METHODOLOGY
3.1 Materials:

Linoleic acid (LA) and Linolenic acid (ALA), Soybean Lipoxygenase, Trans-2-hexenal, Cis-3-hexenol, Jasmonic acid were obtained from sigma chemical company, St.Louis, USA. Standard molecular weight markers and Trizol reagent were procured from Genei, Bangalore, and HPLC solvents like n-hexane, propane-2-ol, acetic acid, and methanol were of high quality and obtained from India. Other chemicals used in this study were of high quality analytical/reagent grade procured from standard Indian chemical companies.

Phytophthora palmivora (code: 09-23) was obtained from Indian Institute of Spice Research (IISR) Calicut, Kerala. Enterobacter cloacae (509) and Erwinia herbicola (3609) were obtained from Indian Institute of Microbial technology (IMTech) Chandigarh. Pseudomonas aeruginosa and Xanthomonas axonopodis were obtained from Regional Agricultural Research Station (RARS) Tirupathi.

3.2 Plant material:

Different varieties of Carica papaya namely Pusa dwarf and Coorg Honey dew were obtained from Indian Institute of Horticulture research, Bangalore. Red lady was obtained from Indian Agricultural Research Institute, New Delhi. Coimbatore No.6 was obtained from Tamil Nadu agricultural university, Coimbatore. The papaya seeds were surface sterilized with 0.1% mercuric chloride, for few minutes, washed with autoclaved water. The above treatment was repeated for two to three times. For germination studies, the seeds were allowed to grow under constant light and ambient temperature and the germinated seedlings were harvested at 48hr for LOX activity assay.
3.3 Preparation of Crude Extract:

Papaya seedlings (10gm) were homogenized with a mortar and pestle in 50 ml of extraction buffer containing 50 mm Tris-HCl, pH 8.0 containing 10mM Kcl, 500 mM sucrose, 0.5mM PMSF. The homogenate was centrifuged at 10,000 rpm for 30min at 4°C and the supernatant was used for enzyme assay.

3.4 Spectrophotometric assay of Lipoxygenase Activity:

Lipoxygenase activity was measured spectrophotometrically by using Schimadzu 1601 UV-VIS spectrophotometer (Schimizu et al., 1984). The standard assay mixture consists of 10µl of linoleic acid or linolenic (sigma) and 50µl of Tween 20 (Sigma) in 10ml of 0.1M phosphate buffer, pH 9.0. The reaction was initiated by the addition of 0.1ml of LOX enzyme extract to 1ml of standard assay mixture. LOX activity was measured by monitoring the change in absorbance at 234nm over a period of 3min and activity was calculated using the following equation.

\[
\text{Lipoxygenase activity} = \frac{\text{Volume of reaction mixture} \times \text{absorbance difference per minute}}{\varepsilon \times \text{Volume of enzyme in ml}}
\]

Where \(\varepsilon\) = (Molar extinction co-efficient of hydroperoxides) = 27,500.

Definition of Enzyme Unit:

One unit of lipoxygenase activity is defined as one µ mole of hydroperoxide formed per minute. Specific activity is expressed as units per mg of protein.

Protein Estimation:

Protein content present in the crude enzyme preparation at various stages of both seed germination and purification was determined by the method of Lowry et al., (1951) using BSA as standard. In case of all chromatographic fractions, the protein content was estimated spectrophotometrically by taking absorbance at 280 nm on
Schimadzu 1601 UV-VIS spectrophotometer using quartz cuvette with 1 cm path length. The concentration of the protein was calculated by considering 1 O.D unit equivalent to 1 mg protein.

For pH dependency of the LOX, the buffers employed were 100mM Citrate buffer ranging from 4 to 5.5, 100mM Potassium phosphate buffer ranging from 6.0 to 7.0 and 100mM Tris HCl buffer for pH ranging from 8.0 to 9.0. The distribution of LOX activity in various parts of Papaya plant (flower, leaf, fruit) was also measured.

**3.5 Assay of Hydroperoxide Lyase:**

Hydroperoxide lyase was assayed by the loss in absorption at 234nm of the hydroperoxide as described by Vick and Zimmerman (1976). Hydroperoxide substrate solution for this assay was prepared by incubating 0.6ml of 8mM linoleic acid substrate solution with 1.2 mg of soybean lipoxygenase (8200 units/mg) in 30ml of distilled water for 30 minutes. The reaction mixture in a final volume of 3ml contained 0.5ml of the hydroperoxide substrate solution, 0.02 to 0.1 ml of enzyme solution, 0.1M Tris HCl pH 8.5 and add 10µl of NDGA to inhibit the lipoxygenase activity. The reaction was initiated by addition of the enzyme solution and run at 25°C for 2 minutes. Based on the rate of decrease in absorbance at 234nm, enzyme activity was calculated using the formula.

\[
\text{HPL Activity} = \frac{(\text{Vol of reaction mixture}) \times (\text{absorbance difference per minute})}{\Delta \text{Vol of enzyme in ml}}
\]

Where \(\epsilon\) = (Molar extinction co-efficient of hydroperoxides) = 27,500.
3.6 Extraction and Purification Lipoxygenases:

Preparation of Crude Extract:

A 20% crude extract was prepared by homogenizing the Papaya seedlings in extraction buffer containing 50mM Tris-HCl pH 8.0 extraction buffer containing 10mM Kcl, 500mM sucrose, 0.5mM PMSF. The homogenate was passed through four layers of cheese cloth and was centrifuged at 10,000 rpm for 30 min at 4°C. The pellet was discarded and the resulting supernatant was used for further purification of LOX enzyme.

3.7 Ammonium Sulphate Fractionation:

The crude extract of Papaya seedlings was subjected to ammonium sulphate precipitation initially to 20% saturation by adding solid ammonium sulphate crystals under continuous stirring at 4°C. The preparation was centrifuged at 10,000 rpm for 30min at 4°C. The pellet with no activity was discarded and the resulting supernatant was brought to 20-60% saturation by further addition of solid ammonium sulphate. The sample was again centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant with no substantial activity was discarded and the pellet was resuspended in 100mM Tris HCl buffer pH 8.5. This suspension was dialyzed for 24 hr at 4°C. The dialysate was centrifuged at 10,000rpm for 30min and after centrifugation; the clear supernatant was subjected to further purification.

3.8 DEAE-Cellulose Anion Exchange Chromotography:

The dialyzed and concentrated fraction was subjected to anion chromatography. The DEAE-cellulose (2gm) was suspended in 50ml of equilibration buffer of 100mM Tris HCl pH 8.5, washed and degassed thoroughly for 30 minutes and poured into column tube (15mm x 30 cm). The gel beads were allowed to settle
gently and the column was connected to the reservoir having the same buffer. Pre-equilibration of the prepared column was carried out for about 12hr at a flow rate of 1ml/min with 100mM Tris HCl buffer pH 8.5. The dialysate was centrifuged at 10,000 rpm for 30 minutes and the supernatant was loaded on to DEAE-52 column and washed thoroughly to eliminate all unbound proteins and bound proteins were eluted with a linear gradient of 0.1M Tris Hcl -0.3M Tris Hcl gradient and 2ml fractions were collected for 35 min. Eluted fractions were screened for protein and LOX activity. The pooled enzyme was concentrated and subjected to further purification.

3.9 HPLC Analysis:

Shimpack PA-DEAE (8mmX100mm) column was equilibrated with 100mM Tris HCl buffer pH 8.5 at a flow rate of 1ml/min. The concentrated enzyme protein was loaded onto the column and the proteins were eluted using 30mM ammonium acetate and 20mM acetic acid (pH 7-8.5) at the flow rate of 1ml/min using Shimadzu L6-6 AD pumps. The effluent was continuously monitored at 280nm on a Schimadzu UV/VIS detector. Peaks with activity were collected and the purity of enzyme was checked on SDS-PAGE.

3.10 SDS-Poly Acrylamide Gel Electrophoresis:

Vertical gel electrophoresis was carried out according to the method of Laemmli (1970). The samples containing 15µg of protein was dissolved in sample buffer containing 5% SDS, 2%Mercapto ethanol (V/V) and 0.02% Bromophenol blue in 0.0625M Tris HCl, pH 6.8. Samples were boiled at 100°C for 5min and subjected to electrophoresis. The Stacking gel contained 4% Polyacrylamide in 0.125M Tris-HCl pH 6.8 and resolving gel contained 10% Polyacrylamide in 0.375M Tris HCl, pH
8.8. The ratio of acrylamide to N, N', N'-methylene bis acrylamide was 29.2:0.8. Proteins were electrophoresed at 120V for 6h with an electrode buffer containing 0.025M Tris-HCl, 0.192 M glycine and 0.1% SDS with pH 8.5. After completion of the electrophoresis, the gel was fixed in 7.5% acetic acid solution for 1hr and stained with coomassie brilliant blue containing 0.05% coomassie brilliant blue in water: methanol: glacial acetic acid (65:35:5). Later the gel was destained in solution containing water: methanol: glacial acetic acid (75:20:5). The molecular weight of the purified lipoxygenase was calculated by comparing with standard molecular weight marker.

3.12 Reverse Phase HPLC Analysis of LOX Products:

Hydroperoxy products of linoleic and linolenic acid were separated on Shimadzu HPLC using reverse phase silica column (CLC-Sil, 25X0.46cm). LOX products generally are unstable and undergo degradation rapidly. Slight acidic medium and anaerobic conditions increase the stability of these compounds. Hence methanol: water: glacial acetic acid (85:15:0.1) were used as mobile phase. The flow rate was set at 1ml/min for the analytical column. The eluted products were continuously monitored on Schimadzu SPD-6AV UV-VIS detector at 235nm.

3.13 Preparation of Standard HPODs/HPOTs:

Commercial Soybean LOX was used to generate 13-HPOD and 13-HPOT. Potato LOX was used for the generation of 9-HPOD and 9-HPOT.

3.14 Generation of 13-HOD and 9-HOD:

The products of Papaya LOX with LA as substrate were reduced with sodium borohydride. The reaction was terminated by adding 1ml of 6NHCl. The products formed were extracted in to two volumes of hexane: ether (1:1) thrice. The organic
solvent was separated from the aqueous layer in a separating funnel. Finally the organic phase was evaporated on a rotary evaporator to total dryness. The dried products, redissolved in solvent system containing methanol: water: glacial acetic acid (85:15:0.1) are used as 13-HOD. Similarly the products of Potato LOX and Soy LOX with LA as substrate were reduced with Sodium borohydride and the experiment was repeated as above. The dried products, redissolved in solvent system containing methanol: water: glacial acetic acid (85:15:0.1).

3.15 Generation of 13-HOT and 9-HOT:

The products of Papaya LOX with ALA as substrates were reduced with sodium borohydride. The reaction was terminated by adding 1ml of 6N HCl. The products formed were extracted in to two volumes of hexane: ether (1:1) thrice. The organic solvent was separated from the aqueous layer in a separating funnel. Finally the organic phase was evaporated on a rotary evaporator to total dryness. The dried products, redissolved in solvent system containing methanol: water: glacial acetic acid (85:15:0.1) are used as 13-HOT. Similarly the products of Potato ALA as substrate were reduced with Sodium borohydride and the experiment was repeated as above. The dried products, redissolved in solvent system containing methanol: water: glacial acetic acid (85:15:0.1) are used as 9-HOT.

3.16 Effect of Phytophthora palmivora infection on LOX activity of Papaya Seedlings:

The papaya seedlings (Red lady resistant to Phytophthora blight and Coimbatore No.6, susceptible to Phytophthora blight) were surface sterilized with 1% sodium hypochlorite for 5 minutes and washed three times with sterile distilled water to remove the surface borne microorganisms. Inoculation with Phytophthora palmivora.
The spore suspension of Phytophthora palmivora was prepared from 7-day old culture grown on Carrot broth and was diluted to contain approximately 6.5X10^4 spores/ml. The papaya seed were grown in autoclaved river bed sand placed in 15cm pots and incubated aseptically in an incubator under light at 25^0 C for 7 days. The seedlings were removed from sand; the root system was washed with sterilized distilled water. The seedlings were inoculated with Phytophthora palmivora by root dip inoculations for 30 min. The healthy and inoculated seedlings were harvested at 48hr interval for the measurement of LOX activity for fourteen days.

3.17 Lipid Peroxidation:

Lipid peroxidation was monitored by measuring the conversion of lipid hydroperoxides to malondialdehyde (MDA) by the Thiobarbituric acid reaction, with the minor modification of Dhinsda et al (1981). A 20% crude extract was prepared by homogenizing the Control and infected Papaya seedlings in 0.1% trichloro acetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 30 minutes at 5^0 C. The supernatant (67µl) was mixed with 933µl of 20% Trichloroacetic acid containing 0.5% Thiobarbituric acid in 1.5 ml centrifuge tubes and incubated at 95^0 C for 30 minutes. The tubes were cooled immediately in an ice and centrifuged at10,000 rpm for 10 minutes. After centrifugation, the absorbance of the supernatant solution was measured at 532nm. The value of non specific absorption was read at 600nm and substracted. The concentration of MDA was calculated using its extinction coefficient of 155mM^{-1} protein.

\[
\text{MDA/mg} = \frac{(\text{Vol of reaction mixture}) \times (\text{absorbance at 532nm - non specific absorption at 600mn})}{\varepsilon \times \text{Vol of enzyme in ml}}
\]

Where \(\varepsilon\) = (Molar extinction co-efficient of hydroperoxides) =155mM^{-1} cm^{-1}. 

3.18 Endogenous Product Analysis OF Lipoxygenase by HPLC:

Endogenous levels of LOX products were analyzed by homogenizing papaya seedlings in 100mM Tris Hcl pH 8.5 buffer. The homogenate was passed through four layers of cheese cloth and centrifuged at 10,000g for 30 minutes and the supernatant was collected. The LOX products were extracted twice with an equal volume of hexane: ether (50:50, V/V) and the phase evaporated to dryness on a rotator vaccum evaporator. The residue was dissolved in HPLC mobile phase methanol: water: glacial acetic acid (85:15:0.1).

3.19 Extraction of Total RNA:

To determine the effect of Phytophthora palmivora infection on LOX mRNA levels, the total RNA was collected from healthy and infected papaya seedlings of resistant variety by using Himedia RNA-Xpress™ Reagent according to the manufacturer’s directions, followed by RNAse-free DNase treatment. Total RNA concentration were measured at 260nm and 280nm by NanoDrop™ 1000 spectrophotometer (USA) and the purity of the total RNA extracted was determined as the ratio of optical density at 260nm to that at 280nm (OD$_{260}$ / OD$_{280}$) with values between 1.8 and 2.1. Isolated RNA was dissolved in 50µl of RNase free H$_2$O and stored at -80°C (Feng et al., 2009). The integrity of total RNAs was determined by electrophoresis on 1% (w/v) agarose gel.

3.20 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

Reverse transcription was performed using 2µg of reaction mixture according to Fermentos RevertAid™ First Strand cDNA Synthesis Kit (Code: #K1621, #1622). The reaction mixture contained 10XRT buffer, 25XdNTP mix, oligo dT primers and reverse transcriptase enzyme. The RNA samples were amplified in a thermo cycler at
25°C for 10 min, 42°C for 15 min, 99°C for 5 min and 5°C for 5 min. The resulting cDNA was used for amplification. The volume of the PCR mix was 10µl and contained 1µl of cDNA, 400nM forwarding primer 5’-CGGCAGGGA GAATGAAAATA-3’ and reverse primer 5’-TACCGCCTGATTGCTCTTCT-3’ and one unit of Taq polymerase. Samples were amplified for 3 min at 93°C for 30 sec, 58°C for 1 min, 72°C for 2 min for 35 cycles. After last cycle, PCR products were then separated by electrophoresis on 1.5% agarose gels in 0.5X Tris borate EDTA buffer and stained with ethidium bromide. The signals were integrated with scion Imaging software available on http://www.scioncorp.com.

3.21 Phylogenetic Analysis:

The partial CP-LOX cDNA (211bp) and its deduced protein sequence was first aligned with other LOX sequences (Arabidopsis thaliana L., Glycine max (L.) Merr, Nicotiana tabacum L., Hordeum distichum L., Cucumis sativus L. Gossypium hirsutum L. solanum tuberosum L, Arachis hypogea L, Sesbania rostrata L, Lycopersicum esculentum L, Zea mays L, Citrus jarnbhiri, L, Oryza sativum L, ) by means of Clustal X 2.0.11. Phylogenetic tree based on amino acid sequence was constructed using and Mega version 4.0 software.

3.22 Native Gel Electrophoresis:

To determine the expression of LOX isoenzymes, the proteins were electrophoresed on Polyacrylamide gel under non-denaturing conditions (native PAGE). The resolving gel contained 10% Polyacrylamide in 0.375M Tris HCl pH 8.8. The ratio of acrylamide to N, N’ methylene bis acrylamide was 30:0.8. The separating gel was casted on vertical slabs. The protein samples collected from healthy and infected papaya seedlings of both resistant variety (Red lady) and susceptible variety
(Co-6) were loaded onto separating gel. Soybean LOX was used as marker along with standard native protein markers. The proteins were electrophoresed at 80V for 3hr at 4°C. After electrophoresis, the gel was washed and stained with o-diansidine hydrochloride.
3.23 In-gel Staining:

Active lipoxygenase was stained according to the published procedures of the Hydeck and Schewe (1985). The LOX protein samples were loaded onto 10% native gel and after completion of electrophoresis, the gel was immediately washed with 0.1M Tris HCl buffer pH 9.0 for 15 minutes at 4°C. The gel was then incubated with substrate solution prepared by mixing one volume of 80µM substrate (linoleic acid) with equal volumes of methanol and 0.53M potassium hydroxide to give potassium linoleate or linolenate. This potassium linoleate or linolenate was then mixed with 100 volumes of fresh 0.1M Tris HCl buffer pH 8.5 along with 0.1% sodium cholate. The gel was incubated for exactly 5 minutes at 25°C. After incubation, the gel was washed quickly with 0.1M Tris HCl buffer pH 8.5 or with double distilled water to remove excess of the substrate attached to the gel. The washed gel was later stained with O-diansidine hydrochloride in 0.1M Tris HCl buffer pH 8.5. The above said procedure was carried out using 0.1 M phosphate buffer at pH 6.5

3.24 2-D Gel Electrophoresis:

The proteome profile of control and infected seedlings of resistant papaya variety was analyzed by using 2D-Gel electrophoresis. 2D-Gel analysis of the infected Papaya sample was carried out by using Bio-Rad’s Ready Prep kit.

3.25 Proteomic Analysis: sample preparation:

The healthy and infected papaya seedlings (6th day of post inoculation) of resistant variety were homogenized on ice in lysis buffer (40mM Tris, 8M Urea, 4%CHAPS and a mixture of protease inhibitors). The homogenate was centrifuged at 1000g for 30 min at 4°C. The final supernatant was collected (Saravitz and Seidow, 1995) and the protein concentration was determined as per the Lowry method.
3.26 Two-dimensional Electrophoresis:

Samples of approximately 10mg were applied on immobilized pH 3 to 10 linear gradient strips (Bio-rad) in rehydration buffer (8M urea, 2%CHAPS, 65mM dithiothereitol, 1mM PMSF and 0.2%pI ampholytes) and focused at 8000V for 2hr 30min. An equilibration buffer was prepared having 50mM Tris-HCL, pH8.8, 6M Urea, 30%glycerol and 2% SDS. Immobilized pH gradient strips were equilibrated in 2% dithiothereitol and 2.5% iodoacetamide for 15 min respectively.

3.27 Second Dimension Separation by SDS-PAGE:

Second dimension seperation was carried out by using SDS PAGE. The Stacking gel contained 4% Polyacrylamide in 0.125M Tris-HCl pH 6.8 and resolving gel contained 12% Polyacrylamide in 0.375M Tris HCl, pH 8.8. The ratio of acrylamide to N, N, N’-methylene bis acrylamide was 29.2:0.8. Proteins were electrophoresed at 90V for 35-40 min with an electrode buffer containing 0.025M Tris-HCl, 0.192 M glycine and 0.1% SDS with pH 8.5. After the completion of electrophoretic run the gel was stained using Colloidal silver staining (Van Oostveen et al., 1997; Kovarik et al., 1999) method followed by destaining.

Image Analysis:

The gels were quantified by using PD Quest 7.4.1 Software. Spot detection and the matching the gels were identified by Alpha easer 1.0.1 software.

3.28 MALDI-TOF/MS:

The silver stained G-250 stained protein band was excised from the SDS-PAGE and subjected to in-gel digestion with trypsin. The tryptic peptides were extracted with 60% acetonitrile and 5% trifluroacetic acid on to a MALDI target plate.
Mass spectra were recorded on a Bruker Daltonics Maldi-TOF mass spectrophotometer. Protein identification was performed with Mascot search engine and Clustal W (http://WWW.ebi.ac.uk.clustalW).

3.29 Production of LOX Polyclonal Antiserum:

The antibodies were raised against the LOX isozyme (LOX-2) expressed in resistant infected seedlings at pH 9.0. The area of Polyacrylamide gel corresponding to CP LOX-2 isozyme was extracted from the native gel by passive elution. In order to perform passive elution, the gel was excised and immersed in 1 ml of elution buffer containing 50mM Tris HCl, 150mM Nacl and 0.1mM EDTA pH 7.5 and incubated overnight at 30°C in a rotatory shaker. After overnight incubation, eluted gel was centrifuged at 10,000rpm for 10min and supernatant was collected carefully into microfuge tube. The protein content of the supernatant was estimated as per the following equation. An aliquot of the supernatant was tested for the presence of protein by subjecting to SDS-PAGE followed by silver staining.

\[ \text{mg protein/ml} = \left( \frac{A_{280}}{1.55} - \frac{A_{260}}{0.76} \right) n \]

where ‘n’ is the dilution factor.

3.30 Immunization of Rabbit:

Primary immunization of New Zealand female rabbit was done with 150µg of lipoxygenase protein with Freund’s incomplete adjuvant (FIA) subcutaneously with micro syringe. After one week, booster dose was made with 150µg of protein with Freund’s incomplete adjuvant (FIA). After 15 days second booster dose was given with same concentration of protein with Freund’s complete adjuvant (FCA). Test bleed was done by ear vein and blood was collected to isolate antiserum. Aliquots of serum were stored at freezing temperature and lyophilized.
3.31 Immuno Reactivity:

Radial Immuno Diffusion:

1% (w/v) agar was dissolved in phosphate buffered saline (KH$_2$PO$_4$-6.315gm, K$_2$HPO$_4$-1.09gm, Nacl-8.5gm and H$_2$O-100ml) by heating to 90°C on a water bath with constant stirring. To the molten Agarose 0.01% sodium azide and 20µl of LOX-2 antiserum was added and the mixture was coated on to the slide and kept for hardening. With help of a cork borer five wells were prepared using a template. Plugs were removed with the help of a needle and bottom was sealed with molten agarose. Two fold dilutions of the LOX extract (Antigen solution) was prepared separately and added to the first four wells (20µl each) and to the last well control sample was added. The gel plate was kept in a humidity chamber at a constant temperature (between 16°C to 20°C) for 18-24hr for precipitin ring formation. The slides were stained with coomassie brilliant blue R-250 in water: methanol: glacial acetic acid (50:40:10). Alternatively the gel was destained in solution containing water: methanol: glacial acetic acid (50:40:10). The gel was stained for 10-15 min with coomassie brilliant blue dye and was destained for appropriate period to visualize the precipitin rings clearly.

3.32 Western Blot Analysis:

The differential expression of LOX protein in resistant and susceptible genotypes after inoculation with phytophthora palmivora was determined by western blot analysis. The papaya seedlings were homogenized in extraction buffer containing protease cocktail (Leupeptin, PMSF and Apoprotein) and centrifuged at 10,000 rpm for 10 minutes. The resulting supernatants were collected and the protein concentrations were measured using Lowry method. Protein extraction and western blots were performed as per the standard methods.
LOX proteins were resolved on 10% SDS-PAGE and electro transferred to nitrocellulose membrane at 100v for 90 min. The membrane was washed with TBST buffer (0.2M Tris-HCl pH 7.6, Nacl 0.1% Tween-20) for 15 min with 5 min interval. The blot was immersed in blocking buffer (5% non-fat milk in TBST) for 30 min at room temperature. The blot was again washed with TBST for 30 min with 10 min interval. The membrane was probed with 1:500µl dilution of primary LOX-2 antibody in blocking buffer and incubated for overnight at 4ºC on a shaker. The blot was again washed for three times, 10 min each with min with 5min interval. After washing the membrane was probed with secondary antibody (1:1000) in blocking buffer and incubated for 1hr at room temperature. The bands were visualized by enhanced chemiluminiscence assay kit (Pierce) according to the manufacturer's instructions. To normalize protein loading, blot was stripped with stripping buffer (Genei Banglore) and reprobed with anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) antibody.

3.33 Screening papaya plants for Resistance against Phytophthora blight:

The ELISA plates were coated with polyclonal antibody (1 µg) raised against CP-LOX-2 and the plates were incubated overnight at 0ºC. The crude extracts (200µl) of both resistant and susceptible seedlings was added to the ELISA plate and was incubated at 37ºC for 3hr. After incubation the plate was washed for three times and CPLOX-2 polyclonal antibody was diluted (1:1,000) with the ELISA buffer, added to the wells and incubated at 37ºC for 3hr. After washing, conjugate composed of goat-anti-rabbit immunoglobulin coupled with alkaline phosphatase diluted to 1:1,000 was added to plates and incubated 1hr at room temperature. O-nitrophenyl phosphate (1mg/ml) was then added and incubated for 1hr at room temperature and the absorbance of enzyme – substrate complex was measured at 405 nm in ELISA reader.
3.34 Antimicrobial studies:

Antifungal Activity of Papaya LOX Hydroperoxides:

Antifungal activity of hydroperoxides of LA (13-HPOD, 9-HPOD) and ALA (13-HPOT, 9-HPOT) was determined by microtitre plate method (Broekaert et al., 1990) The spore suspension of Phytophthora palmivora was obtained by culturing the fungi in carrot juice broth for 5-6 days in reciprocal shaker at 20°C. Tests were performed with 10µl of test solution (13-HPOD, 9-HPOD, 13-HPOT and 9-HPOT 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°C. Controls were maintained by adding 10µl of 10% ethanol and 90µl of spore suspension. Fungal growth in the presence of hydroperoxy fatty acids was monitored at 490 nm after 12 hr of incubation. For each treatment, three replicates were maintained. The percent growth inhibition of Phytophthora palmivora was calculated using the following formula.

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

Abs of corrected control= Abs of control at 12hr-Abs of control at 30 minutes.
Abs of corrected test=Abs of test at 12hr-Abs of test at 30 minutes.

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.

**Effect of Antimicrobial Activity of Papaya LOX Hydroperoxides and Hydroxides:**

Antifungal activity of hydroperoxides of LA (13-HPOD, 9-HPOD) and ALA (13-HPOT and 9-HPOT) products of LOX against the test fungi (*Colletotrichum gleosporoides, Aspergillus niger, Aspergillus flavus and Fusarium oxysporum*) 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 1ml of spore suspension (2.5X10⁴) was pipette onto potato dextrose agar plates and evenly distributed with a glass spreader. To test the effect of hydroperoxides (13-HPOD, 9-HPOD, 13-HPOT and 9-HPOT) 14mmX2mm filter discs (Whatman No1), were placed centrally on the agar surface of previously seeded potato dextrose agar plates. Different LOX hydroperoxides (13-HPOD, 9-HPOD, 13-HPOT and 9-HPOT) dissolved in 10µl of 10% ethanol were pipette on to the filter discs on petriplates. Control plates were prepared by pipetting 10µl of 10% ethanol on to the filter discs. Plates were incubated at 25°C for 72hr. Plates were examined for zones of growth inhibition around each disc. The lowest concentration of hydroperoxide that produced, detectable zone of inhibition was considered as minimum inhibitory concentration (MIC) and is expressed as micrograms of hydroperoxide per disc. The experiment was repeated for 3 minutes. Antifungal activity of papaya LOX LA and ALA products (13-HPOD and 13-HPOT) was tested against various fungal pathogens which included
Colletotrichum gleosporoides, Aspergillus and Fusarium oxysporum. The antifungal activity was performed as described above and observed for the zones of inhibition. Similarly antibacterial activity of hydroperoxide and hydroxides of papaya LOX (13-HPOD, 9-HPOD, 9-HPOT, 13-HOD, 9-HOD, 13-HOT and 9-HOT) was assayed against the test organism i.e., Xanthomonas axonopodis, Erwinia herbicola Enterobacter cloacae and pseudomonas aeruginosa on the nutrient agar medium. Various concentrations of hydroperoxides and hydroxides (1.5µg, 3µg, 4.5µg and 6 µg) dissolved in 10% ethanol were added to each filter disc and three replicates were maintained and 10% ethanol was used as control. Plates were incubated at 37°C for 24hr and observed for zone of growth inhibition around each disc. The lowest concentration of hydroperoxide that produces 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 for 3 times.

Determination of Minimum Bactericidal Concentration of Oxylipins

To determine the minimum bactericidal concentrations, the graded levels of Cis-3-hexenol, Trans-2- hexenal and Jasmonic acid were added to the sterile 150ml conical flasks containing 50 ml nutrient broth. The flasks were inoculated with an overnight culture of Erwinia herbicola, Enterobacter cloacae, Pseudomonas aeruginosa, and Xanthomonas axonopodis and incubated at 37°C for 24hr. Growth was indicated by turbidity and measured at 660nm in a colorimeter. Control was maintained by adding 10% ethanol. The experiment was repeated thrice. When growth was observed, the oxylipins under study was considered as bacteriostatic and the oxylipins which inhibited the growth in the liquid culture were treated as bactericidal.
Antifungal activity of Oxylinps of 13-Lipoxygenase

The antifungal activity of the Cis-3-hexenol, Trans-2- hexenal and Jasmonic acid were determined against *Phytophthora palmivora*, *Colletotrichum gleosporoides*, *Aspergillus niger*, *Aspergillus flavus* and *Fusarium oxysporum*. The flasks were inoculated with the above test fungi and incubated at 37°C for 5-6 days. Different flasks of Potato dextrose broth was inoculated with the above fungi having the varying concentrations of the oxylinps (1µM, 5µM, 10µM and 15µM) and incubated on a reciprocal shaker at 37°C for 3-4 days for the growth of the fungi. After the incubation period the flasks were observed for the growth of the fungi.