DISCUSSION
The best strategy to control Phytophthora blight of Papaya would be to reduce the infection incidence by targeting the vital regulators involved in the growth and development of *Phytophthora palmivora*. A common group of metabolites playing a prominent role in pathological responses of plants are oxylipins generated by oxygenation of fatty acids. The biosynthesis of Plant oxylipins is initiated by lipoxygenases. 13-LOX and their products have gained attention as potential defense signaling compounds. However, their significance was confined to *Leguminaceae* members. In the present study, we demonstrated the role of 13-LOX in resistance of papaya to *Phytophthora* blight.

Seedlings of papaya were categorized into two major groups resistant and susceptible against *Phytophthora* blight. Based on the preliminary screening of papaya for lipoxygenase activity, Red Lady (resistant to *Phytophthora* blight which showed maximum activity was selected for the present study. The other variety of Papaya, Coimbatore No.6 (susceptible to *phytophthora* blight) was selected for comparative study with the previously mentioned resistant variety. The lipoxygenase extracted in the present study was active at pH 6.5 and 9.0 (Venere et al., 2003; Gardner et al., 1996). Most lipoxygenases have been classified as two types based on pH profile (Seidow, 1991). Type-1 lipoxygenase has an optimum activity at pH 9.0-10.0. Type -2 lipoxygenase generally have pH optima of 6.0 -7.0. Most plant lipoxygenases belong to type-2 form, Soybean lipoxygenase-1 appearing to be an exception (Galliard and Chan, 1980). The pH profile of papaya lipoxygenases studied showed an optimum pH of 6.5 and 9.0. Based on its optimum pH, papaya LOX may be classified as having both Type-1 and Type-2 LOX. Lipoxygenase had been estimated in different stages of Leaf, flower and fruit. Among the three maximum lipoxygenase activity was recorded in fruit, leaf followed by flower.
During germination in papaya seedlings showed an increase in LOX activity within 9-13 days of germination followed by an equally substantial decrease in LOX activity over the subsequent hours. The rapid increase in LOX activities observed shortly after germination indicates a role for LOXs in regulating cellular activities during plant growth and development. Increase in lipoxygenase activity soon after germination and during early stages of seedling growth have been reported for a number of species including rice (Ohta et al., 1986), Pigeon pea (Uma et al., 2000) and chilli (Sucharitha et al., 2010). The highest LOX activity was observed in soybean during leaf growth (Saravitz et al., 1995) and LOX activity level was reported as being positively correlated with the rate of elongation of an organ (Seidow et al., 1991). Potato LOX1 is expressed during tuber growth, and its antisense suppression resulted in smaller tubers (Kolomiets et al., 2001). Maize LOX3 (ZMLOX3) knockout mutants showed shorter roots and increased senescence (Gao et al., 2008). The olive 9/13-LOX gene is predominantly expressed during fruit ripening and is believed to be associated with senescence in the plant (Palmieri-Thiers et al., 2008). A similar observation was made in kiwifruit LOXs (Zhang, 2009). Some LOXs, such as r9-LOX1 and OsLOX1 in rice (Mizuno et al., 2003; Wang et al., 2008) and PdLOX in almond (Mita et al., 2001), were found to be involved in germination.

Hydroperoxide lyase (HPLS) is a membrane bound enzyme found in higher plants. Hydroperoxide lyase is expressed at various levels in tissues or organelles of higher plants in soybean, HPLS was found both in the seeds (Matsui et al., 2006) and Leaves (Kallenbach et al., 2011). Papaya seedlings showed maximum HPLS activity in fruits compared to leaf and flower. In cucumber seedlings, on the other hand root possessed the highest activity (Shoresh et al., 2005). In maize HPLS activity was
found in leaves and it appears to be absent in seeds due to the predominance of hydroperoxide dehydrase (Nemchenko et al., 2006). In maize leaves, the enzyme was mainly located in chloroplast located in chloroplast membranes. In pigeon pea, the hypocotyls-root section possessed the highest activity and is absent in leaves.

LOXs have been isolated and purified from many sources in plant system. The most extensively studied plant LOXs are from soybean (Axelrod, 1981; Galliard and Chan, 1980; Shibata et al., 1987) and potato (Seikya et al., 1977; Schimizu et al., 1984; Reddanna et al., 1990). In the present study, Papaya LOX was purified with an overall purification fold of 4.50 with specific activity of 53.2 U/mg. The purification profile exhibited the similarity with chilly LOX (Sucharitha et al., 2010). The enzymes from Pea, Cow Pea, winged bean, cucumber and brinjal reported to have molecular weights in the range of 65.80 kDa, 95kDa and 95 kDa respectively (Veronico et al., 2006). The purified papaya LOX resolved had a molecular weight of approximately ~68kDa. Generally, most of the plants contain lipoxygenases with molecular weight in the range of 94-104kDa (Shibata & Axelrod, 1995). Lorenzi et al (2004) reported that lox purified from Olive fruit had a molecular mass of 98kDa. The molecular mass of soluble LOX from Tomato fruit was 95kDa (Suurmeieijer et al., 1998) and LOX from pea seeds, 93kDa (Szymanowska et al., 2009). But also plant LOXs with lower molecular weight were reported for example, Pearl millet lipoxygenase isozymes had molecular masses 83kDa, 77kDa and 73kDa (Babitha et al., 2004).

From the product profile of Papaya lipoxygenase it can be observed that lipoxygenase preferentially oxygenates at 13th position and producing 13-hydroperoxy fatty acids from LA and ALA as substrates. In addition, it also
oxygenates at 9th position but to a lesser extent. The ratio of 13-hydroperoxides to 9-hydroperoxides was found to be 8:2. Papaya lipoxygenase thus produces 13-hydroperoxyfatty acids, which appears to be different from potato and others which oxygenate at the 9th position majorly and similar results were reported in soybean, which exhibited both 13- and 9-hydroperoxides in 9:1 ratio at pH 9.0. Barley lipoxygenase has an optimum pH of 6.5 and yields predominantly 9-hydroperoxides (Sharma et al., 2005). Maize LOX-2 active in a pH range from 6.0 to 9.0 and catalyzes the formation of 9-hydroperoxides (Gao et al., 2008).

Plants, in response to attack by microbial pathogens, activate a complex and highly coordinated set of defense responses (Tumlinson et al., 2008). In a successful defense response, plants typically undergo hypersensitive reactions leading to small necrotic lesions at the site of infection, which prevents the growth of the pathogen (Mukthar et al., 2008). These hypersensitive responses initiated by specific recognition event, between pathogen derived elicitor and a host receptor, triggers the induction of a variety of defense related genes (Mueller et al., 2008). A major difference between resistance and susceptibility to pathogens is in the timing of induction of these host defense response and more slowly, of at all, in a disease reaction.

In the present work, a high level expression of LOX activity in the resistant papaya cultivar and low level expression of LOX activity in the susceptible variety were observed. In the resistant seedlings, the increase in LOX activity upon pathogen infection was within 48hr of inoculation and reaching maximum on the day 6 of inoculation. The resistant variety showed about 0.8 fold increase in LOX activity on day 6 of post inoculation. In susceptible seedlings, on other hand, the LOX activity
decreased after inoculation with the fungal pathogen. Thus, resistant and susceptible variety showed differential expression of lipoxygenase activity in response to the pathogen infection. Increased LOX activity has been observed in several incompatible host-pathogen combinations (Chehab et al., 2008; Chandra Shekara et al., 2007; Dangl and Jones, 2001). Similar results were observed in pearl millet infected with *Sclerospora graminicola*. The LOX activity increased in the resistant seedlings compared to respective uninoculated controls. In pigeon pea infected with Fusarium udum, the LOX activity increased 10 fold in the resistant cultivar over a period of 4 days following infection (Uma et al., 2000). Other examples include, the induction of lipoxygenase activity up to 7fold in tobacco leaves over a period of 11 days following infection with *Erysiphe cichoracearum* (Lupu et al., 1980). In potato tubers also, LOX activity in response to infection might be an indication that a defense mechanism and is initiated in resistant genotypes (Zhao et al., 2010) Reported an increase in LOX activity associated with a bacterial induced hypersensitive reaction in cucumber cotyledons.

Increased LOX activity and rapid lipid peroxidation are a general response to biotic and abiotic stresses. The increase in LOX activity has been observed in plant tissues and cells in response to infections with bacterial, fungal and viral pathogens and to application of elicitor preparations. One possible role for the involvement of LOX metabolism in plant protection can be production of compounds participating in signal transduction mechanisms. Avdiushko and Kuc (1993) showed that the content of polyunsaturated fatty acids (linoleic and linolenic) decreased as the LOX activity increased in the cucumber leaves following infection. Systemic protection and biochemical responses induced by localized infection or treatment with chemicals suggest the presence of a systemically translocated signal in induced resistance. Plant
LOX metabolites such as jasmonic acid, methyl jasmonates and other volatile hexenals, nonenals, etc., could be transported systemically and regulate the defense mechanisms in the upper leaves of plants (Avdiushko et al., 1993). LOX may also have a direct role in the protection of plant tissues. Lipid peroxidation is considered to be of major importance in some aspects of pathogen resistance, particularly in hypersensitive response (Keppler and Novacky., 1987). LOX activity produces hydroperoxides which can generate the reactive oxygen species (ROS) capable of initiating enzyme-independent lipid peroxidation (Anderson, 1989). Our results support the findings of Keppler and Novacy (1986 and 1987) who reported that an increase in LOX was accompanied by peroxidation measured as an increase in level of melandialdehyde after inoculation of cucumber cotyledons with *Pseudomonas syringe pv pisi*. They suggest that free radical induced lipid peroxidation might cause the membrane damage leading to host cell necrosis followed by the attack of free radicals and lipid peroxides on the invading pathogen. In fact in the present study increased rate of lipid peroxidation was observed in the highly resistant papaya immediately after inoculation, suggesting such a possibility.

The relative ratio of 13-HPOD and 13-HPOT in healthy papaya seedlings was 2:1. In infected conditions the relative ratio of 13-HPOD and 13-HPOT was 1:3. 13-HPOT is the major LOX product formed in response to infection which is the precursor of Jasmonic acid or traumatic acid indicating the operation of a new pathway of LOX mediated defense responses in papaya seedlings. This may be possible with either the specific release of ALA from membrane phospholipids in response to infection or increased LOX specificity to ALA during infection. LOX metabolites of octadeca dienoic acids may be involved in mediating the physiological responses, while octadecatrienoic acid metabolites may be mediating defense
responses under stress conditions in papaya seedlings. Similar results were obtained in pigeon pea seedlings infected with Fusarium udum, (Uma et al., 2000; Fliegmann et al., 2003; Wilson et al., 2004).

Differential gene expression and activity of LOX in compatible and incompatible interactions reinforce the hypothesis of a relationship between LOX and resistance (Veronesi et al., 2006). The isolated papaya LOX cDNA allowed us to study LOX gene expression in Papaya during the interaction with the fungal pathogen Phytophthora palmivora. LOX gene expression was investigated in several plant-pathogen interactions and LOX transcripts were shown to accumulate in rice plants upon inoculation with the fungus M. grisea (Zhou 2009) and in tomato, bean, and Arabidopsis thaliana plants inoculated with Pseudomonas (Delaplace et al., 2009; Meier et al., 1993; Han et al., 2001; Bieza and Lois 2001). The mRNA profiles of the present study indicate high levels of expression in infected seedlings when compared with healthy seedlings, suggesting that pathogen perception plays important role in plant defense mechanism (Slusarenko et al., 1993; Lee et al., 2003).

The phylogenetic tree analysis deduced using amino acid sequences from databases (Clustal W and Mega version 4.0 software) showed homology pattern of papaya lipoxygenase with other lipoxygenases indicating the existence of 13-lipoxygenases in carica papaya (Feussner and Wasternack, 2002; Marmey et al., 2007).

Multiple forms or isozymes of lipoxygenase have been detected in both animal and plant species (Baysal and Dermirdoven, 2007; Skrzypczak et al., 2000 & 1997; Seidow et al, 1991). Although multiple isozymes of lipoxygenase have been identified in many plant species, the physiological role of any specific plant lipoxygenase
isozyme has yet to be established. Given the presence of multiple isozymes of lipoxygenase in plants, it is possible that individual lipoxygenase isozymes within a plant may have distinct physiological roles. Induction in host lipoxygenase activity and individual lipoxygenase isozymes after infection with bacterial and fungal pathogens have been observed for several plant species. In the present study, the LOX isozyme analysis was done by activity staining of the day 6 of post inoculated resistant seedlings which revealed the presence three of LOX isozymes designated as LOX-1, LOX-2 and LOX-3. Lipoxygenase isozymes operated in both wound and pathogen induced defense signal transduction pathways (Feussner, 2002) while others, like POTL-3 and tobacco LOX1 (Kolomiets 2001) appear to have a specialized function in pathogen induced defense responses only. Pea seed lipoxygenases are characterized by two major isoforms of 95kDa and two less abundant isozymes (Leone et al., 2001; et al., Veronico et al., 2006). Barley lipoxygenase has only one isozyme localized in the germ (Lee et al., 2003).

To test the effect of blight infection on papaya seedling protein expression, 2D-electrophoresis technique was carried out. Approximately thirty spots (each in Healthy and Infected seedlings of Papaya) were observed in 2D gels from healthy and infected seedlings. The relative levels of 9 proteins were elevated and a new protein with a molecular size of 116 kDa and at pI 9.0 was expressed in infected seedlings. Other examples include, in soybean seeds lipoxygenase isozymes have been characterized by IEF and pI values of 5.68, 6.25 and 6.15 have been observed for Lipoxygenase-1, -2 and -3 respectively(Akram et al., 2008). In tobacco cell suspension, the LOX isozyme was found to have a pI of approximately 5.1 and a molecular weight of 96 kDa (Hamberg et al 2003). In maize, the pI of LOX-1 was found to be 6.4 with a molecular weight of 100kDa and that for LOX-2, the pI was
found to be 5.5-5.7 with a molecular weight of 90kDa (Dimitrios et al., 2005). Barley lipoxygenase has a molecular mass of approximately 90kDa and an isoelectric point of almost 5.2 (Hirota et al., 2005; Gausing K. 2000).

The identity of the induce protein (CPLOX-2-116kDa) form 2D gel was carried out with MALDI-TOF/MS analysis. This study shows that peptide mass fingerprinting especially in combination with MS/MS, is an excellent tool to identify proteins. Rep et al 2002, applied two dimensional PAGE to allow more extensive coverage of the proteome of xylem sap of infected tomato. The protein of interest were punched out of gel and destained. The tryptic peptides were extracted with 60% acetonitrile and 5% trifluoroacetic acid on to a MALDI target plate. The MALDI-TOF/MS analysis was performed on Bruker Doltonics Company. The resulting peptide spectra were used to search data bases to yield more comprehensive results. The search reveals the highly established homology of the protein to papaya lipoxygenase. The specific LOX isozyme (LOX-2) induced during the infected condition was used for the production of antibodies.

The production of antiserum is a very important step for the immunological experiments. The induction of LOX protein in resistant papaya seedlings may be confirmed by anti-LOX antibody. The developed polyclonal antibody showed the cross reactivity with the LOX antigen in the form of precipitin ring (Peterman and Siedow, 1985) found that extracts from roots, hypocotyls and leaves did not form precipitin lines in double diffusion tests. Antibodies raised against Soy LOX1 did not cross react with commercial Dolichos biflorus lectin or with the bean lipoxygenase. The expression of LOX proteins was analyzed in resistant and susceptible genotype after inoculation with Phytophthora palmivora by western blot analysis. In the
incompatible interaction which was associated with hypersensitive response, a single band with relative molecular weight approximately 116 kDa was revealed by probing nitrocellulose blots of enzyme extracts with antiserum raised against LOX-2 isozyme. In susceptible extracts, the protein band was not detected. This provides the evidence for the involvement of LOX in the disease resistance. Indirect ELISA was conducted to test the susceptible and resistant varieties using the anti CPLOX-2 antibody. In the susceptible varieties (Co1,Co2 and Co6) no color indication suggest the absence of CPLOX-2 protein, on the other hand, in resistant varieties(Red Lady, Surya and Know-YOU) yellow color indicates the presence of CPLOX-2 protein.

LOX may also have a direct role in the protection of plant tissues. LOX metabolites acts as an 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 papaya LOX products of both LA and ALA were screened for their antimicrobial effects by spore germination method, microtitre plate method and filter-disc method.

Microscopic visualization approach was used to investigate the effect of Hydroperoxy fatty acids on germination of Sporangia. The LOX products showed potential effect on the development of sporangia, an important stage in life cycle of Phytophthora palmivora. This 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.
The antimicrobial activity of Papaya LOX products (13-HPOD, 9-HPOD, 13-HPO, 9-HPOT, 13HOD, 9-HOD, 13-HOT, and 9-HOD) were tested against different fungal and bacterial pathogens. The filter disc assay (Sucharitha et al., 2010; Muni kumari et al., 2011) showed that both hydroperoxides and hydroxides of LA and ALA were effective against Colletotrichum gleosporoides, Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Erwinia herbicola, Enterobacter cloacae, Xanthomonas axonopodis and pseudomonas aeruginosa. The hydroperoxides of LA and ALA showed inhibition at concentration of 3µg/disc and hydroxides of LA and ALA showed inhibition at a concentration of 1.5µg/disc. Among all the papaya products, ALA products showed maximum inhibition of the organisms in comparison to the LA products.

Similarly Cis-3-hexenol, Trans-2-Hexenal and Jasmonic acid, the metabolites of 13-lipoxygenase pathway, are tested for their antimicrobial activity against different bacterial and fungal pathogens like Colletotrichum gleosporoides, Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Erwinia herbicola, Enterobacter cloacae, Xanthomonas axonopodis and pseudomonas aeruginosa. Trans-2-Hexenal, the highly potential antimicrobial agent prevented the growth of all bacteria and fungi at low concentration (1µM). Cis-3-Hexenol and Jasmonic acid showed inhibition starting from 5µM concentration. A direct relationship between amount of oxylipin (Cis-3-Hexenol, Trans-2-Hexenal and Jasmonic acid) and retardation of bacterial growth as well as fungal growth was observed. From these observations, it appears that ALA metabolites of LOX pathway might be involved in mediating the defense responses of the plant against the Phytophthora blight.
Similar inhibitions in germinations of conidia of rice blast, *Pyricularia oryzae* by LOX hydroxides (13-HPOT and 9-HPOT) (Shimura et al., 1983) and Aspergillus niger by LOX hydroperoxides (13-HPOD and 9-HPOD) (Sailaja et al., 1997) and inhibition of cyospore germination of *Phytophthora capsici* by 9- and 13HPOTs as well as the hydroxy derivatives of arachidonic acid were reported (Ricker and Bostock, 1994).

It can be concluded that LOX activity is higher in the resistant variety of the Papaya which further induced in response to infection with *Phytophthora palmivora*. Also the LOX hydroperoxides formed from ALA are potent antimicrobial agents and the ALA pathway is quite operative in the pathogen infected conditions, suggesting their possible involvement in the development of resistance. The resistance development appears to be mediated by the hydroperoxy derivatives of ALA. The present study indicates that both 13 and 9-LOX metabolism is involved in Papaya seeds against *Phytophthora palmivora*. Direct pathogen control via antimicrobial oxylipins in plants suggests that 13-LOX derived oxylipins might indeed be important contributors to the outcome of give plant-microbe interactions. The production of antibodies against LOX protein is useful in screening the resistant varieties of the papaya by observing the cross reactivity between the LOX antigen and the LOX polyclonal antisera. The present study provides correlative evidence for induction of LOX activity and expression of isozymes in resistant papaya seedlings. Hence LOX can be used as a diagnostic tool for resistance in screening different varieties of papaya.