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Lipoxygenase as a Diagnostic Marker for Resistance to Anthracnose in Chilli (*Capsicum Annum*)

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Abstract

In this paper, biochemical and immunochemical evidence for the induction of Lipoxygenase (LOX) during plant-fungal interaction is presented. LOX activity was studied in seedlings of chilli (Capsicum annum) genotypes resistant and susceptible to Anthracnose pathogen *colletotrichum capsici.* An increase in LOX activity was observed in incompatible interaction where as the activity decreased in compatible ones. Resistant chilli genotype showed 3.5 fold increase in LOX activity after 9 days of inoculation with the pathogen. Native-gel assay revealed three isozymes LOX-1, LOX-2 and LOX-3 in both susceptible and resistant genotypes. Additional isozymes LOX-4 and LOX-5 were unique only to resistant genotype after inoculation. Immnoassay by Western blot also showed an induction of LOX protein in the infected resistant chilli genotype.

Keywords: *Capsicum annum*, *Colletotrichum capsici*, 9-HPODE, 9-HPOTrE, Lipoxygenase.

Introduction

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

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physiological functions in plants, and among these are the generation of oxylipins (Hamburg and Gardner, 1992) that can serve in signaling functions during episodes of stress. When plant tissues are injured by insects or pathogens, lipid-degrading enzymes are activated and released (Naravaez- Vasquez et al. 1999). This provides the necessary polyunsaturated fatty acids and the hydroperoxides thus generated can be further metabolized to biologically active compounds like jasmonic acid and traumatic acid. These compounds evoke a variety of cellular responses (Farmer and Ryan, 1990; Farmer et al., 1992; Rosahl, 1996).

Literature is replete with many examples showing induction of LOX during plant-microbe interactions. While the list is exhaustive, we have reviewed a few such as 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), potato infected with *Rhizoctonia solani* (Reddy et al., 1992), tomato, infected with Powdery mildew (Kato et al., 1992), bean inoculated with *Pseudomonas syringae* (Croft et al., 1990) and pigeon pea infected with *Fusarium udum* (Uma et al., 2000). Keeping the importance of LOX in defense responses, the present study has been carried out to establish LOX as a biochemical marker for disease resistance.

Materials and Methods

Chilli Seeds and Fungal culture

Chilli (*Capsicum annum*) seeds, susceptible (CA-960, Sindhur) and resistant (G4, Bhagya Lakshmi) to Anthracnose, were collected from LAM farm, Department of Agriculture, Guntur, Andhra Pradesh. An isolate, *Colletotrichum capsici* of ITCC No.2041 was obtained from Indian Agricultural Research Institute, New Delhi.

Seedling inoculation and sampling

Seeds of chilli varieties, G4 and CA-960, resistant and susceptible to Anthracnose respectively, were surface sterilized and grown under constant light at 25ºC for a period of 15 days. The spore suspension of *Colletotrichum capsici* was prepared from 7-d old culture grown on Potato dextrose broth and was diluted to contain approximately $6.5X10^4$ spores/mL. Each chilli variety weighing 25g of seeds was soaked for 30 min in 100mL of spore suspension. These seeds were later rolled in germination towels and kept under constant light at 25ºC until germination. The germinated seedlings were harvested at 48h interval for 15 d. The seeds soaked in distilled water were taken as controls.

Determination of LOX Activity

The seedlings (1g) were homogenized with 100mM potassium phosphate extraction buffer (pH 7.0). The homogenate was passed through four layers of cheesecloth and was centrifuged at 10,000 *rpm* for 30 min at 4ºC. The resulting supernatant was used as the enzyme source. The LOX activity of the chilli seedlings was assayed at 48h interval for 15 d by UV-Spectrophotometer at 235ηm (Schimizu et al., 1984). The reaction mixture consists of 2.9mL of 0.1M potassium phosphate buffer pH 6.5 and

100µL of enzyme. Reaction was initiated by the addition of 10µL of substrate (linoleic or α-linolenic acid) to give a final concentration of 250µM. The enzyme activity was expressed as units/mg protein wherein one unit is defined as 1µ mole of hydroperoxide formed per minute. Protein content was estimated according to the method of Lowry et al., (1951) using bovine serum albumin as standard. Specific activity was calculated as units /mg protein.

LOX Isosymes pattern on Native-PAGE

The crude extracts of harvested chilli seedlings were electrophoresed through 10% native-PAGE under cold conditions. On completion of electrophoresis, the gel was washed and incubated with 0.53 M potassium linoleate and 1 % sodium cholate for 5 min. at 25°C. After incubation, the gel was washed with 100mM potassium phosphate buffer and stained with O-diansidine hydrochloride for colour development (Heydek and Schewe, 1985).

Raising of Antibodies

The antibodies were raised against the lipoxygenase isozyme (L4) expressed in infected seedlings at pH 6.5. Primary immunization of mouse was done with 75µg LOX protein with Freund's Complete Adjuvant (FCA) subcutaneously into four sites with micro syringe. After 12 to 15 days, booster dose was made with 75 µg protein in Phosphate Buffer Saline. Test bleed was done by tail bleed and blood was collected from heart puncture to isolate antiserum. Aliquots of antiserum were stored at freezing temperature and used for Western Blot Analysis.

Western Blot Analysis

Resistant (G4) and susceptible (CA-960) chilli seedlings were inoculated with *Colletotrichum capsici* and harvested after 9 days of infection. The cytosolic proteins (30µg) of harvested chilli seedlings were resolved by electrophoresis on 10% SDS-PAGE. After electrophoresis, the proteins were electro transferred to nitrocellulose membrane (Bio-Rad) and blocked with 10% non fat milk in TBST (10mM Tris-HCl pH 8.0, 150mM NaCl, 0.05%Tween-20). The levels of LOX protein were detected using a 1:500 dilution of polyclonal anti-mouse LOX antibody, followed by incubation with a 1:5000 dilution of a horseradish peroxidase-conjugated IgG secondary antibody. The membrane was rinsed with TBST between all hybridizations, and the secondary antibody was visualized using Enhanced ChemiLuminescence (ECL) assay kit (Pierce). Glyceraldehyde 3-phosphate(GAPDH) was used as loading control. Monoclonal antibody for mouse anti–GAPDH (Chemi-Con International Inc.) and appropriate Peroxidase-conjugated antimouse secondary antibodies (Sigma chemicals) were used. The membranes were also stained with Ponceau Red to verify equal loading and uniform transfer of proteins.

Protein Estimation

The protein content was measured by the Bradford method (Bradford, 1976) with the Bio-Rad protein assay using crystalline Bovine Serum Albumin (BSA) as a standard.

Statistical Analysis

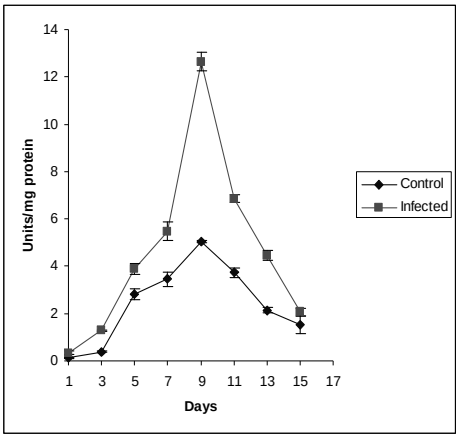
All the experiments were repeated three times. The relation between the time interval and LOX activity was expressed as mean ± standard error.

Results and Discussions

Differential induction of **LOX** activity during infection of resistant (G4) and susceptible (CA-960) chilli genotypes were studied with LA and ALA as substrates. The constitutive LOX activity was more in resistant chilli seedlings than susceptible genotype. Resistant genotype, upon infection, showed a steady increase in LOX activity from day 1 of infection and reached maximum by day 9 compared to uninoculated control. The maximum induction of LOX activity (2.6 and 3.5 fold) was recorded on day 9 of infection with AL and ALA as substrates respectively (Figure I-A and I-B). In suspectible seedlings, the LOX activity decreased at all intervals in comparison with uninoculated control seedlings (Figure II-A and II-B). Similar results were reported in other plant-microbe systems (Koch et al., 1992; Melan et al., 1993; Uma et al., 2000). The resistant and susceptible chilli genotypes showed differential pattern in the activity levels of LOX in response to pathogen infection.

The isozyme pattern of LOX in chilli genotype is characterized by native gel analysis. The native gel showed different banding pattern in resistant and susceptible genotypes (Figure3). The native gel showed the presence LOX-1 and LOX-2 isozymes both in control and inoculated susceptible genotype. The LOX-1 isozyme nearly corresponded to the soya LOX. LOX-3 isozyme is constitutive both in control and inoculated seedlings of resistant genotype. But, inoculated resistant genotype revealed the induction of LOX-4 and LOX-5 which were not detected in any other sample. However, the band representing LOX-4 was darker than LOX-5 isozyme **.** The highest LOX activity recorded in the 9 d inoculated seedlings may be due to the induction of these new LOX isozymes, L4 and L5. The induction of LOX-4 isozyme was more prominent than LOX-5. These findings indicate that the expression of new isozyme LOX-4 is involved in defense against the pathogen**.**

We analysed the expression of LOX protein in resistant and susceptible genotype after inoculation with *colletotrichum capsici* by Western Blot Analysis. In the incompatible interaction which was associated with hyper sensitive response, a single band with relative molecular wt. approximately 85 KDa was revealed by probing Western Blots of enzyme extracts with antiserum raised against LOX-4 isozyme (Figure 4). In susceptible extracts, this band was not detected. This provides the evidence for the involvement of LOX in the disease resistance.



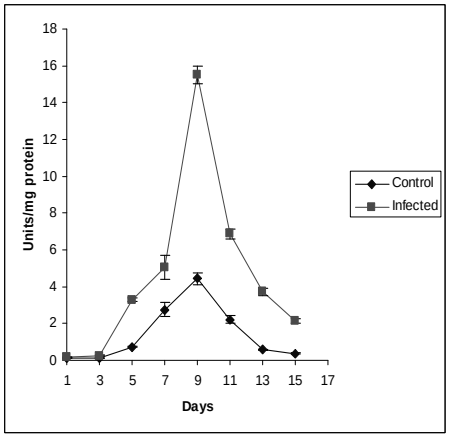
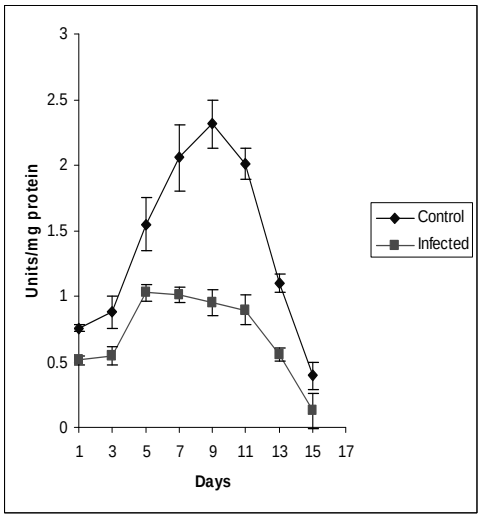
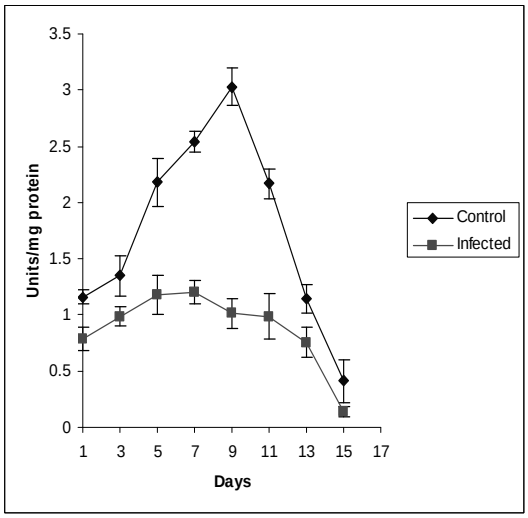


Figure 1-A Figure I-B

Figure 1: Lipoxygenase activity in control and *colletotrichum capsici* inoculated seedlings of resistant genotype (G4) at different time intervals with a) LA and b) ALA as substrates. Data are the mean ± standard error from three independent experiments.





2-A 2-B

Figure 2: Lipoxygenase activity in control and *colletotrichum capsici* inoculated seedlings of susceptible genotype (CA-960) at different time intervals with a) LA and b) ALA as substrates. Data are the mean ± standard error from three independent experiments.

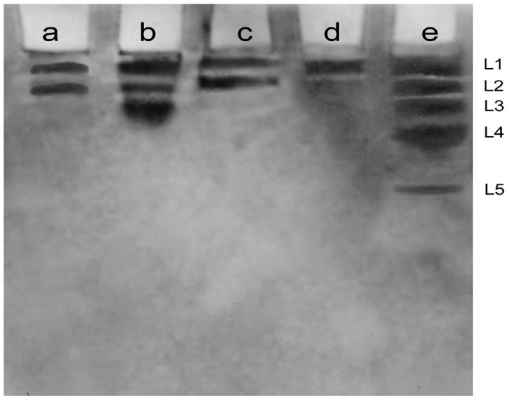


Figure 3: Characterization of lipoxygenase isozymes using non-denaturing polyacrylamide gel electrophoresis (10%). 30 µg protein of the crude extract was loaded per lane and stained with 0-diansidine hydrochloride for lipoxygenase activity. (Lane-a) Susceptible control seedlings, (Lane-b) Resistant control seedlings, (Lane-c) Inoculated susceptible seedlings, (lane-d) soy lipoxygenase, (Lane-e) Resistant inoculated chilli seedlings.

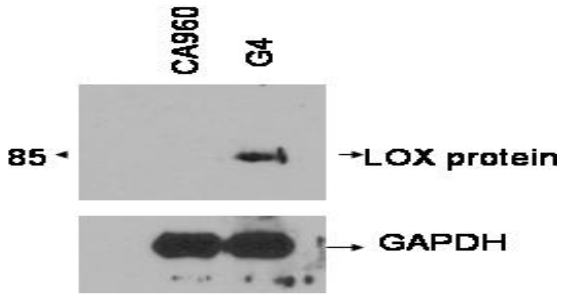


Figure 4: The expression levels of LOX protein in G4 (resistant) and CA-960 (susceptible) chilli seedlings by Western Blot Analysis. Proteins (30 µg) separated on 10% PAGE were Electro blotted onto nitrocellulose membrane. The blot was blocked with 10% milk and treated with anti LOX primary antibody (1:500) over night and finally with antimouse secondary antibody (1:5000) for 1 h. The bands were visualized by enhanced chemiluminescence (ECL) assay kit (Pierce). GAPDH shows equal protein loading.

Conclusion

The present study provides correlative evidence for induction of LOX activity and disease resistance. Hence, LOX can be used as a diagnostic tool for resistance in screening different varieties of chillies against Anthracnose.

Acknowledgements

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References

[1] Bradford M.M., 1976. “A rapid and sensitive method for the qualification microgram of protein utilizing the principle of protein dye binding.” Analytical Biochemistry, 72, pp. 248-254.

[2] Croft, K.P.C., Voisey, C.R., and Slusarenko, A.J., 1990. "Mechanism of Hypersensitive cell collapse: correlation of increased Lipoxygenase activity with membrane damage in leaves of *Phaseolus vulgaris* (L.0 inoculated with an Avirulent race of *Pseudomonas syringae pv. Phaseolicola,"* Physiolgical and Molecular Plant Physiology, 36, pp. 49-62.

[3] Desender, S., Andrivon, D., and Val, F., 2007. "Activation of defense reactions in Solanaceae: where is the specificity?" Cellular Microbiology, 9 (1), pp. 21-30.

[4] Farmer, E.E., Johnson, R.R., and Ryan, C.A. 1992. "Regulation of expression of Proteinase inhibitor genes by Methyl Jasmonate and Jasmonic acid," Plant Physiol**.,** 98, pp. 995-1002.

[5] Farmer, E.E., and Ryan, C.A., 1990. "Interplant communication: Air borne methyl jasmonate induces synthesis of proteinase inhizbitors in plant leaves," Proc. Natl Acad. Sci. USA, 87, pp. 7713-7716.

[6] Fournier, J., Pouenat, M.L., Rickauer, M., Rabinovitch-Chable,H., Rigaud, M. and Esquerre-Tugaye, M.T., 1993. "Purification and characterization of elicitor- induced Lipoxygenase in tobacco cells." Plant J, 3, pp. 63-70.

[7] Gardner, H.W., 1991. "Recent investigations into the Lipoxygenase pathway of plants." Biochim Biophys. Acta, 1084, pp. 221-139.

[8] Hamberg, M., and Gardner, H.W., 1992. "Oxylipin pathway to jasmonates: biochemistry and biological significance." Biochim. Biophys. Acta, 1165, pp. 1-18.

[9] Hamberg, M., and Fahlstadius, P., 1990. "Allene oxide cyclase. A new enzyme in Plant Lipid Metabolism." Arch Biochem Biophys, 276, pp. 518-526.

[10] Heydeck, D., and Schewe, T., 1985. "Improved procedure for the detection of lipoxygenases on Electrophoregrams." Biomed Biochem Acta, 44, pp. 1261-1263.

[11] Kato, T., Maeda, Y., Hirukawa, T., Namai, T., and Yoshika, N. 1992. "Lipoxygenase activity increment in infected tomato leaves and oxidation product of linolenic acid by its in vitro enzyme reaction. Biosci." Biotech. Biochem., 56**,** pp. 373-375.

[12] Koch, E., Meier, B.M., Eiben, H.G and Slusarenko, A.J.,1992. "A lipoxygenase from leaves of tomato (*Lycopersicum esculentum* Mill.) is induced in response to plant pathogen *Pseudomonas*." Plant Physiology, 94, pp. 571-576.

[13] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall R.J., 1951. "Protein measurement with the Folin Phenol reagent." Journal of Biological Chemistry, 193, pp. 265-75.

[14] Melan, M.A., Dong, X., Endara, M.E., Davis, K.R., Ausubel, F.M. and Peterman, T.K., 1993. "An Arabidopsis thaliana Lipoxygenase gene can be induced by pathogens Abscissic acid and methyl jasmonate." Plant Physiol., 101, pp. 441-450.

[15] Naravaez- Vasquez, J., Florin- Christensen, J., and Ryan C.A., 1999. "Positional specificity of a phospholipase A activity induced by wounding,

systemin and oligosaccharide elicitor in tomato leaves." Plant Cell, 11, pp. 2249-2260.

[16] Rance, I., Fournier, J., and Esquerre- Tugaye, M. T., 1998**.** "The incompatible interaction between *Phytopthora parasitica* var. *nicotianae* race 0 and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences." Proc. Natl. Acad. Sci. USA, 95, pp. 6554-6559.

[17] Reddy, G.K., Reddanna, P., Reddy, C.C., and Curtis, W.R., 1992. "11-hydroperoxy eicosatetraenoic acid is the major dioxygenase product of lipoxygenase isolated from hairy root cultures of *Solanum tuberosum.*" Biochem Biophys Res. omm." 189, pp. 1349-1352.

[18] Robertson, E.F., Dannelly, H.K., Malloy, P.J., and Reeves, H.C., 1987. "Rapid isoelectric focusing in a vertical polyacrylamide minigel system." Analytical Biochemistry, 167, pp. 290-294.

[19] Rusteruccci, C., Montillet, J. L., Angel, J. P., Battesti, C., Alonso, B., Knoll, A., Bessoule, J. J., Etienne, P., Suty, L., Blein, J.P., and Triantaphylides, C., 1999. "Involvement of lipoxygenase dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves." J. Biol. Chem., 274, pp**.** 36446-36455.

[20] Ruzicska, P., Gomhos, Z., and Farkas, G.L., 1983. "Modification of the fatty acid composition of phospholipids during the hypersensitive reaction in tobacco." Virology, 128, pp. 60-64.

[21] Sabine Rosahl., 1996. "Lipoxygenases in Plants - Their role in Development and stress response." Verlag der Zeitschrift fur Naturforschung, 51, pp. 123-138.

[22] Saravitz, D.M., and Siedow, J.N., 1995. "The Lipoxygenase isozymes in soybean [*Glycine max* (L.) Merr] leaves; Changes during leaf development, after wounding and following reproductive sink removal." Plant Physiology, 107, pp. 535-543.

[23] Schimizu, T., Radmark, O., and Samuelsson, B., 1984. "Enzyme with dual lipoxygenase activities catalyzes leukotriene A4 synthesis from arachidonic acid." Proc. Natl. Acad.Sci.USA, 81**,** pp**.** 689-693.

[24] Siedow, J.N., 1991. "Plant Lipoxygenase: Structure and Function." Annual Review of Plant Physiology and Plant molecular biology, 42, pp. 145-88.

[25] Suty, L., Petitet, A.S., Lecourieurx, D., Blein, J.P., and Pugis, A., 1996. "Isolation of partial length cDNAs corresponding to early differentially expressed genes during elicitation of Tobacco cells by Cryptogein: use of differential mRNA display." Plant physiol. Biochem., 34, pp. 443-451.

[26] Uma Maheswari Devi P., Srinivas Reddy P., Usha Rani, N.R., Reddy, K.J., Narsa Reddy, M., and Reddanna, P., 2000**.** "Lipoxygenase metabolites of α-linolenic acid in the development of resistance in pigeon pea *Cajanus cajan* (L.) Mill sp. seedlings against *Fusarium udum* infection." European Journal of Plant Pathology, 106, pp. 857-865.