, Legume Pathology, Crop Protection Division, ICRISAT, Hyderabad for providing facilities to carry out part of this work and for providing pigeonpea varieties and the strain of *Fusarium udum*-Butler; and Prof. M.N. Reddy, Head, Department of Microbiology, Sri Padmavati Mahila University, Tirupati, for critically reading the manuscript.

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