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Full Length Research Paper

Antimicrobial properties of chilli lipoxygenase products

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Plant oxylipins, the metabolic products of polyunsaturated fatty acids have been variously implicated in control of microbial pathogens. To get a better insight into the biological activities of oxylipins, *in vitro* growth inhibition assays were used to investigate the direct antimicrobial activities of 9 and 13-lipoxygenase (LOX) products against a set of plant pathogenic bacteria and fungi. LOX hydroperoxides derived from seeds of *Capsicum annum* showed differential antimicrobial activity. It was found that the LOX hydroperoxides, that is, 9-hydroperoxy octadecadienoic acid (9-HPODE) and 9-hydroperoxy octadecatrienoic acid (9-HPOTrE) showed antimicrobial activity against various fungal and bacterial pathogens. LOX hydroperoxides, that is, 9-hydroperoxy octadecatrienoic acid (9-HPOTrE) showed maximum antimicrobial activity than 9-hydroperoxy octadecadienoic acid (9-HPODE). The antimicrobial activity of oxylipins suggests their role in plant defense mechanism.

Key words: Antimicrobial activity, chilli, hydroperoxides, minimum inhibitory concentration (MIC).

INTRODUCTION

Plant oxylipins represent a diverse family of secondary metabolites believed to occur in all higher plants. They originate from oxidation of poly unsaturated fatty acids (PUFAs), predominantly Linoleic (LA18:2) and α-Linolenic acid (ALA 18:3). The biosynthesis of this oxylipins is mainly initiated by the action of LOXs. LOX (Linoleate: oxygen oxidoreductase, E.C 1.13.11.12) are a class of enzymes that catalyze the addition of molecular oxygen to *cis, cis*-1,4 pentadiene motifs of PUFAs. Plant LOXs can be classified as 9-LOXs or 13-LOXs according to the position at which oxygen is incorporated into LA or ALA, the most important substrates for LOX catalysis in plants. (Feussner and Wasternack, 2002). LOX generated 9- and 13-hydroperoxides have been shown to affect plant cell viability and regulate localized cell death during the hypersensitive reaction (Rusterucci et al., 1999). The 13-LOX derived compounds, including 13-octadecadienoic acid (13-HPODE) and 13-hydroxyoctadecatrienoic acid (13-HPOTrE) are regulators of plant defense gene expression (Almeras et al., 2003). However, the defense related functions of 9-LOX are not fully understood. Both9-LOXs and oxidative processes are proposed to be involved in the HR of tobacco induced by the avirulent pathogen pseudomonas syringae (Montellet et al., 2005). The production of free fatty acid hydroperoxides via the 9-LOX pathway in tobacco is crucial for hypersensitive cell death (Rusterucci et al., 1999). The function of LOXs in defense against pathogens is likely to be related to the synthesis of fatty acid hydroperoxides and of volatile products with signaling functions and antimicrobial activity (Croft et al., 1993).

Most of the studies on plant lipoxygenases have been directed at their purification and characterization of the metabolites. However, there is very little information on the physiological role of lipoxygenases in defense responses. Due to the importance of primary oxygenated metabolites of PUFAs, the present study is designed to study the role of primary oxygenated products of LOX and to determine the antimicrobial property of LOX metabolites against various fungal and bacterial pathogens.

MATERIALS AND METHODS

Chilli genotype

The chilli seeds of different genotypes namely CA-960, G5, LCA-206, LCA-235, G4, LCA-334 and Pusa Jwala were collected from

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LAM farm, Guntur, A.P. Among them, the LOX products from the resistant variety (G4) were used for the following studies.

Preparation LOX hydroperoxides

Partially purified chilli lipoxygenase was employed for the generation of hydroperoxides as per the method described by Reddanna et al. (1990). Typical reaction mixture contained 4 mg of enzyme protein in a total volume of 100 ml oxygen saturated 100 mM potassium phosphate buffer of pH 6.5. The reaction was initiated by the addition of linoleic or α-linolenic acid to give a final concentration of 200 µM and incubated for 2 min at room temperature with constant shaking. The reaction was terminated by acidifying the reaction mixture to pH 2.5 with 6 N HCl. The products formed were extracted into two volumes of hexane: ether (1:1) twice. The organic solvent separated from the aqueous layer in a separating funnel was passed through anhydrous granular sodium sulphate. Finally, the organic phase was evaporated on a rotatory evaporator to total dryness. The dried products, redissolved in HPLC mobile system containing n-hexane, propane-2-ol and glacial acetic acid (1000:15:1) were separated on straight phase HPLC.

Antimicrobial activity of LOX metabolites-filter disc method

Antifungal activity of hydroperoxides of LA (13-HPODE and 9-HPODE) and ALA (13-HPOTrE and 9-HPOTrE) products of LOX against the test fungi (*Colletotrichum capsici*) was assayed by filter disc assay (Bailey and Scott et al., 1990). The organisms were maintained on potato dextrose broth on a reciprocal shaker for four days at 25°C and the conidial suspensions were obtained. Approximately 1 ml of spore suspension (2.5×10^4) was pipetted onto potato dextrose agar plates and evenly distributed with a glass spreader. To test the effect of hydroperoxides (13-HPODE, 9-HPODE, 13-HPOTrE and 9-HPOTrE) on the growth of above mentioned organism on potato dextrose agar, sterile 14 x 2 mm filter discs (Whatman No.1), were placed centrally on the agar surface of previously seeded potato dextrose agar plates. Different LOX hydro peroxides (13-HPODE, 9-HPODE, 13-HPOTrE and 9-HPOTrE) dissolved in 10 µl of 10% ethanol were pipetted on to the filter discs on petriplates. Control plates were prepared by pippetting 10 µl of 10% ethanol onto filter discs. Plates were incubated at 25°C for 72 h. Plates were examined for zones of growth inhibition around each disc. The lowest concentration of hydro peroxide that produced a detectable zone of inhibition was considered as minimum inhibitory concentration (MIC). The experiment was repeated 3 times. Antifungal activity of chilli LOX LA and ALA products (9-HPODE and 9-HPOTrE) was also tested against various fungal pathogens which included *Colletotrichum gleosporoides, Aspergillus flavus, Aspergillus niger, Fusarium oxysporum.* The antifungal activity was performed as described above and observed for the zones of inhibition. Similarly, anti bacterial activity of hydroperoxide products of chilli LOX was assayed against the test organisms (*Xanthamonas campestris, Erwinia sp,* and *Pseudomonas sp*). The bacterial strains were inoculated into nutrient broth and incubated at 37°C for 24 h. 1 ml of the culture was spread onto the previously prepared nutrient agar plates. Hydroperoxides were loaded onto the filter discs and then incubated for 24 h at 37°C and studied for the antimicrobial property of HPODES and HPOTrES by measuring the zone of inhibition.

Antimicrobial activity of hydroperoxides- agar diffusion method

Antifungal activity of hydroperoxides of chilli LA (9-HPODE) and ALA (9-HPOTrE) products of chilli LOX against the test fungi (*C. capsici, C. gleosporoides* and *A. niger*) was assayed by agar cup method (Benson, 1990; Bailey; Scott et al., 1990). Potato dextrose agar plates were prepared aseptically and wells of 10 mm diameter were made by using sterile cork borer. Each plate consisted of 3 wells, of which 2 wells were filled with different chilli hydroperoxide product and one with 10 µl of ethanol which was maintained as control. The plates were incubated at 27°C for 72 h and observed for zone of growth inhibition.

Antifungal activity of hydroperoxides: Micro-titre plate assay

Antifungal activity of hydroperoxides (13-HPODE, 9-HPODE, 13-HPOTrE and 9-HPOTrE) was assayed by microtitre plate assay (Broekaert et al., 1990). Conidial suspension of *C. capsici* was obtained by culturing the fungus in potato dextrose broth on a reciprocal shaker for 4 days at 25°C. Test were performed with 10 µl of test solution (13-HPODE, 9-HPODE, 13-HPOTrE and 9-HPOTrE ranging from 5 to 20 µg in 10% ethanol) and 90 µl of spore suspension of *C. capsici* in either half strength potato dextrose broth or broth supplemented with 1 mM $CaCl_2$ and 50 mM KCl. Effects on germination were recorded after 12 h incubation with varying concentration of hydroperoxides. Controls were maintained by adding 10 µl of 10% ethanol and 90 µl of spore suspension. For each treatment, three replicates were maintained. Reported data are the means and standard deviations of the values obtained in at least three independent experiments. Percent growth inhibition 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 = Absorbance of control at 12 h - Absorbance of control at 30 min.
Absorbance of corrected test = Absorbance of control at 12 h - Absorbance of test at 30 min.

RESULTS

The antifungal activity of the LOX hydroperoxides of LA and ALA was tested against *C. capsici.* Of all the compounds tested, maximum inhibition of fungal growth was observed with 9-HPOTrE and 13-HPOTrE followed by 9-HPODE and 13-HPODE. The LA hydroperoxides (9-HPODE and 13-HPODE) also showed inhibitory effect on *C. capsici* but less significantly than the ALA hydroperoxides (9-HPOTrE and 13-HPOTrE). In control filter discs, however, there was no inhibition of the fungal growth (Figure 1).

The effect of chilli LOX hydroperoxides (9-HPODE and 9-HPOTrE) on the growth of various fungal pathogens namely *C. gleosporoides, A. flavus, A. niger* and *Fusarium oxysporum* were also tested. The chilli LOX ALA products showed maximum zones of inhibition compared to the chilli LA products against the tested fungal pathogens (Table 1) (Figure 2). Similarly, the antibacterial activity of chilli LOX hydroperoxides of LA and ALA (9-HPODE and 9-HPOTrE) was also tested on different bacterial strains (*X. campestris, Erwinia* spp. *and*

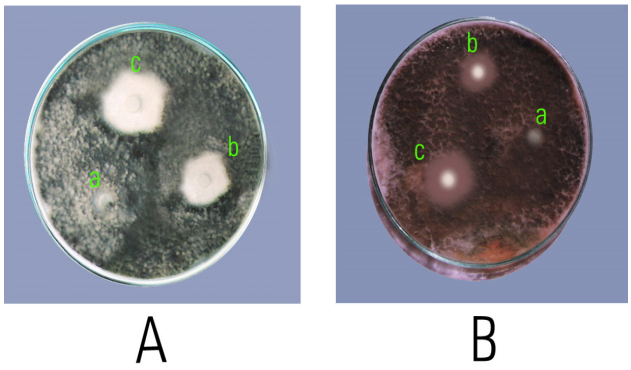

Figure 1. Antifungal activity of chilli LOX products by filter disc assay against *C. capsici*. Control (10 µl 10% ethanol) (a), 5 µg/ml 9-HPODE (b), 5 µg/ml 9-HPOTrE (c). A, LOX ALA products; B, LOX LA products.

Table 1. Minimum inhibitory concentration (MIC) of the chilli LOX hydroperoxides against various fungal pathogens-disc diffusion method.

Organism	Inhibitory concentration	
	5 µg/disc	mm
C. gleosporoides	Control (10 µl 10%ethanol)	-
	(9-HPODE)	4
	(9-HPOTrE)	8
A. niger	Control (10 µl 10% ethanol)	-
	(9-HPODE)	6
	(9-HPOTrE)	10
A. flavus	Control (10 µl 10% ethanol)	-
	(9-HPODE)	8
	(9-HPOTrE)	10
F. oxysporum	Control (10 µl 10%ethanol)	-
	(9-HPODE)	6
	(9-HPOTrE)	10

Pseudomonas sp.) and the zones of inhibition was calculated (Table 2). Chilli LOX ALA products showed maximum growth inhibition of the bacterial pathogens tested compared to the LA products (Figure 3). The effect of chilli LOX hydroperoxides on the growth of various fungal pathogens was also performed by agar diffusion method against the following test organisms (*C. capsici, C. gleosporoides* and *A. niger*). The chilli LOX hydroperoxides of ALA and LA (9-HPOTrE and 9-HPODE) inhibited fungal growth on potato dextrose agar plates previously seeded with the fungal cultures. The chilli LOX ALA products showed maximum inhibition of fungal growth compared to the LA products tested. In control wells, however, there was no inhibition of the

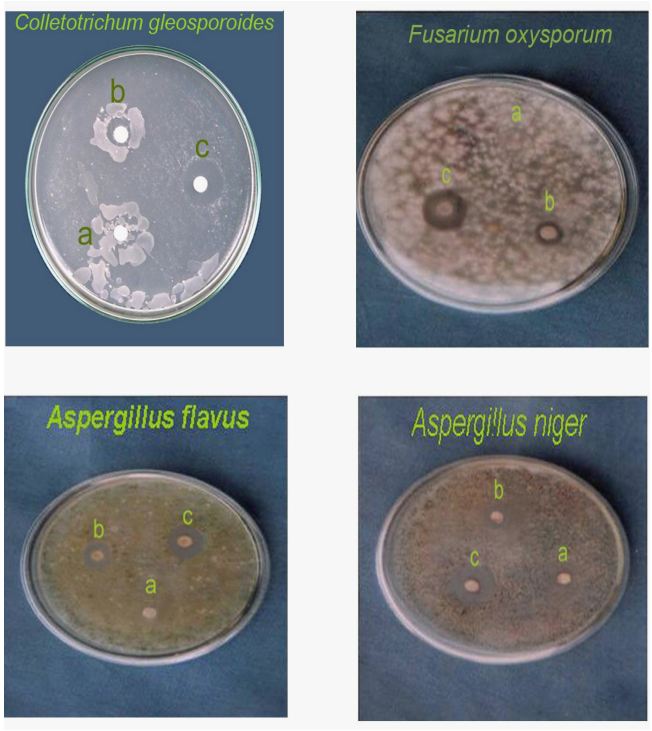

Figure 2. Antifungal activity of chilli LOX products by filter disc assay. Control (10 µl 10% ethanol) (a), 5 µg/ml 9-HPODE (b), 5 µg/ml 9-HPOTrE (c).

Table 2. Minimum inhibitory concentration (MIC) of the chilli LOX hydroperoxides against various bacterial pathogens-disc diffusion Method.

Organism	Inhibitory concentration	
	5 µg/disc	mm
X. campestris	Control (10 µl 10%ethanol)	-
	(9-HPODE)	6
	(9-HPOTrE)	12
Erwinia spp.	Control (10 µl 10%ethanol)	-
	(9-HPODE)	6
	(9-HPOTrE)	10
Pseudomonas spp.	Control (10 µl 10%ethanol)	-
	(9-HPODE)	6
	(9-HPOTrE)	10

fungal growth (Figure 4). The antimicrobial assays thus indicate the possible role of chilli LOX ALA products in defense responses against the pathogens. From these studies, it can be suggested that the LOX metabolites of ALA are specifically involved in inducing defense response against the invading pathogen. Growth of *C. capsici* was studied in microtitre plates in the presence of

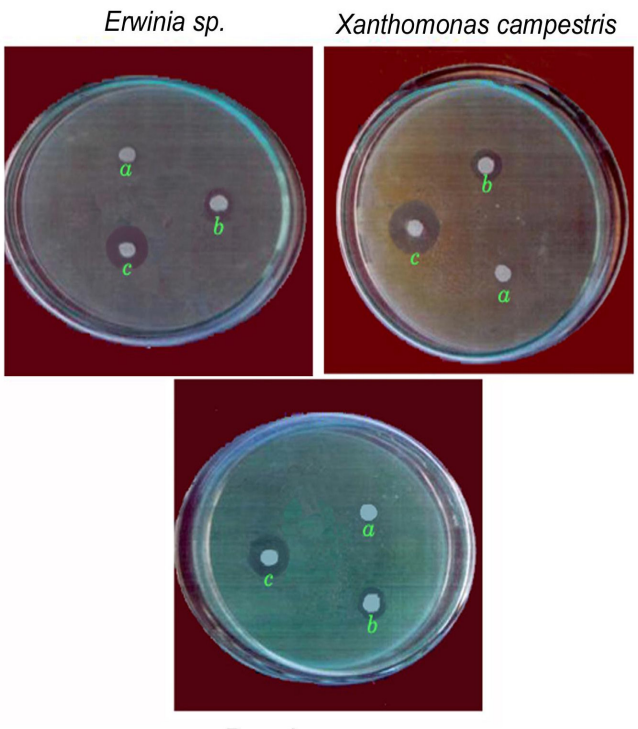

Figure 3. Antibacterial activity of chilli LOX products by filter disc assay. Control (10 µl 10% ethanol) (a), 5 µg/ml 9-HPODE (b), 5 µg/ml 9-HPOTrE (c).

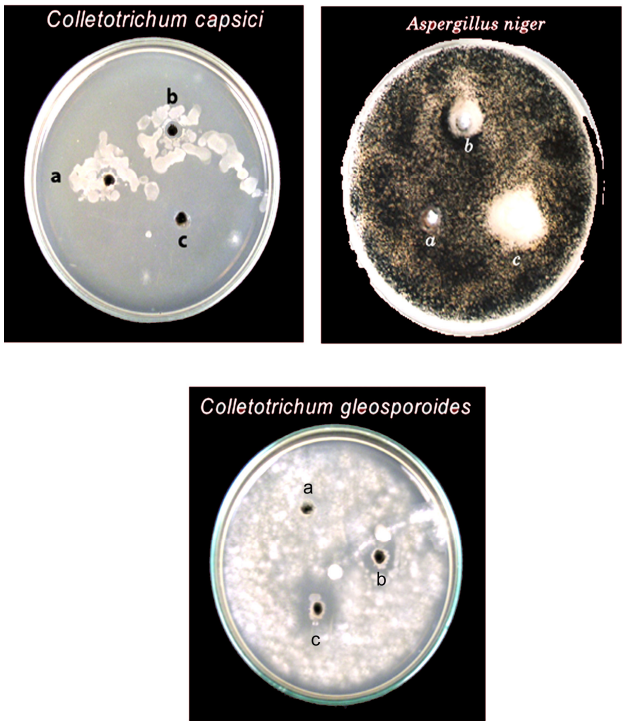

Figure 4. Antifungal activity of chilli LOX products by agar diffusion assay. Control (10 µl 10% ethanol) (a), 5 µg/ml 9-HPODE (b), 5 µg/ml 9-HPOTrE (c).

different concentrations of hydroperoxides of LA and ALA (9-HPODE, 13-HPODE, 9-HPOTrE and 13-HPOTrE) using an enzyme linked immunosorbent assay (ELISA) at 490 nm as described by Broekaert et al. (1990). Percent inhibition of germination of *C. capsici* was recorded after 12 h of incubation in the ELISA plates as described in the methodology. As shown in Table 3, linolenate hydroperoxides, 9-HPOTrE and 13-HPOTrE showed maximum inhibition (86 and 84%, respectively) even at 5 µg/ml when compared with linoleate hydroperoxides (9-HPODE and 13-HPODE).

DISCUSSION

LOX pathways are crucial for lipid peroxidation process during plant defense responses to pathogen infection. LOX metabolites act as antimicrobial compounds leading to the development of resistance. The primary products of LOX, the fatty acid hydroperoxides, are very reactive and cause oxidative damage to membranes, leading to cell necrosis and death (Hildebrand, 1989). In order to test such possibility, different chilli LOX products of both LA and ALA were screened for their antimicrobial effects by filter disc assay and agar diffusion method. Filter disc assay showed that both hydroperoxides of LA (9-HPODE and 13-HPODE) and ALA (9-HPOTrE and 13-HPOTrE) were effective against *C. capsici* as antifungal agents. The ALA hydroperoxides were found to be more effective antimicrobial agents than the LA products. These studies suggest that LOX ALA metabolites exert maximum antimicrobial activity. The antimicrobial activity of the chilli LOX products was tested against different fungal and bacterial pathogens. The chilli LOX ALA products showed that the chilli LOX ALA products maximum inhibition of the organisms is in comparison with the LA products. From these observations, it appears that ALA metabolites of LOX pathway might be involved in mediating the defense responses of the plant against the fruit rot pathogen. Similar inhibitions in germination of conidia of rice blast, *Pyricularia oryzae* by LOX hydroxides (13-HOTrE and 9-HOTrE) (Shimura et al., 1983) and *A. niger* by LOX hydroperoxides (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 the hydroxy derivatives of arachidonic acid were reported (Ricker and Bostok, 1994).

The present study indicates that the 9-LOX metabolism is involved in chilli against *C. capsici.* Direct pathogen control via antimicrobial oxylipins in plants suggests that 9-LOX derived oxylipins might indeed be important contributors to the outcome of given plant-microbe interactions. The formation of biologically active molecules from fatty acid 9-hydroperoxides in plants has not received great attention, but biological role for such compounds in plant defense has been suggested (Caldelari and Farmer, 1998) in potato and tobacco in

Table 3. Antifungal activities of hydro peroxides against *C. capsici* after 12 h of incubation: microtitre plate method.

Treatments	Percent inhibition			
	5 µg	10 µg	15 µg	20 µg
13-HPODE	70.74±1.22	78.705±1.43	83.34±1.002	94.60±1.02
13-HPOTrE	83.923±1.18	85.550±1.30	91.68±1.00	95.31±0.94
9-HPODE	75.783±1.17	78.784±1.34	88.724±1.002	98.074±0.748
9-HPOTrE	85.934±0.71	88.524±0.62	92.253±0.807	99.059±1.001

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

response to pathogen attack as a result of formation of colneleic acid (CA) and colnelenic acid (CnA) (Weber et al., 1999). The divinyl ethers (CA and CnA) of the 9-LOX pathway act as antimicrobial compounds.

Conclusion

The primary products of LOX, the fatty acid hydroperoxides, are very reactive and cause oxidative damage to membranes, leading to cell necrosis and death (Hildebrand, 1989). LOX metabolites in this study exerted antimicrobial activity against various fungal and bacterial pathogens. The LOX hydroperoxides formed from ALA are potent antimicrobial agents and the ALA pathway is operative in the pathogen infected conditions, suggesting their possible involvement in the development of resistance.

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