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Antifungal Potential of *Papaya* Lipoxygenase Metabolites against *Phytophthora palmivora*

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Partially purified *lipoxygenase* (LOX) protein from papaya seedlings was incubated with linoleic acid and α-linolenic acid and the resulting hydroperoxides were monitored by high performance liquid chromatography. Invitro characterization of the LOX enzyme activity showed that it possesses both 9- and 13-LOX specificity, with a predominance for the 13-LOX function. The relative ratio of 13-hydroperoxy fatty acids to 9- hydroperoxy fatty acids was found to be 8:2. The hydroperoxy fatty acids were screened for their antifungal activity against *phytophthora palmivora*, the causative agent of phytophthora blight in papaya. The 13-hydroperoxides showed pronounced antifungal activity by inhibiting both the germination of sporangia as well as the growth of the mycelium.

Key words: Lipoxygenase, 13-HPODE, *Phytophthora palmivora*, Antifungal activity.

Lipoxygenases, LOXs (linoleate:oxygen oxidoreductase, EC. 1.13.11.12) constitute a large family of non-haem, iron-containing dioxygenases widely diffused both in plant and animal kingdoms[1]. They catalyse the addition of molecular oxygen to polyunsaturated fatty acids [PUFAs] containing a (Z,Z)-1,4-pentadiene system to produce the corresponding fatty acid hydroperoxides. In plants, the most common substrates for LOX are linoleic acid (LA) and α-linolenic acid (ALA), which are the major PUFAs of plant membrane phospholipids[2]. LOX isoenzymes catalyzes the formation of hydroperoxy fattyacids in stereo specific manner to yield 9-hydroperoxy octadecadienoic acid (9-HPODE) and /or 13-S hydroperoxy octadecadienoic acid (13-HPODE) form LA and 9 or 13-hydroperoxy octadecatrienoic acid (9-HPOTE/13-HPOTE) from ALA[3]. LOX from tobacco[4] and chilly produces predominantly 9-hydroperoxides from LA and ALA[5]. In Soybean[6] and pigeon pea[7], 13-hydroperoxide is the major product of LOX activity .LOX generated 9-and 13-hydroperoxides have been shown to affect plant cell viability and regulate localized cell death during the hypersensitive reaction[8]. The 13-LOX derived compounds, including 13-octadecadienoic acid (13-HPODE) and 13hydroxyoctadecatrienoic acid (13-HPOTE) are regulators of plant defense gene expression[9].

In plants, LOX gene expression is associated with a number of developmental events and induced by environmental changes, notably pathogen challenge. LOXs have been hypothesized to play a role in response to plant pathogens[10]. The function of LOX in defense against pests seems to be related to the synthesis of antimicrobial compounds[11], the signaling compounds[12], and found to be regulators of the hypersensitive response (HR)[13].

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The goal of the present study is to determine the antifungal potential of Papaya LOX metabolites against *Phytophthora palmivora*, a widely distributed necrotrophic fungus that parasitizes more than 138 plant species. It is particularly important as a pathogen of Papaya as it attacks the fruit, stem, and roots of papaya. Germinating spores of *Phytophthora palmivora* attack the lateral roots, causing small reddish-brown spots. These spots enlarge quickly, especially when the soil becomes flooded, and rot the whole root system. The roots turn into a soft, dark brown, stringy mass that can no longer nourish or support the plant. Papaya fruit production reduces because of severe epidemics of *Phytophthora palmivora.*

MATERIAL AND METHODS

LA, ALA and soyabean LOX were obtained from Sigma Fine Chemicals, USA. HPLC Solvents were procured from SD-Fine Chemicals, India. *Phytophthora palmivora* (code No: 09-23) was obtained from Indian Institute of Spice research, Kerala, India. Papaya seeds (variety: Red Lady) were procured from Indian Institute of Horticulture Research, Bangalore, India.

Generation of LOX Metabolites

The LOX enzyme was extracted from Papaya seedlings (Red lady) and subjected to ammonium sulphate fractionation (40%) and dialysis for partial purification. Partially purified papaya LOX was employed for the generation of hydroperoxides[14]. Typical reaction mixture contained 3mg of enzyme protein in a total volume of 100ml of oxygen saturated 0.1M Tris HCl (pH 8.5) buffer. The reaction was initiated by the addition of LA and ALA to give a final concentration of 200µM and incubated for 2 min at room temperature with constant stirring. The reaction was terminated with the addition of 6N HCl to acidify the reaction mixture to pH 2.5. The products were extracted with Hexane: Ether (1:1) twice and organic phase was separated from aqueous layer using a separating funnel. Finally, the organic phase was evaporated on a rotary evaporator and the dried products were utilized for HPLC analysis.

HPLC Analysis of LOX Metabolites

Hydroperoxides of LA and ALA were separated on Shimadzu HPLC Reverse Phase Silica column (CLC-SIL, 25X0.4) using methanol, water and glacial acetic acid (85:15:1) as the mobile phase[15]. The flow rate was 2ml/min. and the eluted products were continuously monitored at 235nm. The identification of LOX products was done by Co-chromatography using soybean and potato LOX products as standards. The major soybean products with LA and ALA as substrates are known to be 13-HPODE and 13-HPOTE and that of potato are 9-HPODE and 9-HPOTE.

Antifungal Activity of Papaya LOX Hydroperoxides

Antifungal activity of hydroperoxides of LA (13-HPODE, 9-HPODE) and ALA (13-HPOTE, 9-HPOTE) was determined by microtitre plate method[16]. The spore suspension of *Phytophthora palmivora* was obtained by culturing the fungi in carrot broth for 5-6 days in reciprocal shaker at 20°C. Tests were performed with 10µl of test solution (13-HPODE, 9-HPODE, 13-HPOTrE and 9-HPOTE ranging from 1, 5, 10, 15 and 20 µg in 10% ethanol) and 90 µl of spore suspension of *Phytophthora palmivora* in carrot broth and the plates were incubated for 12hrs. at 20^0C. Controls were maintained by adding 10 µl of 10% ethanol and 90µl of spore suspension. Fungal growth in the presence of hydroperoxy fattyacids was monitored at 490 nm after 12 hrs. of incubation. For each treatment, three replicates were maintained. Reported data contain the means and standard deviations of the values obtained in at least three independent experiments. The percent growth inhibition of *Phytophthora palmivora* was calculated using the following formula.

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

Absorbance of corrected control= Abs. of control at 12hr. – Abs. of control at 30 min.

Absorbance of corrected test = Abs. of test at 12hr. – Abs. of test at 30 min.

Spore Germination Assay

The germination of sporangia in the presence of hydroperoxy fatty acids was assessed in 96-well micro plates in water. The spore suspension of *Phytophthora palmivora* was obtained by culturing the fungi in carrot broth for 5-6 days in reciprocal shaker at 20°C. Hydroperoxy fatty acids in ethanol were added in each well to a

final concentration of 100µM together with 5,000 spores in a total volume of 100 µl and the plates were incubated for 12 hrs. The controls were maintained by adding 10µl of 10% ethanol. Triplicates were maintained for each treatment. The plates were observed in an inverted microscope (Optics) and for each well 2 to 4 non-overlapping pictures were acquired with camera and 200 to 300 spores were assessed for germination status.

RESULTS AND DISCUSSION

The product profile of partially purified papaya LOX was determined with LA and ALA as substrates. After incubation of papaya LOX with LA, the products were separated on Reverse Phase HPLC and the products were monitored at 235nm. Two peaks, with UV absorption maxima at 235nm were resolved with retention time (RT) 7.395 min. and 10.793 min. on RP-HPLC (Fig. 1). The major peak with RT 7.395 min. was identified as 13-HPODE and minor with 10.793 min. as 9-HPODE after Co-chromatography with soybean and potato standards respectively. The relative ratio of 13-HPODE to 9-HPODE is 8**:**2. Similarly, papaya LOX with ALA exhibited the presence of 13-HPOTE at 8.725 min. and 9-HPOTE at 11.366 min. (Fig. 2). The results inferred that papaya LOX potentially oxygenates both at 13th and 9th position of LA/ ALA and result in the generation of both 13- and 9- hydroperoxy fatty acids. Similar results were reported in soybean, which exhibited both 13- and 9-hydroperoxide in 9:1 ratio at pH 9.0[17].

The fatty acid Hydroperoxides are very reactive and cause oxidative damage to membrane leading to cell necrosis and death[18]. In order to test such possibility, different papaya LOX products of both LA and ALA were screened for their antifungal activity against *Phytophthora palmivora*. Percent inhibition of *Phytophthora palmivora* was recorded after 12hr of incubation in microtitre plate. As shown in table 1, all the Hydroperoxides showed more than 50% inhibition at a concentration of 5µg/ ml onwards. The 13-hydroperoxides of both LA (13-HPODE) and ALA (13-HPOTE) showed maximum inhibition of 97.2% and 99.07% respectively at 20 µg/ml whereas 9-hydroperoxides of both LA (9-HPODE) and ALA (9-HPOTE) showed inhibition of 93.3% and 95.4% respectively. These studies suggest that 13-LOX metabolites exert maximum antifungal activity.

Microscopic visualization approach was used to investigate the effect of Hydroperoxy fatty acids on germination of Sporangia, an important development stage in the life cycle of fungi. Usually, Sporangia germinate directly in a nutrient medium by producing germ tubes that develop into mycelial masses. The control, in the presence of water, showed the formation of sporangia which are papillate and ovoid with the widest part close to the base and release of zoospores from the sack of germinating sporangia and the detached sporangium contains a short pedicel (Fig. 3).

The formation of spore sac is impeded in the presence of 9-HPODE (Fig. 4) where as 9-HPOTE restricted the formation of mycelium (Fig. 5). The initial fragmentation of mycelium was observed in the presence of 13-HPODE (Fig. 6) and complete fragmentation of mycelium was found in the treatment with 13-HPOTE (Fig. 7). This

Table 1. Antifungal activities of hydro peroxides against *Phytophthora palmivora* after 12 h of incubation: microtitre plate method. Percent Inhibition was calculated as per the equation given in the methodology. Date presented as mean ± S.D. of three independent experiments. All the values are significant at P<0.05 compared to control values

Treatment	Percent Inhibition				
	1µg	5 µg	10 µg	15 µg	20 µg
13-HPODE	42.5 ± 0.07	68.74 ± 0.092	76.09 ± 0.14	84.24 ± 0.04	97.2 ± 0.09
13-HPOTrE	44.6 ± 0.19	73.26 ± 0.12	84.2 ± 0.05	91.35 ± 0.07	99.07 ± 0.02
9-HPODE	39.3 ± 0.13	59.28 ± 0.21	71.26 ± 0.18	75.6 ± 0.09	93.31 ± 0.07
9-HPOTrE	41.8 ± 0.06	61.36 ± 0.23	75.3 ± 0.07	81.9 ± 0.19	95.4 ± 0.03

study clearly indicates that the LOX products are potential not only to inhibit the germination of sporangia but also the growth of mycelium. The results suggest the involvement of LOX in the defense mechanism of papaya against *Phytophthora palmivora* infection.

CONCLUSIONS

The LOX metabolites of LA (13-HPODE and 9-HPODE) and ALA (13-HPOTE and 9-HPOTE) proved to be potential antifungal agents against *Phytophthora palmivora* by inhibiting the formation of sporangia as well as growth of

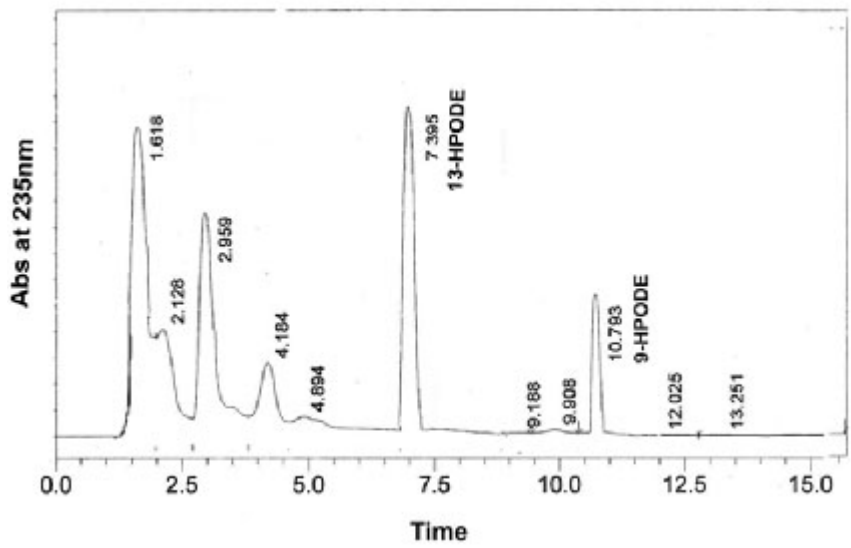


Fig. 1. HPLC Seperation of LOX products extracted from papaya seedlings with LA as substrate on Reverse Phase Silica CLC-SIL column. The products were identified as 13-HPODE and 9-HPODE based on Co-Chromatography with soybean and potato standards respectively

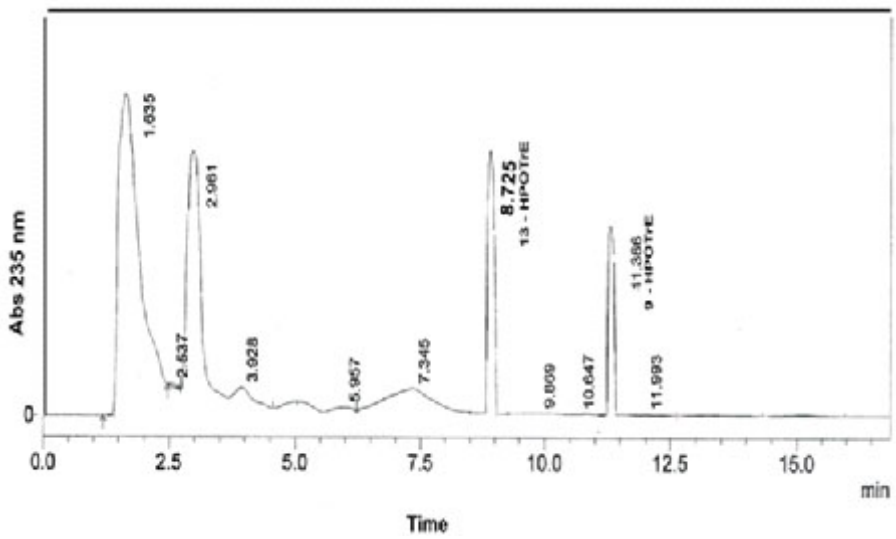
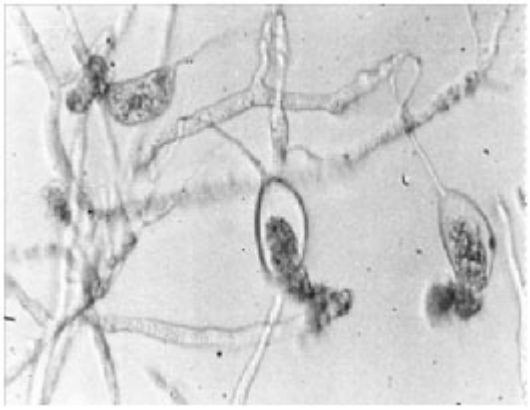
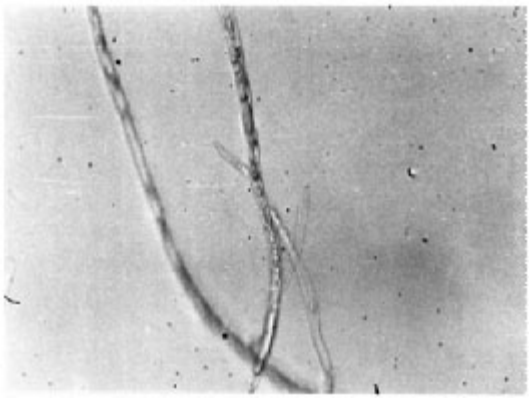


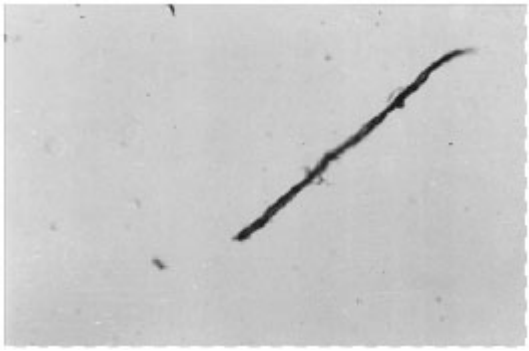
Fig. 2. HPLC Seperation of LOX products extracted from papaya seedlings with ALA as substrate on Reverse Phase Silica CLC-SIL column. The products were identified as 13-HPOTE and 9-HPOTE based on Co-Chromatography with soybean and potato standards respectively



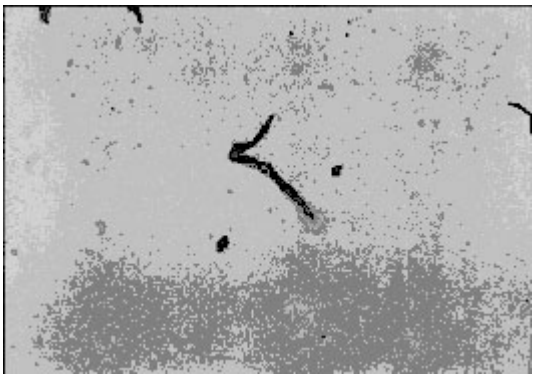
A (Control)



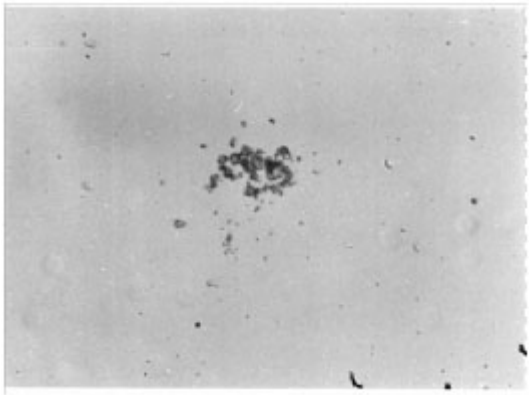
B (9-HPODE)



C (9-HPODE)



D (13-HPODE)



E (13-HPOTE)

Fig. 3. Microscopic Visualization of Germination of Sporangia Magnified Under 40X. The Spores of Phytophthora palmivora were incubated for 12 hrs. in Microtitre Plates

Fig. 3A. Formation of Sporangium and release of zoospores in the presence of water (Control)
Fig. 3B. Inhibition of Sporangia Formation with 100 µM 9-HPODE
Fig. 3C. Restricted growth of Mycelia in the Presence of 100 µM 9-HPOTE
Fig. 3D. Initiation of Mycelium Fragmentation with 100 µM 13-HPODE
Fig. 3E. Complete Fragmentation of Mycelium with 100 µM 13-HPOTE

mycelium. The antifungal activity of 13-hydroperoxy fatty acids suggests the predominance of 13-LOX pathway in papaya that belongs to the family caricaceae. The results indicate the function of 13-LOX in defense mechanism of papaya by contributing to the inhibition of growth of *Phytophthora palmivora*. Further, 13-LOX metabolites may enhance the defense capacity of papaya plant in addition to the initial hypersensitive cell death reaction.

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