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Induction of Lipoxygenase in Downy Mildew Resistant Seedlings of Pearl Millet in Response to Inoculation with *Sclerospora graminicola*

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ABSTRACT

Lipoxygenase (LOX) activity was studied in seedlings of pearl millet genotypes resistant and susceptible to downy mildew pathogen *Sclerospora graminicola*. An increase in LOX activity was observed during the incompatible host-pathogen interaction whereas the activity decreased in compatible ones. Resistant pearl millet seedlings exhibited a 2.4-fold increase in LOX activity after inoculation with the pathogen. The enzyme activity was maximum at 18 h after inoculation. The enzyme activity was maximum in shoot portion of resistant genotype after inoculation. The enzyme activity correlated well with the degree of host-resistance to the pathogen. Substrate-based gel assay revealed five isozymes (LOX- 1, LOX- 2, LOX- 3, LOX- 4 & LOX- 5) in both susceptible and resistant genotype. An additional isozyme (LOX- 6) was unique to only resistant genotype after inoculation.

Key Words: Host-pathogen interaction; Resistance; Susceptibility; Induction; Defense-related enzymes; Linoleic acid

INTRODUCTION

Lipoxygenases (LOX) (Linoleate: oxygen oxidoreductase, EC 1.13.11.12) are a class of non-heme, iron containing dioxygenases that catalyze the oxygenation of polyunsaturated fatty acids with a 1, 4 -cis, cis-pentadiene structure to form conjugated diene hydroperoxide (Holtman, 1996). LOX activity is nearly ubiquitous in the plant kingdom (Eskin *et al*., 1974). Lipoxygenase may play a role in senescence, wounding and pest resistance (Gardner, 1991; Vick, 1993). Chemically induced LOX (WCI- 2) gene expression correlated with the onset of resistance against *Erysiphe graminis* f.sp. *tritici* in wheat (Gorlach *et al*., 1996).

The primary products of LOX, the fatty acid hydroperoxides, are very reactive and may cause oxidative damage to membranes, leading to cell necrosis and death (Hilderbrand, 1989). In some cases, lipid pentadienyl, peroxyl free radicals (Gardner, 1991) or other active oxygen species such as the superoxide anion may also be generated by LOX action (Roy *et al*., 1994). Arachidonic acid and eicosapentaenoic acid, isolated from the mycelium of *Phytophthora infestans*, were shown to elicit phytoalexin accumulation and hypersensitive cell death in potato tissues, probably via LOX action (Ricker & Bostock, 1994). These reactive bursts that occur during the plant defense response are believed to promote hypersensitive cell death (Levine *et al*., 1994). LOX generates three kinds of molecules with different functions: a) hydroperoxides and free radicals that might be involved in localized cell death observed during the hypersensitive response b) signal molecules such as jasmonic acid and its methyl ester that can trigger defense gene expression and amplify the initial response and c) antimicrobial compounds such as 2 -trans-hexenal that constitute a direct defense against pathogen attack (Croft *et al*., 1983).

The downy mildew disease of pearl millet [*Pennisetum glaucum* (L.) R.Br.] caused by *Sclerospora graminicola* (Sacc.) Schroet is one of the major constraints for pearl millet production in the semi-arid tropics. Nagarathna *et al.* (1992) demonstrated the LOX activity in the resistant and susceptible seedlings from 24 h up to five days of post-inoculation. Changes in the lipoxygenase activity in the early stages of pearl millet downy mildew interaction is not available on the early changes. Hence, in the present study the changes in LOX activity during 24 h was monitored at hourly intervals. The specific LOX activity in different tissues of pearl millet seedlings was observed. The LOX activity related to the degree of the resistance of pearl millet and the isoform profile of LOX was studied.

The mechanism of how defense against pathogen helps in breeding for disease resistance. This study was undertaken to relate lipoxygenase activity in pearl millet to downy mildew resistance.

MATERIALS AND METHODS

Collection of seed samples. The seeds downy mildew resistant pearl millet genotypes namely 7042R, IP18292,

IP18293, IP18294, IP18295, IP18296, IP18297, IP18298 and P-310-17 and susceptible genotypes 23B, HB3, 7042S, MBH 110, 700651 and Kalukombu were collected from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India and from the Project Co-ordinator, All India Co-ordinated Pearl Millet Improvement Programme (AICPMIP), Pune, India.

Pathogen and inoculation procedure. Seeds were plated on moist blotter paper discs in petri dishes and germinated for two days at 25 ± 2°C. Downy mildew pathogen *S. graminicola* was maintained on its susceptible host pearl millet (HB3) under greenhouse condition. A suspension of zoospores (40,000 zoospores/mL) of *S. graminicola* was prepared in distilled water and inoculated to two-days-old seedlings by the dip inoculation technique (Safeeulla, 1976). The inoculated seedlings were incubated in a moist, dark chamber. The seedlings were removed at hourly intervals from the zoospore suspension and used for enzyme extraction. Distilled water treated seedlings served as control.

Determination of LOX Activity

Enzyme extraction. Enzyme extracts were prepared from healthy and inoculated seedlings of both resistant and susceptible genotypes. The seedlings (0.5 g) in 5 mL of 0.2 M sodium phosphate buffer (pH 6.5) were homogenized with acid washed sand using mortar and pestle at 4°C. The homogenate was centrifuged at 9,000 g for 20 min at 4°C. The supernatant was used as the enzyme source. The protein content of extracts was estimated by the dye binding method (Bradford, 1976) using bovine serum albumin (Sigma) as the standard.

Enzyme assay. Enzyme activity was measured by following the procedure of Borthakur *et al*. (1987). The activity was determined spectrophotometrically by monitoring the appearance of the conjugated diene hydroperoxide at 234 nm. The substrate for LOX assay was prepared according to the method described by Axelrod *et al*. (1981). Linoleic acid (28 mg) was weighed into a 10 mL glass measuring cylinder and an equal weight of Tween-20 plus 2 mL of distilled water were added. Sufficient amount (50 µL) of 2N NaOH was added to obtain a clear solution. The volume of the solution was made up to 10 mL with distilled water. Each time the substrate was prepared fresh and used for the enzyme assay. The reaction mixture contained 2.7 mL of Sodium phosphate buffer (0.2 M, pH 6.5) and 0.3 mL of substrate. The reaction was initiated by adding the enzyme extract, and the change in absorbance at 234 nm was recorded for three minutes using a Hitachi U-200 spectrophotometer. The enzyme activity was expressed as a change in the absorbance (δ_{234}) /mg protein/min.

Time course study of LOX activity in pearl millet seedlings. One set of seedlings (0.5 g), each of IP18292 (resistant) and 23B (susceptible) was removed at regular time intervals of one hour up to 24 h after zoospore/distilled water treatment and used for enzyme extraction.

Tissue-specific expression of LOX activity in pearl millet. The root and shoot from the seedlings of IP18292 (resistant) and 23B (susceptible) genotypes were excised after 18 h of zoospore inoculation and used as source of enzyme.

LOX activity in genotypes of pearl millet with different degrees of downy mildew resistance. Seedlings of downy mildew resistance/susceptible were removed at 18 h after treatment with zoospore and used for enzyme extraction.

Electrophoretic banding pattern of LOX. LOX was electrophoresed through PAGE by the method of Davis (1964). The LOX activity staining was done by soaking the gels, at room temperature, in a medium containing 100 µL linoleic acid, 20 mg O-dianisidine, and 100 µL 1 M KCN in absolute ethanol and diluting to 100 mL with 0.1 M phosphate buffer (pH 7.0) or with 0.1 borate buffer (pH 8.5), as described by Funk *et al*. (1985). When the orange band pattern is sufficiently visible, the gels were washed with distilled water and stored in a solution containing 15% (v/v) ethanol and 0.1 M borate buffer, pH 8.5.

Statistical analysis. All experiments were repeated three times with three replicates. The relation between the time interval and LOX activity was determined by regression equation. The tissue-specific activity was expressed as mean ± standard errors. The field reaction of pearl millet genotypes to downy mildew and LOX activity was compared by correlation coefficient.

RESULTS

Time course study of LOX activity in pearl millet seedlings. The constitutive LOX activity was more in resistant pearl millet seedlings than in susceptible seedlings (Fig. 1). Resistant seedlings upon inoculation showed a steady increase in enzyme activity from early hours and reached a maximum at 18 h compared to the un-inoculated control. The enzyme activity increased by 2.1-fold at 18 h after inoculation. In susceptible seedlings the enzyme activity decreased at all the intervals when compared to un-inoculated control. The enzyme activity was almost the same at later time intervals i.e. from 18 h on-wards in susceptible and resistant genotypes before and after inoculation.

Tissue-specific expression of LOX activity in pearl millet. The LOX activity in root and shoot region of seedlings of resistant and susceptible genotypes upon inoculation is shown in Table II. The constitutive enzyme activity in un-inoculated seedlings of resistant and susceptible genotype was higher in the shoot region compared to the root. Upon inoculation with *S. graminicola*, LOX activity increased in the shoot tissue of the downy mildew resistant genotype by 2.9-fold. In contrast, there was a decrease in LOX activity in shoot

Fig. 1. Temporal pattern of lipoxygenase activity in pearl millet seedlings of downy mildew susceptible (23B) and resistant (IP18292) genotypes, with or without inoculation of S. graminicola zoospores. Data is expressed as change in absorbance at 234 nm / mg protein/ min and the values are mean of triplicate samples of three separate experiments. The relation between time interval and LOX activity is represented by regression equation

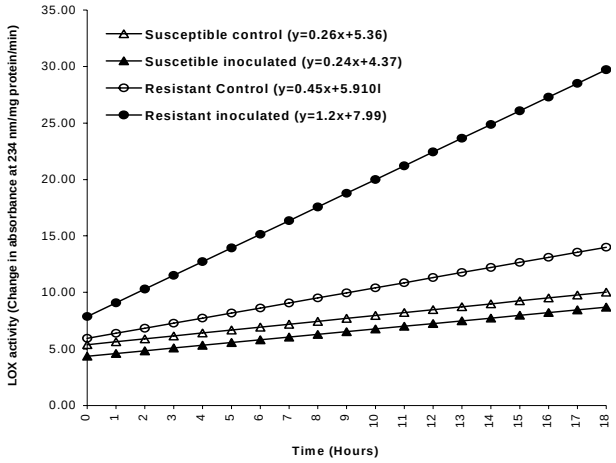


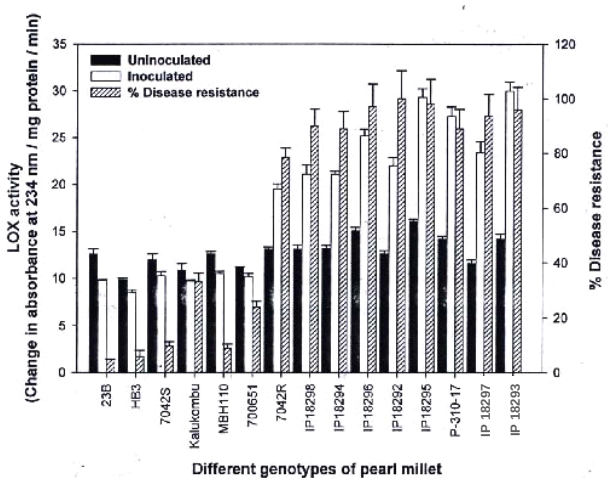
Table I. Tissue-specific expression of lipoxygenase activity in susceptible (S) (23B) and resistant (R) (IP18292) seedlings of pearl millet genotypes, 18 h after inoculation with *S. graminicola*. The enzyme activity was determined and expressed as specific activity (change in absorbance at 234 nm / mg protein / min). Values are the mean ± standard error of triplicate samples of three separate experiments

Tissue of the seedling	LOX activity (Change in absorbance at 234 nm/mg protein/min)			
	Resistant		Susceptible	
	Uninoculated	Inoculated	Uninoculated	Inoculated
Shoot	20.3 ± 0.6	58.9 ± 0.22	14.8 ± 0.72	13.8 ± 0.29
Root	18.3 ± 0.18	26.0 ± 0.17	11.7 ± 0.58	9.0 ± 0.17

and root regions of susceptible seedlings.

LOX activity in genotypes of pearl millet with different degrees of downy mildew resistance. Among the un-inoculated pearl millet seedlings of different genotypes highest enzyme activity was found in 'IP18295' (16.1/mg protein/min) and it was low in 'HB 3' (9.9 mg protein/min) (Fig. 2). Upon inoculation with *S. graminicola* zoospores, a increase in the enzyme activity was recorded over that of un-inoculated control seedlings was recorded in all the resistant genotypes - 7042R, IP18292, IP18293, IP18294, IP18295, IP18296, IP18297, IP18298 and P-310-17. Highest enzyme activity was noticed in IP18293 (30 mg protein/min) and IP18295 (29.3 mg protein/min) after inoculation. In all the other genotypes i.e. 23B, HB 3, 7042 S, Kalukombu, MBH 110,

Fig. 2. Genotype specific expression of lipoxygenase in pearl millet. Two-day-old seedlings were inoculated with zoospore suspension of S. graminicola. After 4 h of inoculation enzyme activity (change in absorbance at 234 nm / mg protein / min) was determined. The values are mean of triplicate samples of three separate experiments and the bars indicate standard errors. The field reaction of pearl millet genotypes to downy mildew and LOX activity was compared by correlation coefficient. Correlation coefficient 'r' (I) disease resitance vs LOX activity in uninoculated pearl millet = 0.6179 (ii) disease resitance vs LOX activity in inoculated pearl millet = 0.935*. *** significant at 0.1% level**



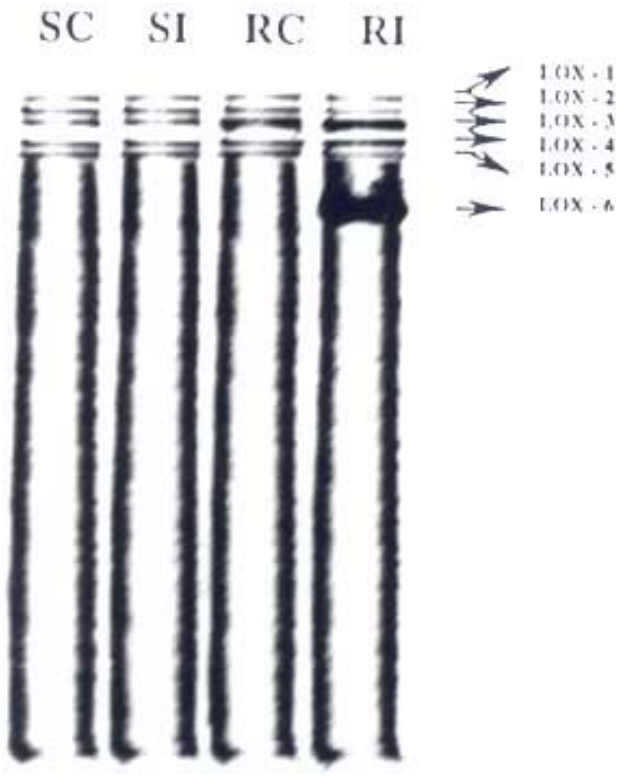
a decrease in enzyme activity was observed after inoculation. A significant correlation coefficient was obtained for the relation between LOX activity and downy mildew reaction of different pearl millet genotypes in field.

Electrophoretic banding pattern of LOX. The profile of major LOX isozymes of pearl millet genotype is characterized by a substrate gel assay (Fig. 4). The banding pattern was similar in inoculated and un-inoculated susceptible seedlings as well as in un-inoculated resistant seedlings. The gel show 3^{ed} five isozymes represented as LOX- 1, LOX- 2, LOX- 3, LOX- 4 and LOX- 5 in both un-inoculated and inoculated susceptible seedlings. However, the band representing LOX- 3 was darker in resistant genotype compared to the susceptible genotype. But inoculated resistant seedlings revealed a new isozyme LOX- 6, which was not detected in any other sample.

DISCUSSION

The defense-related enzymes play a prominent role either directly or indirectly in the resistance responses. The results indicated that LOX activity is induced in resistant genotype of pearl millet seedlings, after inoculation with *S.*

Fig. 3. Polyacrylamide gel electrophoresis profiles of LOX isozymes from the seedlings of pearl millet genotype 23B (susceptible) and IP18292 (resistant) with or without inoculation with the downy mildew pathogen Sclerospora graminicola. 15 µg of crude protein was loaded per lane. SC: susceptible uninoculated control, SI: susceptible inoculated, RC: resistant uninoculated control and RI: resistant inoculated



graminicola zoospores. Such an induction of enzyme activity was more in downy mildew resistant pearl millet genotypes. The possible function of LOX in defense reactions may include membrane lipid peroxidation leading to the irreversible membrane damage during the hypersensitive cell death or the production of antimicrobial compounds (Slusarenko, 1996). LOX activity has been observed in several other incompatible host-pathogen combinations (Croft *et al*., 1990). A number of studies have shown increased LOX activity in plant tissues and cells in response to plant pathogens (Ohta *et al*., 1991). LOX activity has been shown to increase in resistant tissues in several host-pathogen combinations, for example, C*ucumis sativus/Pseudomonas syringae* pv. *pisi* (Keppler *et al*., 1987), Oats/*Puccinia coronata avenae* (Yamamoto, 1986) and rice/*Pyricularia oryzae* (Ohta *et al*., 1988).

In pearl millet, an induction of LOX activity was observed in the shoot of resistant genotypes after inoculation with *S. graminicola*. In the susceptible genotype there was a reduction in enzyme activity after inoculation. This indicated that the pathogen may inactivate the enzymes and/or reduce host protein synthesis due to colonization of the pathogen. The pathogen colonizes the mesocotyl and shoot regions of the susceptible seedling, while in resistant seedling the pathogen did not colonize the tissue (Sharada *et al*., 1995). The present study shows that LOX activity was higher in shoot region of the resistant seedling.

In the present study it has been established that after inoculation, LOX activity increased in the seedlings of all the resistant genotypes, whereas it decreased in the susceptible seedlings, as compared to the respective un-inoculated controls. This increase in the LOX activity in response to infection might be an indication that a defense mechanism has been initiated in the resistant genotypes. A correlation between induction of LOX activity and resistance of the plant has been shown in tobacco infected with *Erysiphe cichoracearum* (Lupu *et al*., 1980), in *Pseudomonas synringae*-inoculated bean (Croft *et al*., 1990), in wheat infected with *Puccinia graminis* (Ocampo *et al*., 1986) and in rice infected with *Magnaporthe grisea* (Ohta *et al*., 1991). In resistant pearl millet seedlings LOX activity significantly increased on the second and third day after inoculation, while in susceptible seedlings, the activity decreased over respective healthy control (Nagarathna *et al*., 1992).

Data presented here shows that LOX activity triggered much earlier and this plays an important role in early defense. Lipoxygenase from leaves of tomato (*Lycopersicon esculentum* Mill.) is induced in response to plant pathogen *Pseudomonas* has been reported by Koch *et al*. (1992). Lipoxygenase activity has been shown to increase in resistant tissues in several host-pathogen combinations of *Cucumis sativa* and *Pseudomonas syringae* pv. *pisi* (Keppler & Novacky, 1986). Rodriguez-Rosales *et al*. (1999) have stated that hydroperoxides formed by the action of lipoxygenase on linoleic acid and linolenic acids lead to changes in membrane fluidity and permeability, ultimately leading to dysfunction in the lipid bilayer. Gardner (1991) reported that polyunsaturated fatty acid hydroperoxides resulting from the action of LOX undergoes a variety of reaction including generation of free radicals, which provoke changes in membrane properties, ultimately leading to dysfuntioning of the lipid bilayer membrane deterioration and senescence. Alternatively, lipoxygenase-derived fatty acid hydroperoxides can be converted into more stable compounds, including jasmonic acid, which participate in the onset of defense reaction to biotic and abiotic stresses.

The isozyme pattern of LOX from resistant genotype suggested that LOX has a role in defense reaction strongly suggested that LOX- 6 was induced in the resistant genotype after inoculation with *S. graminicola*. Both enzyme assay and electrophoresis established that LOX is induced in the resistant genotype after inoculation.

Results obtained here suggest the involvement of LOX in the defense mechanism of pearl millet against *S. graminicola* infection and has a possible use of the enzyme as a biochemical marker of resistance.

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