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Characterization of pigeonpea lipoxygenase in response to *Fusarium udum* infection*

A K MANDAL[1] and P SINHA[2]

Indian Agricultural Research Institute, New Delhi 110 012

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Lipoxygenases (LOX) (linoleate: oxygen oxidoreductase, E.C. 1.13.11.12) are dioxygenases that catalyze the addition of molecular oxygen to polyunsaturated fatty acids containing the group *cis, cis* -1,4–pentadiene. Linolenic acid ($C_{18:3}$) contains such group and is the most abundant fatty acid present in the majority of plant tissues. Linoleic acid ($C_{18:2}$) which also contains the *cis, cis*-1,4–pentadiene group is found in high concentrations in seeds and embryos (Noordermeer *et al.* 2001). Several studies have been conducted aiming to elucidate the physiological role of LOX in soybeans, wheat, tobacco and potato (Noordermeer *et al.* 2001, Garrote *et al.* 2003). These studies suggest that LOX are related to plant growth and development, senescence, plant defense against pathogens and insects, and biosynthesis of regulatory molecules. In soybeans, LOX have been studied and suggested that 10 to 12 genes encoding LOX are present in the genome (An-Fei-Hsu *et al.* 2007). Despite the extensive characterization, physiological roles of these enzymes have not been fully understood. However, recent data show that these enzymes affect the levels of protease inhibitors in seed suggesting that they have an important function during seed development (Noordermeer *et al.* 2001). In leaves, it has been observed that LOX expression increases considerably as a result of reproductive sink removal and mechanical wounding, indicating that LOX can be considered a vegetative storage protein as well as an important component of the plant response system to pathogen and insect attack (Devi *et al.* 2004). Several hypotheses were put in explaining multiple physiological roles, specially their involvement in biosynthesis of several growth regulators such as ascorbic acid, traumatin and jasmonate (An-Fei-Hsu *et al.* 2007). It is reported that increased LOX activity has been directly correlated with plant resistance against pathogens and involved in an active resistance mechanism known as hypersensitive response, a form of programmed cell death (Noordermeer *et al.* 2001). LOX as an expression of defense-related gene studied in some plant species has not been characterized in pigeonpea so far. Present communication aimed at characterization of LOX pool present in leaves and roots of pigeonpea subjected to *Fusarium* infection.

LOX activity has been estimated in 4 resistant and 2 susceptible pigeonpea genotypes. Particulars of the genotypes are given in Table 1. During July – September 2005–07 seeds were surface sterilized with 0.1% $HgCl_2$ solution for 2 min. and sown in plastic pots (6" dia) containing sterilized sand-soil mixture (50:50).

A virulent isolate (AF-13, collected from severely infected pigeonpea field in Alwar district Rajasthan) of *Fusarium udum* was grown on PDA slant for 7 days at 28ºC±1ºC and spore suspension (approx 1.2×10^6 spores/ml) was made in sterile water. Fifteen-days-old seedlings of each genotype were uprooted carefully from the pots, rinsed thoroughly with sterile water and immediately dipped in the spore suspension

Table 1 Particulars of pigeonpea genotypes

Genotype	Selection from	Source	Wilt reaction
'ICP 87119'	cross C11(ICP 7118)×breeding line	ICRISAT, Hyderabad	Resistant
'ICP8863'	Selection ICP 7626 (land race UP)	ICRISAT, Hyderabad	Resistant
'BDN' 2	Selection landrace	IIPR, Kanpur	Resistant
'BSMR' 736	Improved selection landrace	IIPR, Kanpur	Resistant
'Bahar'	Selection landrace	IIPR, Kanpur	Susceptible
'TTB' 7	Improved selection landrace	UAS, Bangalore	Susceptible

*Short note

Based on complete information of M Sc thesis of the first author submitted to IARI, New Delhi during 2007.

[1]Ph D student, (E-mail: asitpat_@rediffmail.com), [2]Senior Scientist, Division of Plant Pathology, (E-mail: sinha_path @iari.res.in)

Table 2 Steady-state kinetic parameters for pigeonpea LOX

Kinetic parameters	Resistant genotype	Susceptible genotype
K_m mM/l	2.33	0.0994
V_{max}	0.1168	0.0492
K_{cat}	22.3	0.8946
K_{cat}/K_m	9.5708	4.7218

for 30 min. Seedlings were then re-transplanted in pots with sand-soil mix and kept in the laboratory condition to avoid transpiration shock and next day transferred in glasshouse condition. For control, seedlings were dipped in sterile water in similar fashion transplanted in pots. Samples of pigeonpea leaf of inoculated and un-inoculated genotypes were collected at 2, 4, 6 and 8 days after inoculation (DAI) and root samples were taken only at 4 DAI and stored at –80°C.

One gram of frozen sample (at – 80ºC) was grinded with liquid nitrogen into powder and stirred for 15 min. in 4 ml of ice-cold phosphate buffer (2 ml of 50 mM, *p*H 6.5 + 2 ml 2% polyvinyl pyroline phosphate). Crude extracts were filtered through muslin cloth and centrifuged at 4ºC for 20 min. at 10,000 rpm. The 2.5 ml of supernatants were then loaded into prepared PD20 columns (pharmacia) and eluted with 3.5 ml phosphate buffer and LOX assay were carried out. Protein content determination was by the bicinchoninic acid assay (Clemente *et al.* 2000). Protein concentration in leaf extracts at 4 DAI were 1.37 - 1.85 and 1.08 – 1.18 mg/ml in inoculated and non - inoculated resistant genotype ('BSMR 736') and corresponding values for susceptible genotype ('TTB 7') were 0.92 – 0.99 and 0.83 – 1.05 mg/ml respectively.

A stock solution of linoleic acid (assay 98%) was prepared with 0.1 ml of linoleic acid in 60 ml of absolute alcohol and volume made up to 100 ml with distilled water. Before use, 1 ml of this stock solution was diluted to 6 ml with sodium-borate buffer (0.2 mM, *p*H 8.5).

LOX catalyzes oxygenation of polyunsaturated fatty acids with a 1, 4–cis, cis pentadiene structure to form conjugated dienehydroperoxides which is measured spectrophotometrically by observing increase in absorbance at 234 nm. Reaction was carried out at 25ºC in quartz cuvettes taking supernatant (75 µl) and substrate solution (2.925 ml) and oxygenating by bubbling air for 3–4 min. Increase in absorbance at 234 nm was measured for 20 min. at 30s intervals at 25°C temperature in a spectrophotometer (UVIKON Spectrometer Model 931). Enzyme activity was expressed as number of unit/mg of protein/min. Here one unit (U) of enzyme activity produced a change in absorbance of 0.001/min. at 234 nm of wavelength.

Enzymes extracted from leaf samples collected at 4 DAI were assayed at different *p*H range 5.0–10.0 with 0.5 intervals using using linolenic acid and linoleic acid in separate sets.

Since enzyme extracts have shown maximum activity at *p*H 9.0 and substrate preference for linoleic acid. Therefore, enzyme assays for the leaf samples collected at 2, 4, 6 and 8 days after inoculation and from roots at 4 days after inoculation were done at *p*H 9.0 using linoleic acid as substrate.

Leaves from 'ICP 8863' (resistant) and 'TTB 7' (susceptible) at 4 days after inoculation were grinded to fine powder and defatted with hexane at room temperature. Extraction and fractionation of protein was performed as described by Clemente *et al.* (2000). Defatted powder (15 g) was extracted with 150 ml of 50 mM sodium phosphate buffer (*p*H 6.8), for 2 hr and centrifuged at 10,000g for 30 min. Supernatant was dialyzed against 40% polyethylene glycol for 16 hr and centrifuged at 25,000g for 20 min. To purify LOX, dialyzed fraction was loaded in Sephadex G 100 gel filtration column (100×2) at a flow rate of 20 ml/hr using the extraction buffer. The fractions containing LOX activity were pooled and applied to DEAE cellulose DE 52 column (3.2×4.2 cm). LOX isoforms were eluted with a gradient linear on 50 mM sodium phosphate (100 ml) to 200 mM (100 ml), *p*H 6.8, at a flow rate of 30 ml/hr. Protein elution was detected at 280nm, and assayed for LOX activity. Changes in absorbance (Δ OD_{234}) were plotted against substrate concentrations [S]. Based on steady-state enzyme kinetics parameters viz Michaelis-Mentent constant (K_m mM), maximum velocity (V_{max} mM/min), catalytic activity (K_{cat} Mm/s) and selectivity ($K_{cat}/K_{m/s}$) estimated from the following equations:

$$dS/dt = K_m [S]/(K_m + [S]) \quad \ldots (1)$$

$$V_{max} = K_2 . E_t \quad \ldots (2)$$

$$K_m = (K_{-1} + K_2)/K_1 \quad \ldots (3)$$

$$K_{cat} = V_{max}/E_t \quad \ldots (4)$$

$$\text{Selectivity} = K_{cat}/K_m \quad \ldots (5)$$

Where, K_1, K_{-1} and K_2 are constants (unit/s), forward rate for substrate binding, reverse rate for substrate binding and

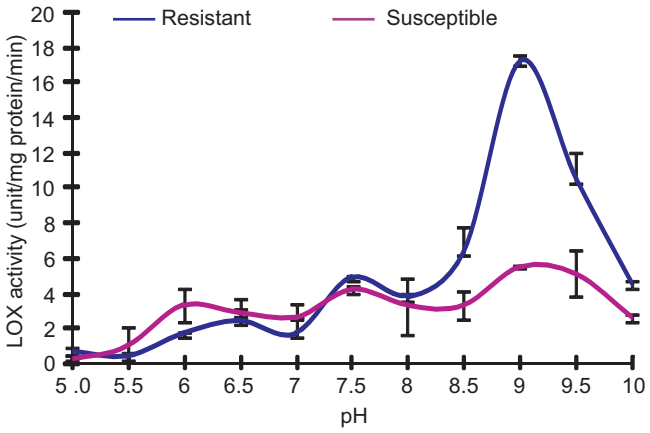

Fig 1 Lox activity (unit/mg protein/min) in resistant and susceptible pigeonpea genotypes in relation to *p*H

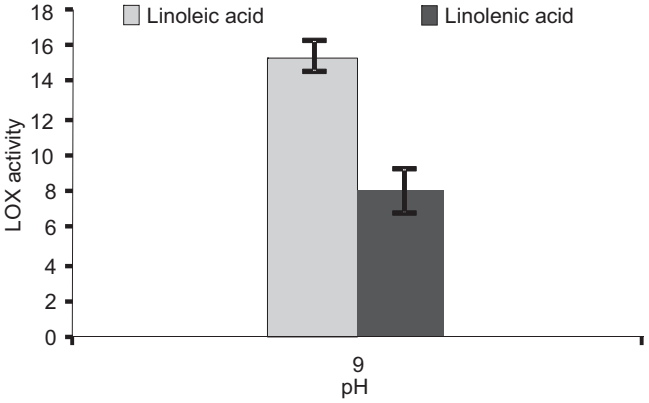

Fig 2 Pigeonpea LOX activity in relation to substrate preference

catalytic rate constant respectively. E_t is the total enzyme in the reaction under assumption (An-Fei-Hsu *et al.* 2007). Substrate concentration [S] and enzyme velocity (V) has been fitted into the equation 1 using SPSS 10.0 software package. Arbitrary values of K_1 and K_{-1} assumed were 10 and 1 respectively for the approximation E_t and K_2 for comparison of resistant and susceptible genotypes.

LOX activity was low at *p*H 5.0 and increased at *p*H 6.5, 7.5 and 9.0 and decreased above *p*H 9.5 in both resistant and susceptible genotype (Fig 1). Initial two peaks at *p*H 6–6.5 and 7.5 in both resistant and susceptible genotypes are not significantly (P=0.05) different as the peak at *p*H 9.0. Enzyme activity at *p*H 9.0 was around 3 times higher in resistant as compared to susceptible genotype. Therefore, it was apparent that pigeonpea LOX had three distinct peaks of activity at around *p*H 6.0 – 6.5, *p*H 7.5 and *p*H 9.0.

Activity measured (*p*H 9.0) was around 15.37 units for linoleic acid as compared to 8.13 units for linolenic acid (Fig 2). Therefore, pigeonpea LOX has substrate preference for linoleic acid as compared to linolenic acid.

All the resistant genotypes inoculated with pathogen have shown an increased LOX activity at 2–8 days after inoculation as compared to the un-inoculated ones (Figs 3, 4). Increased enzyme activity was prominent at 4–days and at 6–days increasing trend was maintained although there was slight decline in activity at 8 days. In susceptible genotypes no clearcut distinction has been marked between inoculated and un-inoculated seedlings (Figs 5, 6).

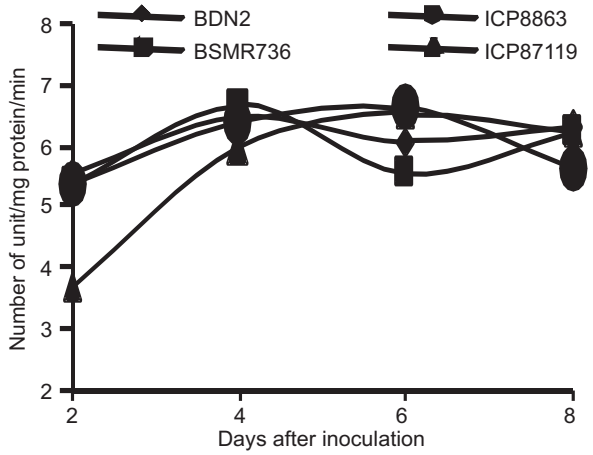

Fig 3 Lox activity in inoculated resistant pigeonpea genotypes

LOX enzyme analyzed from the roots of resistant genotypes at 4 DAI although varied genotype-wise but activity was comparatively higher than susceptible genotypes (Fig 7).

In purified LOX, K_m for resistant and susceptible genotype has been estimated 2.23 and 0.0994 and V_{max} 0.116 and 0.0492 respectively (Table 2). In qualitative analysis, assuming rate constants k_{-1}=1 and k_1=10, and constants parameters like; catalytic activity (K_{cat}) and selectivity (K_{cat}/K_m) have been estimated. LOX kinetics parameter in terms of catalytic efficiency and selectivity were 22.3 and 9.57 in resistant and 0.89 and 4.72 in susceptible respectively.

It was evident that LOX activity was significantly increased in resistant pigeonpea genotypes in response to *Fusarium udum* infection. Increased LOX activity due to pathogen infection has been demonstrated in several plant systems especially beans (Garrote *et al.* 2003), *Phaseolus vulgaris* (Clemente *et al.* 2000) and soybean (An Fei- Hsu *et al*. 2007). Three distinct peaks in response to variable *p*H indicated that pigeonpea LOX might have different isoforms. Higher values of K_m, V_{max}, catalytic efficiency and selectivity were observed in resistant genotype as compared to susceptible one. This difference in steady-state kinetic parameter also indicated that different LOX isoform or isozyme exists in resistant genotypes. In resistant genotype peak at *p*H 9.0 was distinctly prominent one that may be associated with different isoform of the enzyme and is produced in excess quantity in resistant genotype in response to pathogen infection. Otherwise, distinct peak may be due to a totally different isoform that becomes operative during active defense response. LOX has been much studied in soybean where three distinct isoforms that were distinguished by differences *p*H optimum, substrate specificity and products formation (An-Fei-Hsu *et al.* 2007). One LOX isoforms from soybean has been reported to show *p*H optimum at 9–10. Based on *p*H optimum (9.0) in pigeonpea, the distinct isoform looks similar to LOX isoform in soybean. Soybean LOX isoform has been found to have homology with LOX isolated from other plant namely beans (Garrote *et al.* 2003).

Biosynthesis of jasmonic acid that acts as a signal molecule for expression of several defense-related genes is produced by the activation of LOX (Torii 2000). It is now proved that the inducible signalling pathways that regulate 2 proteinase inhibitor genes in tomato, potato, and alfalfa plants, and likely in other plants as well, involve a lipid-based system that results in the production of jasmonic acid as a key signalling molecule which mediates expression of both specific (*R* gene-mediated) as well as basal defense responses (Jalali *et al.* 2006). The

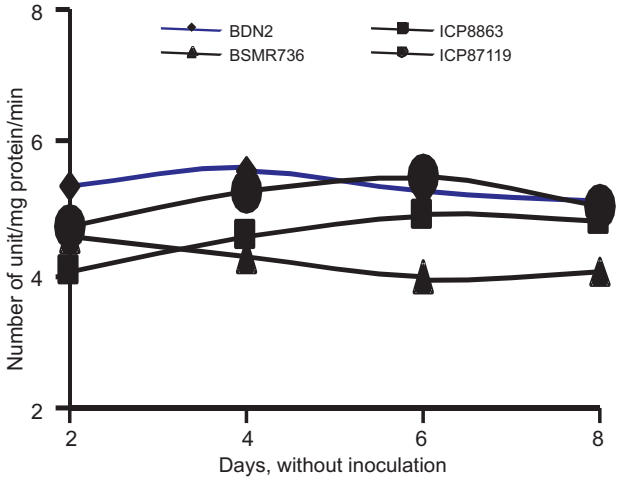

Fig 4 Lox activity in resistant pigeonpea genotypes (non-inoculated)

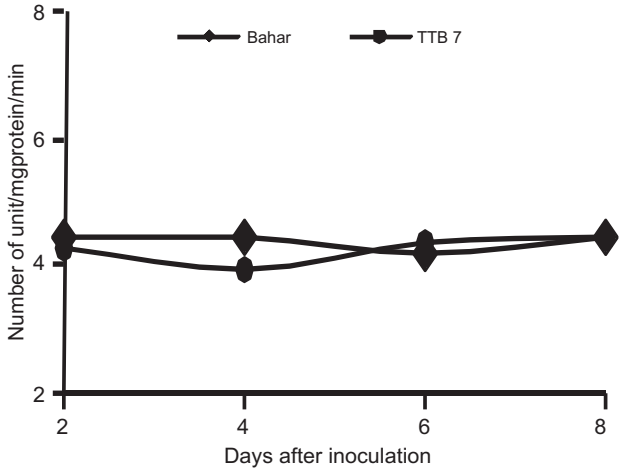

Fig 5 Lox activity in inoculated suscpetible pigeonpea genotypes

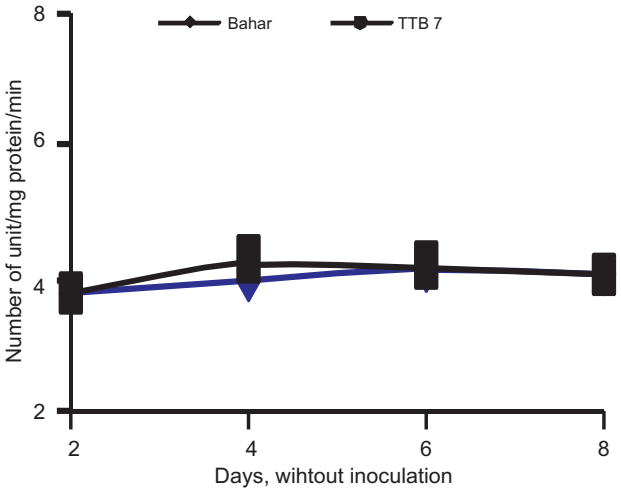

Fig 6 Lox activity in susceptible pigeonpea genotypes (non-inoculated)

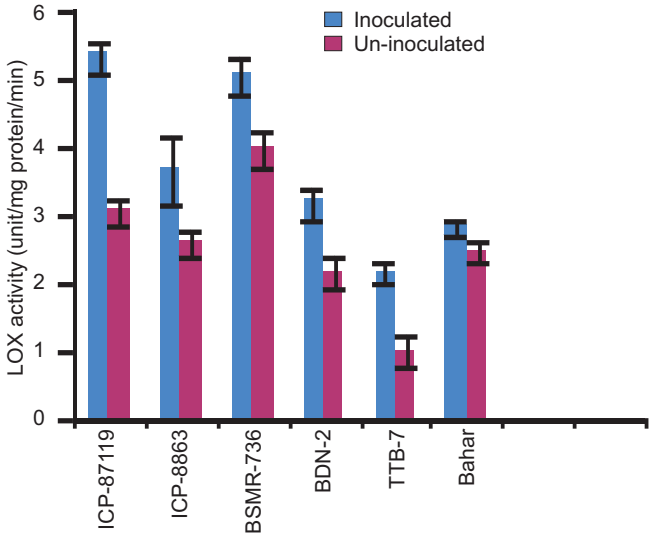

Fig 7 Lox activity at 4 DAI in pigeonpea roots

evidence suggests that pathogen attack produce extracellular signals that interact with the plant cell membrane to release linolenic acid (Kruger *et al*. 2002). Linolenic acid is known to be converted to jasmonic acid through several biochemical steps. Both linolenic acid and jasmonic acid are powerful inducers of the inhibitor genes when placed on leaf surfaces, indicating their involvement in the signalling pathway (Torri 2000, Vaghchhipawala *et al*. 2001). Identification of specific isoform at DNA level and its regulation and expression of proteins may be implicated in pathogen recognition, signal transduction, and the induction of defense-related genes transcription like, MAP and protein kinases, transcription factors, and resistance gene analogs (Torii 2000).

SUMMARY

An experiment was conducted during 2005–07 to characterize lipoxygenase activity in pigeonpea in response to *Fusarium udum* infection. Four known resistant genotypes, viz 'ICP 87119', 'ICP 8863', 'BDN 2' and 'BSMR 736' have shown consistently higher lipoxygenase (LOX) activity in comparison to susceptible genotypes viz 'Bahar' and 'TTB 7'. The *p*H optima for LOX activity were at *p*H 6–6.5, 7.5 and 9.0 exhibited by 3 distinct peaks. Peaks at *p*H 6– 6.5 and 7.5 were almost similar in both resistant and susceptible genotype. Peak of higher activity at *p*H 9.0 was significantly different in resistant in comparison to susceptible genotype. It indicated that pigeonpea LOX system may have at least 3 different isoforms and had substrate preference on linoleic acid. Difference in kinetic parameters like, K_m values, catalytic efficiency and selectivity in purified enzyme has supported the differential kinetic behaviour in resistant and susceptible genotype.

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