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Indices of oxidative stress in cotton (*Gossypium hirsutum* L.) genotypes after infestation of sucking pest

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Abstract
Cotton is a crop of great economic importance in India. In the present study, H_2O_2 content, MDA content and LOX activity were estimated in the leaves (2^{nd} & 6^{th}) of cotton genotypes infected by sucking pests at 50, 60 and 68 days after sowing (DAS). H_2O_2 content, MDA content and LOX activity was found to be maximum in 2^{nd} & 6^{th} leaves of *G. hirsutum* susceptible genotypes followed by *G. hirsutum* resistant genotypes and minimum in *G. arboreum* genotypes. The leaves of resistant genotypes had less production of H_2O_2, MDA and LOX as compared to susceptible cotton genotypes.

Keywords: Cotton, lipoxygenase, malondialdehyde, oxidative stress, pest, resistance

Introduction
Cotton is a crop of great economic importance in India. It is a natural fibre of vegetable origin, like linen, jute or hemp and composed of cellulose. Out of about 50 species of cotton plants in the world, only four have been domestically cultivated for cotton fibres namely *Gossypium hirusutum*, *Gossyium barbedense*, *Gossypium arboreum* and *Gossypium herbaceum*. *Gossypium hirusutum* and *Gossypium barbedense* are the most commonly cultivated species of cotton in the world. *Gossypium hirusutum* variety is the most important agricultural cotton, accounting for more than 90 per cent of world fibre production. As per CAB report (July, 2014) during 2013-14, cotton was grown in India in about 117.27 lakh hectares with a total production of 390 lakh bales and in Haryana, it occupied an area of about 5.66 lakh hectares with annual production of 23 lakh bales (Cotton corporation of India, 2014) [3]. World wide area under cotton production is estimated at around 33.1 million hectares (Johnson *et al.* 2014) [8]. India, China, United State, Pakistan and Brazil are main producers of cotton in the world (Johnson *et al.* 2014) [8] and account for almost 80% of global production. India ranks first in area of cultivation and second in production after China. The sustainability of cotton production worldwide has been affected due to piercing, sucking insect pests and bollworms which are a serious threat to the cotton crop. The cotton ecosystem includes a wide variety of arthropods throughout the world. More than 1326 species of insects have been reported attacking cotton in the world. In India, 162 species have been recorded among which only 15 species consider potential threat to the crop.

When plants are subjected to biotic or abiotic stresses, reactive oxygen species (ROS) such as $\cdot O_2^-$, $\cdot OH^-$ and H_2O_2 are generated in response to stress conditions which are considered to be indicators of oxidative damage (Dat *et al.*, 2000) [4]. ROS can cause oxidative damage to many cellular components, including membrane lipids, proteins, nucleic acids, and chlorophyll. The balance between ROS production and activities of antioxidative enzymes determines whether oxidative signalling and/or damage will occur. Lipoxygenases (LOXs) constitutes another group of anti-oxidative enzymes involved in plant defense against many stresses through octadecanoid pathway (Bruinsma *et al.*, 2009). They catalyze hydroperoxidation of polyunsaturated fatty acids resulting in formation of fatty acid hydroperoxides. The latter are enzymatically and/or chemically degraded to unstable and highly reactive aldehydes, γ-ketols, epoxides, (Bruinsma *et al.*, 2009) and ROS such as hydroxyl radicals, singlet oxygen, superoxide ion and peroxyl, acyl and carbon-centered radicals (Maffei *et al.*, 2007). Therefore, studies on indices of oxidative stress are important to facilitate our understanding of their relationships between the oxidative burst and plant defense responses against insect pest attack.

Material and Methods

The present investigation was carried out at the fields of Cotton Section, Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar during the year 2013-14 and 2014-15. Leaves (2nd & 6th) of nine genotypes *viz*. HD418, HD432, HD503, H1464, H1465, H1098, H1463, H1454 and H1439 were collected before and after the attack of sucking pests. HD418, HD432, HD503 (*G. arboreum*) and H1464, H1465, H1098 (*G. hirsutum*) were the resistant genotypes and H1463, H 1454, H1439 (*G. hirsutum*) were the susceptible cotton genotypes. Sampling was done at 50, 60 and 68 days after sowing (DAS). The leaf samples were collected in ice box containing ice cubes and brought to the laboratory.

Malondialdehyde: Malondialdehyde content was estimated according to the method of Heath and Packer (1968) [7]. To 0.5 ml of supernatant, 2.5 ml of 20% (*w/v*) trichloro acetic acid (TCA) containing 0.5% thiobarbituric acid was added. The mixture was heated in a water bath at 95°C for 30 min and quickly cooled in ice bath. Then the absorbance was recorded at 532 nm and the value of non-specific absorption at 600 nm was subtracted from it. The concentration of malondialdehyde was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Hydrogen peroxide: H_2O_2 was estimated by the method of Sinha (1972) [12]. To 0.4 ml extract, 0.6 ml of 0.1 M phosphate buffer (pH 7.0) and 3 ml mixture of 5% (*w/v*) potassium dichromate and glacial acetic acid (1:3, *v/v*) was added. The mixture was heated for 10 min in a boiling water bath. Colour of solution changed to green due to the formation of chromic acetate. After cooling, absorbance was recorded at 570 nm against the reagent blank without sample extract. The quantity of H_2O_2 was determined from the standard curve 10-160 µmoles of H_2O_2.

Lipoxygenase estimation: The enzyme activity was determined spectrophotometrically at 234 nm by the method of Catherine *et al*. (1998) [2]. The reaction was started by the addition of enzyme. Increase in absorbance was measured at room temperature for 2 min. Lox activity was measured by monitoring the formation of conjugated dienes from linoleic acid. One enzyme unit was defined as the amount of enzyme causing 0.1 unit change in O.D. at 234 min^{-1}.

Results and Discussion

Malondialdehyde (MDA): Results depicted in Fig. 1(a) and Fig. 1(b) shows the MDA content in 2nd and 6th healthy leaves of resistant and susceptible cotton genotypes respectively. The content of MDA was maximum in *G. hirsutum* susceptible genotypes (11.12-12.78 µ moles g^{-1} dry wt.) followed by *G. hirsutum* resistant genotypes (6.73-9.25 µ moles g^{-1} dry wt.) and minimum in *G. arboreum* genotypes (5.18-6.00 µ moles g^{-1} dry wt.). MDA content was higher in susceptible genotypes than resistant genotypes in both 2nd and 6th leaves. In 6th leaf MDA content was 12.94- 14.13 µ moles g^{-1} dry wt. in *G. hirsutum* susceptible genotypes followed by 9.89-12.21 µ moles g^{-1} dry wt. in *G. hirsutum* resistant genotypes and minimum 6.56-8.60 µ moles g^{-1} dry wt. in *G. arboreum* genotypes. All the genotypes differed significantly in MDA content.

Results depicted in Fig. 1(c) shows the effect of pest infection on MDA content in 2nd leaf of resistant and susceptible cotton genotypes and Fig. 1(d) shows the effect of pests infection on MDA content in 6th leaf of resistant and susceptible cotton genotypes. After infection, increase in MDA content was observed in *G. hirsutum* genotypes. In 2nd leaf at 60 DAS, increase in MDA content was 17.29-37.59% in resistant genotypes and 43.37-58.65% in susceptible genotypes. At 68 DAS, however more increase in MDA content was observed and increase was 62.03-101.83% in resistant genotypes and 96.50-117.99% in susceptible genotypes. In 6th leaf increase was 19.01-26.05% in resistant genotypes and 54.38-65.83% in susceptible genotype at 60 DAS stage. At 68 DAS, more increase in MDA content was observed and increase was 37.48-55.22% in resistant genotypes and 97.42-106.56% in susceptible genotypes. Significant increase was observed in all the genotypes. The content increased in all the genotypes but the increase was more in susceptible genotypes as compared to resistant genotypes and all the genotypes differed significantly in MDA content.

A striking early response is the transient accumulation of ROS at the plant surface against pathogenic infection. Prolonged overproduction of ROS can lead to peroxidation of membrane lipids and pigments, denaturation of proteins, damage to DNA and fragmentation of polysaccharides (Rady and Osman, 2012). Lipid peroxidation is measured as the amount of Thiobarbituric acid reactive substances or malondialdehyde (MDA) produced when polyunsaturated fatty acids in the membrane undergo oxidation by accumulation of free oxygen radicals. As lipids peroxidation is the symptom mostly ascribed to oxidative damage, it is often used as an indicator of increased membrane damage (Moller *et al*. 2007). In the present results, higher MDA content was observed in susceptible genotypes as compared to resistant genotypes as shown in fig. 3a & 3b and on pests infection there was increase in MDA content in both 2nd and 6th leaves (fig.1c & 1d) at both 60 DAS and 68 DAS. MDA content was higher in more infected leaves (68 DAS) as compared to less infected leaves (fig. 1c & 1d). Similarly, higher H_2O_2 and MDA concentrations were recorded in bean yellow mosaic virus infected faba bean leaves as compared to corresponding controls (Radwan *et al*., 2010) [10]. Debona *et al.* (2012) [5] observed similar results in wheat varieties inoculated with *Pyricularia oryzae* at vegetative stage showed increase in MDA content in susceptible plants (BR18) and no significant change in partially resistant plants (BRS 229). Whereas, Siddique *et al.* (2014) observed increased level of MDA in resistant genotype (Ravi) of cotton infected with cotton leaf curl Burewala virus.

Hydrogen Peroxide (H_2O_2): Results depicted in Fig. 2(a) and Fig. 2(b) show the H_2O_2 content in 2nd and 6th healthy leaves of resistant and susceptible cotton genotypes respectively. The content of H_2O_2 followed similar trend as MDA content in both 2nd and 6th leaves before infection. In 2nd leaf maximum H_2O_2 content was observed in *G. hirsutum* susceptible genotypes (4.38-4.74 m moles g^{-1} dry wt.) followed by *G. hirsutum* resistant genotypes (3.44-3.52 m moles g^{-1} dry wt.) and minimum was in *G. arboreum* genotypes (2.22-3.16 m moles g^{-1} dry wt.). Similar trend was observed in 6th leaf where maximum content 5.67-5.88 m moles g^{-1} dry wt. was in *G. hirsutum* susceptible genotypes followed by *G. hirsutum* resistant genotypes (3.65-3.93 m moles g^{-1} dry wt.) and minimum was in *G. arboreum* genotypes (2.34-3.24 m moles g^{-1} dry wt.). H_2O_2 content was higher in susceptible genotypes than resistant genotypes. All the genotypes did not differ significantly in H_2O_2 content. Results depicted in Fig. 2(c) show the effect of pests infection

on H_2O_2 content in 2nd leaf of resistant and susceptible cotton genotypes and Fig. 2(d) shows the effect of pests infection on H_2O_2 content in 6th leaf of resistant and susceptible cotton genotypes. Significant increase was observed in all the genotypes.

After infection, increase in H_2O_2 content was observed in *G. hirsutum* genotypes. In 2nd leaf, at 60 DAS stage, increase in H_2O_2 content was 47.93-52.40% in resistant genotypes and 79.78-100.39% in susceptible genotypes whereas 68 DAS stage had more increase in H_2O_2 content and increase was 56.05-143.11% in resistant genotypes and 124.90-146.74% in susceptible genotypes. In 6th leaf increase was 46.34-49.68% in resistant genotypes and 48.63-59.20% in susceptible genotypes at 60 DAS. In 68 DAS, in H_2O_2 increase was 139.77-155-17% in resistant genotypes and 162.34-226.92% in susceptible genotypes. H_2O_2 content increased in all the genotypes but the increase was more in susceptible genotypes as compared to resistant genotypes and all the genotypes differed significantly in H_2O_2 content.

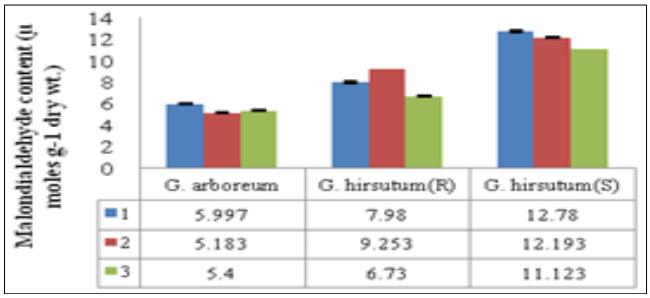
Hydrogen peroxide, a natural toxic plant metabolite is known to increase membrane permeability by attacking membrane lipids. In the present study, H_2O_2 was higher in sensitive genotypes than tolerant genotypes in healthy leaves (fig. 2a & 2b) and pest infection resulted in the increased production of H_2O_2 at both 60 DAS and 68 DAS stages but increase was more in susceptible genotypes than *G. hirsutum* resistant genotypes (fig. 2c & 2d). Similarly, colonization of pea aphids (*Acyrthosiphon pisum*) in pea led to significant insect density and time dependent enhancement in the rate of $\cdot O_2^-$ and H_2O_2 production (Mai *et al.* 2013). Similar results were also observed in Potato (*Solanum tuberosum*) leaves, attacked by green peach aphids (*Myzus persicae*), have nearly twice the H_2O_2 content than uninfested leaves (Kerchev *et al.*, 2012) [9]. Debona *et al.* (2012) [5] observed that wheat varieties inoculated with *Pyricularia oryzae* at vegetative stage resulted into increased H_2O_2 content in susceptible plants (BR18) and no significant change in partially resistant plants (BRS 229). El-Beltagi *et al.* (2012) [6] observed increased level of H_2O_2 and MDA in tomato plants infected with *Meloidogyne incognita* as compared to uninfected leaves and this increased content of MDA and H_2O_2 accounted for the defense mechanism against the invasion by this root-knot nematode.

Lipoxygenase (LOX): Results depicted in Fig. 3(a) and Fig. 3(b) shows the LOX activity in 2nd and 6th healthy leaves of resistant and susceptible cotton genotypes respectively. The activity of LOX in 2nd leaf before infection was maximum in *G. hirsutum* susceptible genotypes (147.74-151.89 units mg^{-1} protein) followed by *G. hirsutum* resistant genotypes (131.96-143.37 units mg^{-1} protein) and minimum in *G. arboreum* genotypes (124.03-131.12 units mg^{-1} protein). In 6th leaf maximum activity (156.81-162.13 units mg^{-1} protein) was in *G. hirsutum* susceptible genotype followed by *G. hirsutum* resistant genotypes (143.32-156.81 units mg^{-1} protein) and minimum in *G. arboreum* genotypes (136.43-142.93 units mg^{-1} protein). LOX activity was higher in susceptible genotypes than resistant genotypes. All the genotypes did not differ significantly in LOX activity.

Results depicted in Fig. 3(c) show the effect of pest infection on LOX activity in 2nd leaf of resistant and susceptible cotton genotypes and Fig. 3(d) shows the effect of pests infection on LOX activity in 6th leaf of resistant and susceptible cotton genotypes. After infection increase in LOX activity was observed in *G. hirsutum* genotypes. In 2nd leaf, at 60 DAS, increase in LOX activity was 7.53-16.19% in resistant genotypes and 10.86-26.21% in susceptible genotypes whereas at 68 DAS, more increase in LOX activity was observed and increase was 17.32-31.88% in resistant genotypes and 23.58-40.47% in susceptible genotypes. In 6th leaf increase was 10.90-13.49% in resistant genotypes and 13.36-20.72% in susceptible genotypes at 60 DAS and 68 DAS stage had 22.60-27.87% increase in resistant genotypes and 30.74-36.57% in susceptible genotypes. Significant increase was observed in all the genotypes. The activity increased in all the genotypes but the increase was more in susceptible genotypes as compared to resistant genotypes and all the genotypes differed significantly in LOX activity.

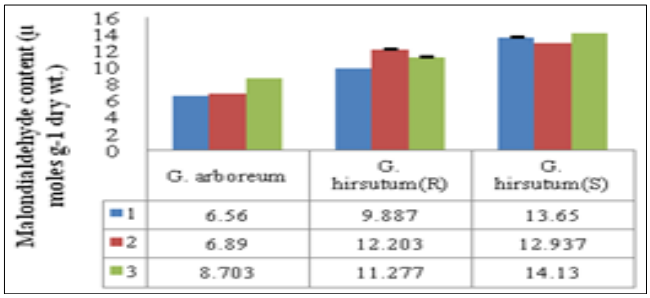
Lipoxygenases (LOXs) are non-heme-iron-containing enzymes, which catalyze the oxidation of polyunsaturated fatty acids containing a cis, cis-1, 4-pentadiene site. LOXs are another group of anti-oxidative enzymes involved in plant defense against many stresses through octadecanoid pathway. LOX activity was higher in sensitive genotypes in healthy leaves (50 DAS) as compared to tolerant genotypes (fig. 3a & 3b). Increase in activity was observed in all the genotypes on pest infection in both 2nd & 6th leaves at both 60 DAS and 68 DAS stages (fig. 3c & 3d). Similar results were observed by Bi *et al.* (1994) [1] in soyabean when infected by herbivore corn earworm *H. zea*. Zhao *et al.* (2009) [13] observed increase in LOX activity in wheat when infected by *Sitobion avenae* (F.). They catalyze hydroperoxidation of polyunsaturated fatty acids resulting in formation of fatty acid hydroperoxides.

The leaves of resistant genotypes had less production of H_2O_2, MDA and LOX as compared to susceptible cotton genotypes suggesting that these components play important role as indices of oxidative stress on sucking pests infection in cotton genotypes studied in the present investigation.



In G. arboretum 1=HD 418 2=HD503 3=HD432
In G. hirsutum (R) 1=H1464 2=H1465 3=H1098
In G. hirsutum (S) 1=H1463 2=H1454 3=H1439
CD at 5%: (a) Genotypes=0.07

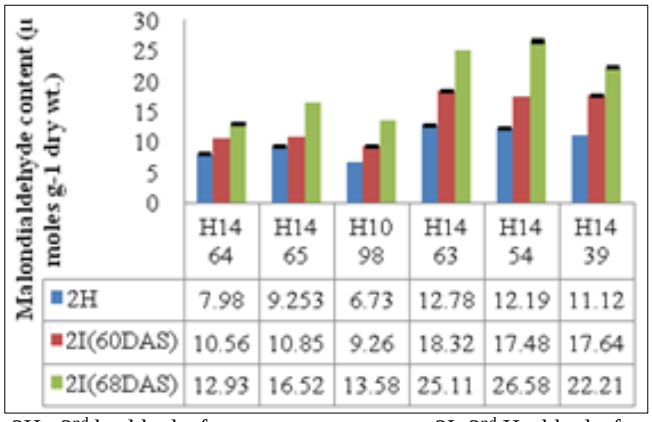
(a)

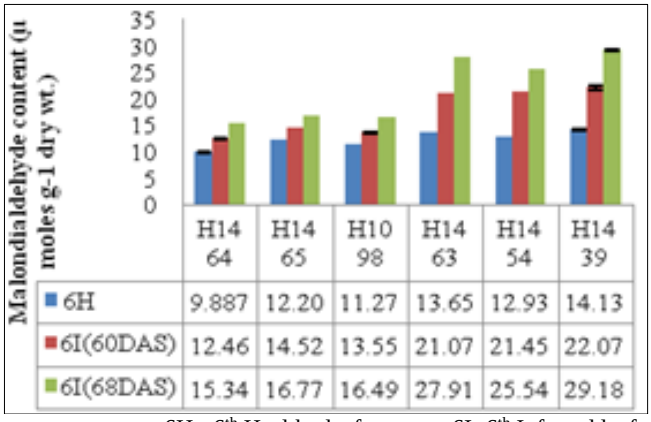


(b) Genotypes=0.07

(b)

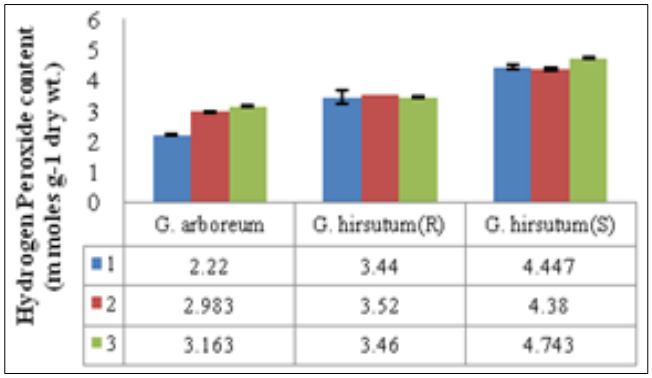
Fig 1(a, b): Malondialdehyde content (µ moles g^{-1} dry wt.) in (a) 2nd and (b) 6th leaves healthy leaf of resistant and susceptible cotton genotypes

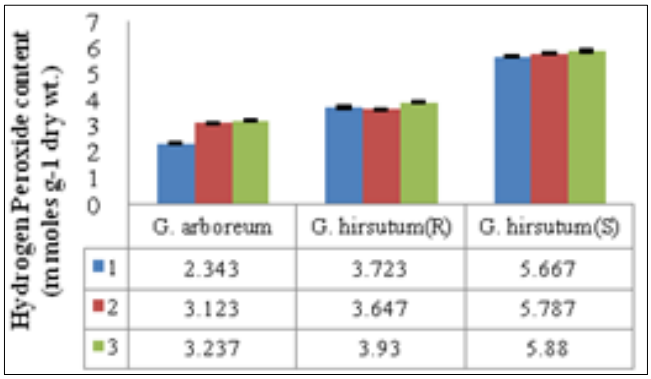




2H= 2nd healthy leaf 2I=2nd Healthy leaf 6H= 6th Healthy leaf 6I=6th Infected leaf
(c)H, I (60DAS) H, I (68DAS) (d) H, I (60DAS) H, I (68DAS)
Genotypes=0.05 Genotypes=0.19 Genotypes=0.25 Genotypes=0.07
Treatment=0.03 Treatment=0.11 Treatment=0.14 Treatment=0.04
Genotypes × Treatment=0.08 Genotypes × Treatment=0.28 Genotypes × Treatment=0.35 Genotypes × Treatment=0.09
(c) (d)

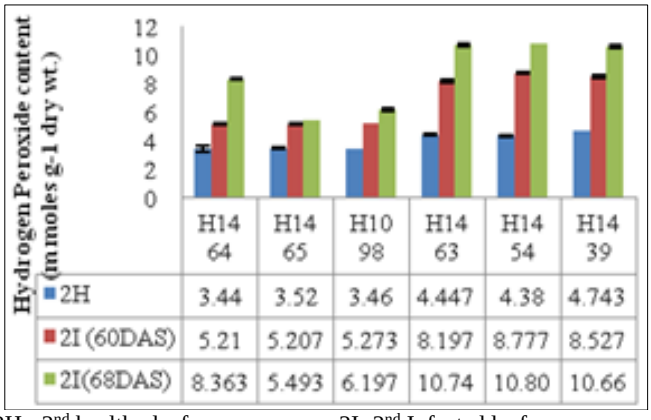
Fig 1(c, d): Effect of pests infection on Malondialdehyde content (µ moles g^{-1} dry wt.) in (c)2nd and (d) 6th leaves of resistant and susceptible cotton genotypes

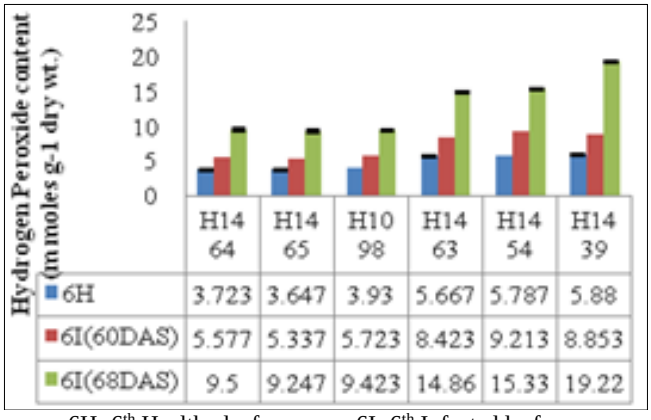




In *G. Arboreum* 1=HD 418 2=HD503 3=HD432
In *G. hirsutum* (R) 1=H1464 2=H1465 3=H1098
In *G. hirsutum* (S) 1=H1463 2=H1454 3=H1439
CD at 5%: (a) Genotypes=0.24 (b) Genotypes=0.11
(a) (b)

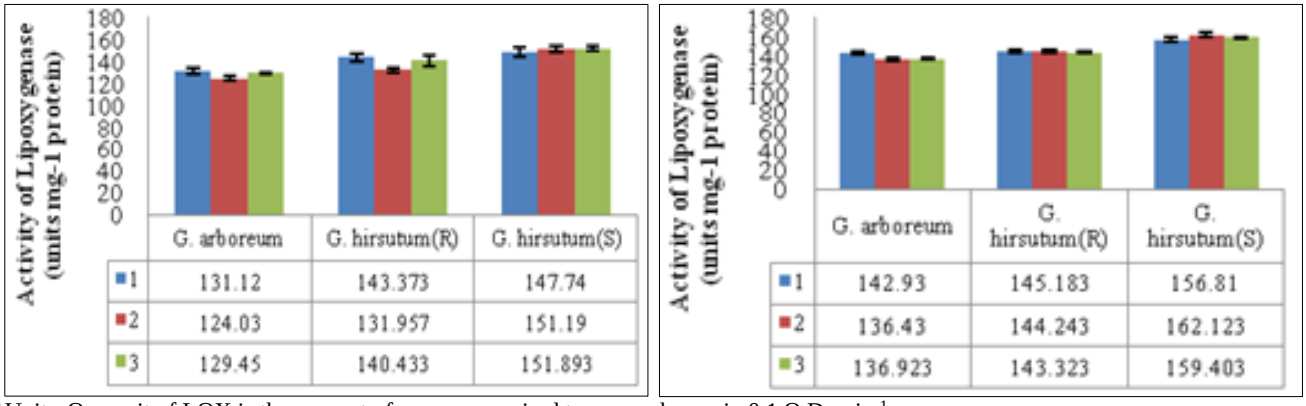
Fig 2(a, b): Hydrogen Peroxide content (m moles g^{-1} dry wt.) in (a) 2nd and (b) 6th healthy leaves of resistant and susceptible cotton genotypes





2H= 2nd healthy leaf 2I=2nd Infected leaf 6H=6th Healthy leaf 6I=6th Infected leaf
(c) H, I (60DAS) H, I (68DAS) (d) H, I (60DAS) H, I (68DAS)
Genotypes=0.17 Genotypes=0.18 Genotypes=0.09 Genotypes=0.16
Treatment=0.10 Treatment=0.10 Treatment=0.05 Treatment=0.09
Genotypes × Treatment=0.25 Genotypes × Treatment=0.25 Genotypes × Treatment=0.13 Genotypes × Treatment=0.23
(c) (d)

Fig 2(c, d): Effect of pests infection on Hydrogen Peroxide content (m moles g^{-1} dry wt.) in (c) 2nd and (d) 6th leaves of resistant and susceptible cotton genotypes



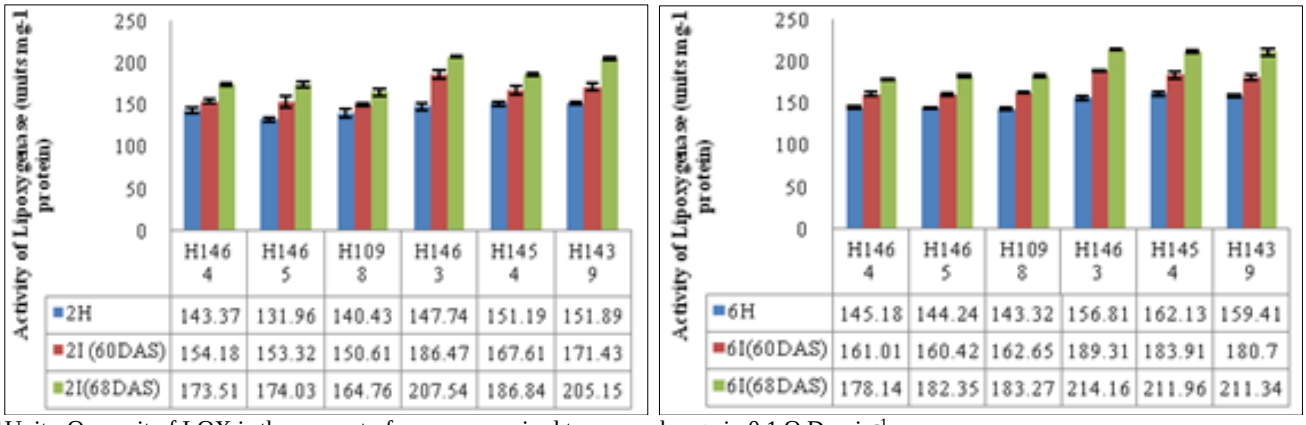
1Unit= One unit of LOX is the amount of enzyme required to cause change in 0.1 O.D. min^{-1}.

In *G. Arboreum*	1=HD 418	2=HD503	3=HD432
In *G. hirsutum* (R)	1=H1464	2=H1465	3=H1098
In *G. hirsutum* (S)	1=H1463	2=H1454	3=H1439

CD at 5%: (a) Genotypes=3.68 (b) Genotypes=2.43

(a) (b)

Fig 3(a, b): Lipoxygenase activity (Units mg^{-1} protein) in (a) 2nd and (b) 6th healthy leaves of resistant and susceptible cotton genotypes



1Unit= One unit of LOX is the amount of enzyme required to cause change in 0.1 O.D. min^{-1}.

2H= 2nd healthy leaf	2I=2nd Infected leaf	6H=6th Healthy leaf	6I=6th Infected leaf
(c) H, I (60DAS)	H, I (68DAS)	(d) H, I (60DAS)	H, I (68DAS)
Genotypes=8.45	Genotypes=6.13	Genotypes=4.79	Genotypes=4.37
Treatment=4.88	Treatment=3.54	Treatment=2.76	Treatment=2.53
Genotypes × Treatment=11.94	Genotypes × Treatment=8.67	Genotypes × Treatment=6.77	Genotypes × Treatment=6.19

(c) (d)

Fig 3(c, d): Effect of pests infection on Lipoxygenase activity (units mg^{-1} protein) in (c) 2nd and (d) 6th leaves of resistant and susceptible cotton genotypes

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