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Differential biochemical and physiological responses to cotton leaf curl virus infection in contrasting cotton genotypes

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Abstract
Cotton production is tremendously impacted by the cotton leaf curl virus (CLCuV) in the Indian subcontinent. Viral infection modifies physiological and biochemical processes in plants, which leads to the appearance of symptoms. Hence, the current study was to discern the quantifiable changes in physiological and biochemical parameters in leaves of cotton plants graft inoculated with CLCuV on different days after infestation (DAI). The results revealed that in the CLCuV-infected plants, the relative water content (RWC) and total soluble protein were decreased. Some enzymes involved in the activities like anti-oxidative and defence mechanisms such as polyphenol oxidase (PPO), catalase (CAT), phenylalanine ammonia-lyase (PAL), and peroxidase (POX) were increased as the infestation period progressed with a concomitant increase in gossypol and total phenolic level. Contents of total soluble sugar, chlorophyll (Chl) along with hydrogen peroxide (H_2O_2) were increased initially and decreased afterwards in all genotypes. Correlation analysis elucidates that RWC is negatively associated with electrolyte leakage (EL) and malondialdehyde (MDA) content. In contrast, Chl content showed a positive correlation with total soluble proteins, secondary metabolites like phenol and gossypol, anti-oxidative and defence enzymes. Three principal components (PCs) were produced by the principal component analysis (PCA) contributing 87.33% of the total variability in various physiological and biochemical traits of different genotypes. Phenolics profiling reveals that concentrations of rutin hydrate in the infected sample increased by 49.6% compared to the control one. Overall, the findings imply that CLCuV infection causes substantial changes in enzyme levels that result in the development of unrectifiable symptoms in susceptible genotypes.

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Introduction

Cotton (*Gossypium hirsutum* L.) commonly called "White Gold" is a significant crop grown for food, fuel, fiber, and feed in 80 different nations throughout the world (Chakravarthy et al. 2014). It has been placed 1st for fibre and 2nd among oil seed crops globally since it contributes 45% and 10%, respectively, of the world's fibre and edible oil (Gul et al. 2014; Rathore 2002). Cotton is India's second-largest *Kharif* crop and is mainly cultivated in Karnataka, Madhya Pradesh, Punjab, Maharashtra, Haryana, Tamil Nadu, Gujarat, Rajasthan and Andhra Pradesh. Cotton holds the top spot in the country's agriculture as well as in Haryana due to its significance as a cash crop.

Cotton farming is imperilled due to numerous unforeseen stresses such as the attack of pathogens and pests that negatively impact the crop's growth. Some of the most

devastating and commercially significant diseases of crop plants, particularly in tropical and subtropical areas, are caused by insect-transmitted viruses (Inoue-Nagata et al. 2016). The *Geminiviruses* are responsible for many of these diseases. Cotton leaf curl virus (CLCuV) is an economically critical monopartite *geminivirus* culpable for the cotton leaf curl disease (CLCuD) (Humza et al. 2016; Leke et al. 2015). It belongs to the second-largest plant virus family, viz*., Geminiviridae. Bemisia tabaci* is solely responsible for the circulative and persistent transmission of CLCuV (Khan et al. 2015; Zerbini et al. 2017).

CLCuD is the most devastating disease because it results in the loss of millions of dollars annually throughout the world. Infected cotton plants display a range of symptoms such as upward or downward leaf curling, stunting and darkening of the veins, and enations on the vein which usually transform into structures that resemble cup-shaped leaves (Akhtar et al. 2008). Following the outbreak of CLCuD in Nigeria in 1912, the disease has since spread to a number of cotton-growing nations, including India, the United States, China, and Pakistan (Rahman et al. 2017). In the Indian Subcontinent, CLCuD reduces the production of cotton by causing up to 80% losses in states of North India (Azhar et al. 2010; Mansoor et al. 2003; Sattar et al. 2013). Recently, cotton in the Indian subcontinent has been under major threat from the cotton leaf curl burewala virus (CLCuBuV). Among the most prevalent and economically devastating diseases, CLCuD caused by this viral infection has caused major cotton economic losses in three northwest Indian states Rajasthan, Punjab and Haryana (Biswas et al. 2020; Siddique et al. 2014; Sonika et al. 2017).

Several studies have revealed that virus proliferation inside plant cells affects various biochemical components and interferes with the physiological functions of infected plants, which impairs growth and yield (El-Dougdoug et al. 2014; Mushtaq et al. 2014; Radwan et al. 2007; Tajul et al. 2011). Numerous defence mechanisms have been evolved by plants either in anticipation of or as a result of diseases (Czosnek et al. 2013). Plants have intricate antioxidant metabolism to repair the destruction caused by reactive oxygen species (ROS). Peroxidase (POX) is among the initial enzymes to react and provides prompt defence against plant-affecting pathogens. Numerous plant oxidative enzymes, including polyphenol oxidase (PPO) and POX, catalyze the synthesis of oxidative phenols that strengthen cell structure during pathogen invasion, regulation of cell wall, wound healing, and oxidation of phenols to produce more reactive radicals that can interact with biomolecules. Thus, plant oxidative enzymes create an unfavourable pathogen development environment (Maksinov et al. 2014; Mohamed et al. 2012). Phenylalanine ammonia-lyase (PAL) is the key enzyme in the metabolism of phenylpropanoids and is essential for the synthesis of many secondary compounds

Plate 1 Graft inoculation of cotton plant with CLCuV containing plant parts

involved in defence, including lignin and phenolics (Hemm et al. 2004; Tahsili et al. 2014).

The invigoration of a wide array of defensive mechanisms in plants at certain stages of the host–pathogen interactions is associated with disease resistance that slows down or halts the infection process (Vanitha et al. 2009; Zhao et al. 2008). By nature, plants protect themselves by producing many secondary metabolites, including phenolics, defence-related enzymes, viz. those involved in phenol metabolism and antioxidative enzymes (Nair and Umamaheswaran 2016). Therefore, despite interactions that do not result in plant disease or death, any pathogen attack reduces crop yields (Berger et al. 2011; Yazaki 2006).

CLCuD-resistant cotton development is essential for lessening the potential risks to the crop, especially in the Indian

subcontinent. Understanding fundamental physiological and biochemical alterations in response to disease incidence would therefore aid in the successful exploitation of the resistance sources, providing characteristics for the breeding of CLCuD-resistant varieties (Zafar and Athar 2013). Our understanding of the physiology and biochemistry of viruses is limited because they cannot be cultivated in vitro. Therefore, in the present study, the physiological and biochemical changes in cotton plants infected with CLCuV were investigated by determining electrolyte leakage (EL), relative water content (RWC) and chlorophyll (Chl), total phenolic content, total soluble protein content, malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and total soluble sugar content and activities of PAL, POX, catalase (CAT) and PPO.

Materials and methods

In the present study, three contrasting genotypes were used for the analysis including highly tolerant, highly susceptible and resistant cotton genotype, i.e. GCH 3, HS 6 and HD 432, respectively. These genotypes were grown under protected conditions during the kharif season (2017) in the cotton division of the Department of Genetics and Plant Breeding at the CCS Haryana Agricultural University, Hisar. After 40 days of sowing, i.e. 0 days after infestation (DAI), the genotypes were inoculated with the CLCuV by grafting the CLCuV containing cotton plant parts (Plate 1) based on the procedure outlined by Akhtar et al. (2013). The control and inoculated plants were maintained in an insect-free environment

in separate cages with a temperature of 23.6–27.5 °C (minimum) and 32–35 °C (maximum) together with a 16 h period of light and 8 h period of dark. The relative humidity ranges from 61 to 78.5% during the morning and 39.3 to 56.8% during the evening. The disease severity index value presented in Table 1, delineated and proposed by Farooq et al. (2011); Akhtar et al. (2010), was used to record data regarding CLCuV symptoms. Using the following formula, the disease scale of CLCuD was determined:

$$\text{Percent disease index (PDI)} = \frac{\text{Total of all the disease ratings}}{\text{Number of plants examined}} \times 16.66$$

CLCuV-infected leaves were collected separately in triplicates from each genotype at four intervals, i.e. at 0, 15, 30 and 45 DAI. Studies for physiological parameters were carried out immediately after sampling. The samples were crushed in liquid nitrogen, and the powdered form of the sample was then used as per necessity to examine the different biochemical constituents. All the chemicals used were of the highest analytical grade and purity and were bought from Merck and Sigma Chemical Co. (USA), Loba Chemicals.

Measurement of physiological parameters

Freshly excised leaves were weighed immediately (FW) and then put in distilled water. After 24 h, the leaves were reweighed (TW) and then dried to constant weight (DW) according to Barrs and Weatherley (1962). RWC of leaves was calculated using the formula:

$$\text{RWC}(\%) = \frac{\text{Fresh weight (FW)} - \text{Dry weight (DW)}}{\text{Fully turgid weight (TW)} - \text{Dry weight (DW)}} \times 100$$

Table 1 Symptom severity scale for CLCuV

Prodromes	Rating of Disease	Disease vulnerability
Asymptomatic	0	Not vulnerable
A plant with 10 or less leaves that either has small veins thickening or leaf enations	1	Highly resistant
A small cluster of veins become thicker	2	Resistant
All veins thicken but no curling of leaves	3	Moderate resistant
Severe leaf curling and thickening of vein on upper one-third of the plant	4	Moderately susceptible
On one half of the plant; there is severe leaf curving and vein thickening	5	Susceptible
Severe leaf curling and thickening of the vein with decreased yield, stunted growth of the plant	6	Highly susceptibility

For estimation of Chl content, 10 ml of dimethyl sulphoxide was added to 100 mg of the samples and kept in the dark for 12 h. According to Hiscox and Israelstam (1979), absorbance measurements at 645 and 663 nm were made against a reagent blank. The following formula was used to calculate the total Chl content:

$$\text{Total Chl content (mg g}^{-1}\text{Fwt)} = \frac{(20.2 \times \text{A645}) + (8.02 \times \text{A663})}{1000 \times \text{W}}$$
$$\times \text{V}$$

For determining EL (in terms of relative stress injury, RSI), Dionisio-Sese and Tobita's (1998) methodology was applied. At room temperature, leaf discs were left in 10 ml of deionized water. After 5 h, the surrounding solution's electrical conductivity (ECa) was determined. The samples were held in a bath of boiling water and the solution's conductivity was once more measured (ECb). The calculation of RSI was done by the use of the formula:

$$\text{RSI}(\%) = \frac{\text{EC}_a}{\text{EC}_b} \times 100$$

Measurement of biochemical parameters

For MDA and H_2O_2 determination, samples were homogenized in 1% and 5% tricarboxylic acid (TCA), respectively. The supernatant obtained after centrifugation was used for estimation. For MDA, 500 µl supernatant and 2 ml of 20% TCA (with thiobarbituric acid in it) were heated at 95 °C as long as 30 min and chilled directly. The supernatant obtained after centrifugation was used for the absorbance at two wavelengths (532 and 600 nm). The value at 600 nm (non-specific absorption) was deducted from the absorbance at 532 nm (Heath and Packer 1968). The extinction coefficient was used to determine the MDA concentration. For H_2O_2, a mixture of 0.5 ml extract, 3 ml mixture of potassium dichromate (5% (w/v)) and glacial acetic acid mixed in a ratio of 1:3 (v/v) and 0.5 ml of 0.1 M phosphate buffer having pH 7.0 was placed in a water bath for 10 min at 90–95 °C (Sinha 1972). After cooling, absorbance at 570 nm was measured in comparison to the reagent blank. The amount of H_2O_2 was calculated using a standard curve prepared by treating different concentrations of H_2O_2 with dichromate acetic acid.

The sample was macerated in 0.1 M phosphate buffer (pH 7.6) for total soluble protein and heated in 80% ethanol for total soluble sugar, total phenolic and gossypol content. After centrifuging the homogenate, the supernatant was utilized for the estimation of total soluble protein. The absorbance of the mixture containing 0.1 ml of supernatant, 0.9 ml of phosphate buffer (pH 7.5) and 5 ml of Bradford reagent was measured at 595 nm against a reagent blank (Bradford 1976). Bovine serum albumin was used to generate a standard curve which was used to calculate the total soluble protein concentration. Total soluble sugar was estimated by adding 5 ml of concentrated sulphuric acid to aliquots (0.5 ml) containing 2% phenol (2 ml) later at 490 nm absorbance was documented against a corresponding reagent blank (Dubois et al. 1956). The standard curve prepared using dextrose was used to determine the total soluble sugar concentration in samples. For total phenolic content estimation, 2 ml 20% sodium carbonate, extract (0.5 ml) and 1N Folin–Ciocalteau reagent (0.5 ml) was used. Using a UV spectrophotometer (Shimadzu UV1900i), the blue colour generated was measured at 650 nm against a blank reagent (Bray and Thorpe 1954). Bell's (1967) method was used for gossypol quantification. 15 ml of diethyl ether was added to the supernatant after adjusting its pH to 3, and the ether phase was then concentrated in a centrifuge. Later, 3 ml of 95% ethanol was used to re-dissolve the residue. For estimation, 1 ml extract with phloroglucinol reagent (0.5 ml) and 1 ml of concentrated HCl was kept at 30 °C for 30 min. The final volume was made 10 ml using ethanol. Against a reagent blank, a UV spectrophotometer was used to detect absorbance at 550 nm.

Anti-oxidative and defence enzyme

For activities of defence enzymes, i.e. PPO, PAL and anti-oxidative enzymes CAT, POX leaf samples were hand homogenized in buffers (sodium phosphate buffer having pH 6.5 for PPO, Tris–HCl buffer with pH 8.0, 7.6, 8.8 for CAT, POX, PAL respectively). The homogenate was subjected to centrifugation at 2 °C, and the supernatant was used for enzyme assays. For determining CAT activity (EC 1.11.1.6), 0.4 ml phosphate buffer (0.1 M and pH 7.0), 0.2 ml enzyme extract and H_2O_2 (0.4 ml) were incubated at 37 °C. Potassium dichromate (5%) and acetic acid in a volume of 2 ml were added to the reaction mixture in a 1:3 ratio and the enzyme activity was stopped. After cooling, the absorbance (at 570 nm) was measured (Sinha 1972). The activity of POX (EC 1.11.1.7) was assessed at 470 nm for 4 min by following the method of Shannon et al. (1966). 200 µl of 0.1% o-dianisidine, 3.68 ml sodium phosphate buffer (0.1 M at pH 7.0), 0.2% H_2O_2 (100 µl) and 20 µl enzyme extract made up the reaction mixture. Spectrophotometric analysis was used to determine the activity of PPO (EC 1.10.3.1) at 410 nm for 4 min. 0.9 ml sodium acetate buffer (0.2 M at pH 4.0), 20 mM catechol (2 ml) and enzyme extract (0.1 ml) made up the reaction mixture (Ying and Zhang 2008). For PAL (EC 4.3.1.25), a mixture of enzyme extract (1 ml), 0.02 M L-phenylalanine (1 ml) and 0.05 M borate buffer at pH 8.8 (2 ml) was incubated at 30 °C for 1 h. To stop the reaction, 10% trichloroacetic acid (0.5 ml) was added, and activity was measured at 290 nm (Ngadze et al. 2012).

Profiling of phenolic compounds

The method described by Adom and Liu (2002) was used to extract phenolic components from healthy and diseased leaves (2.0 g) of GCH 3. With a few minor adjustments to the technique described by Irakli et al. (2012), the extracted materials were further cleaned using Oasis HLB 6CC cartridges and Sep-Pak C18. Nylon syringe filters (0.22-micron) were used to filter the methanol-dissolved material. The phenolic composition of leaves extract of GCH 3 was analysed using the UPLC system following the method of Mandhania et al. (2019).

Statistical analysis

Statistical computing of data was done by OPSTAT and SPSS software (IBM SPSS Statistics version 26). Analysis of the experimental data was done by applying a two-factor randomized block design utilizing the OPSTAT program, which is accessible at the CCSHAU homepage. In addition, correlation analysis and principal component analysis (PCA) were done using SPSS.

Results

Table 2 displays the evaluation outcomes of cotton genotypes against CLCuV following graft inoculation. Grafting success rates across all genotypes were 100%.

Genotypes GCH 3 and HS 6 showed diseased symptoms with PDI values ranging from 3.78 to 12.99 while HD 432 was asymptomatic. PDI scores increased with time based on the genotype's genetic makeup except for HD 432. HS 6 had PDI ranging from 6.11 at 15 DAI to 12.99 at 45 DAI.

Physiological parameters

A significant decrease in RWC has been recorded in all virus-infected genotypes in this study. The decline rate was more between 15 and 45 DAI in the HS 6 genotype (9.467%) compared to GCH 3 and HD 432 (0.89–3.53%). At the same time, an increase in EL was reported in all genotypes post-incursion of the virus. The maximum increase was found in HS 6 (18.65–59.77%) followed by highly tolerant genotype GCH 3 (0.99–6.9%) from 0 to 30 DAI.

The increase was found to be the least in resistant genotype HD 432. The total Chl content followed the different trends in different genotypes after 15 DAI. It reduced in highly susceptible and tolerant genotypes with progress in the infestation period while increasing in the resistant genotype. There was an 18.51% increase in Chl content between 15 and 45 DAI (Table 3).

Biochemical parameters

With the progression of infection, enhancement was observed in MDA content in all genotypes. However, a significantly higher amount of MDA was reported in HS 6 at all DAI compared with GCH 3 and HD 432. The MDA content differs significantly among the genotypes at the same stages of infestation (Fig. 1a). CLCuD initially led to an increase in H_2O_2 content from 0 to 15 DAI; afterwards, it decreased in

Table 2 Evaluation of plants of different genotypes against per cent disease index (PDI) of cotton leaf curl disease caused by CLCuV through grafting

DAI	Genotype	PDI
0	HS 6	0.00 ± 0^a
	GCH 3	0.00 ± 0^a
	HD 432	0.00 ± 0^a
15	HS 6	6.11 ± 0.111^a
	GCH 3	3.78 ± 0.111^b
	HD 432	0.00 ± 0.193^c
30	HS 6	10.11 ± 0.111^a
	GCH 3	7.98 ± 0.192^b
	HD 432	0.00 ± 0.00^c
45	HS 6	12.99 ± 0.192^a
	GCH 3	10.89 ± 0.111^b
	HD 432	0.00 ± 0.00^c

Different small alphabets in the column indicate a significant differences ($p < 0.05$) between genotypes at the same DAI

Values are means $\pm$SE of three replicates

Table 3 Effect of CLCuV infestation on EL (electrolyte leakage), Chl (total chlorophyll) content and RWC (relative water content) in leaves of cotton genotypes at different DAI (days after infestation)

DAI	Genotype	RWC	EL	Chl
0	HS 6	78.95 ± 0.226^a	9.85 ± 0.266^a	1.69 ± 0.031^a
	GCH 3	82.53 ± 0.381^b	8.83 ± 0.489^b	1.85 ± 0.057^b
	HD 432	80.49 ± 0.601^c	7.79 ± 0.223^c	2.01 ± 0.025^c
15	HS 6	77.73 ± 0.286^a	12.69 ± 0.557^a	2.03 ± 0.038^a
	GCH 3	81.62 ± 0.303^b	8.92 ± 0.494^b	2.13 ± 0.151^a
	HD 432	79.61 ± 0.482^c	7.86 ± 0.233^c	3.14 ± 0.053^b
30	HS 6	73.18 ± 0.51^a	15.74 ± 0.594^a	1.90 ± 0.166^a
	GCH 3	78.37 ± 0.357^b	9.44 ± 0.425^b	2.07 ± 0.145^b
	HD 432	78.68 ± 0.456^b	8.06 ± 0.088^c	3.67 ± 0.148^c
45	HS 6	69.02 ± 0.753^a	20.32 ± 0.62^a	1.84 ± 0.05^a
	GCH 3	74.29 ± 0.777^b	10.18 ± 0.309^b	1.98 ± 0.098^b
	HD 432	78.26 ± 0.309^c	8.33 ± 0.079^c	3.73 ± 0.119^c

Different small alphabets in each column indicate a significant differences ($p < 0.05$) between genotypes at the same DAI

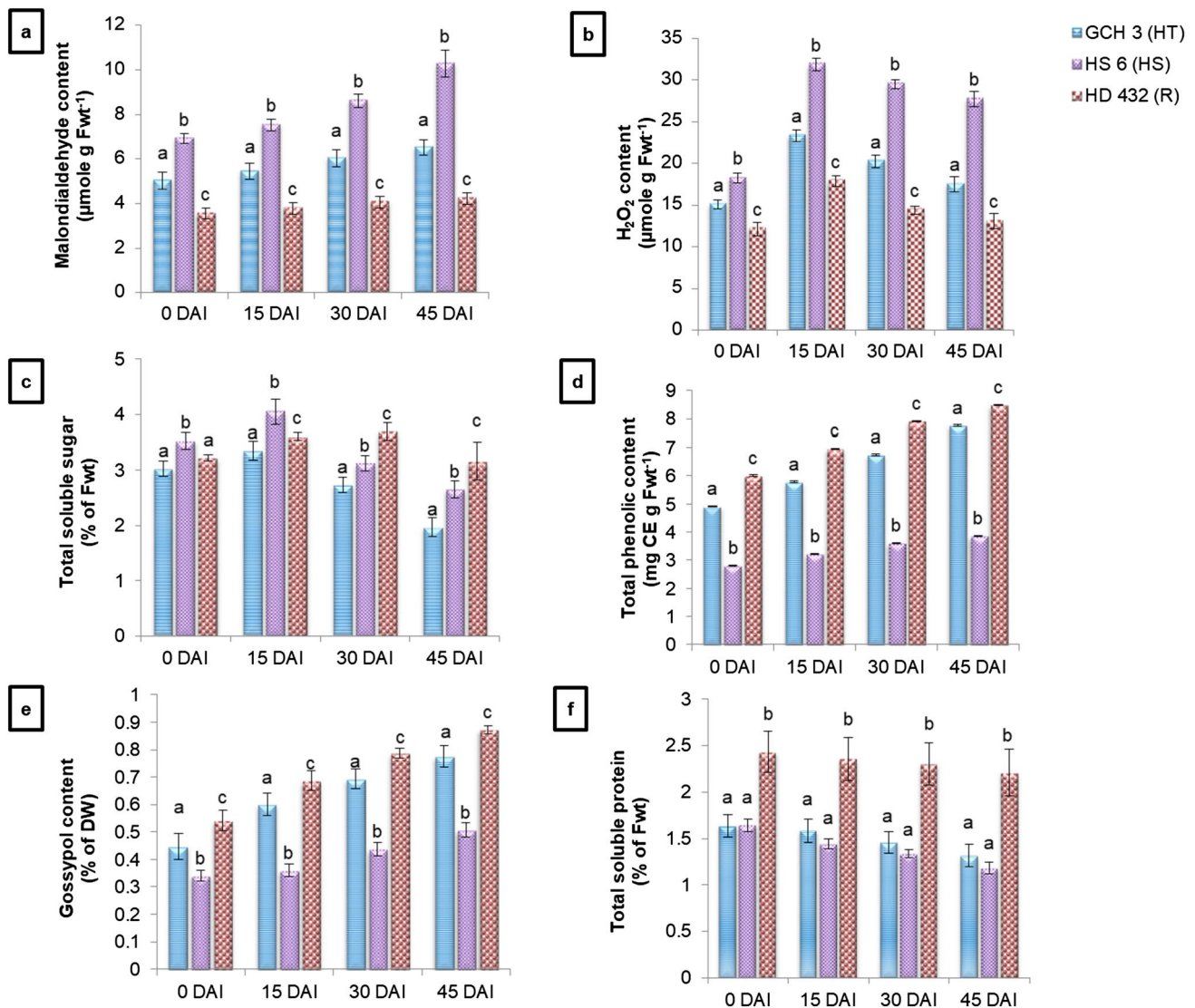Values are means $\pm$SE of three replicates

Fig. 1 Effect of CLCuV infestation on: **a** Malondialdehyde (MDA) content; **b** hydrogen peroxide (H_2O_2) content; **c** total soluble sugar content; **d** total phenolic content; **e** gossypol content; **f** total soluble protein content in leaves of cotton genotypes at different days after infestation (DAI). Different small alphabet letters on each panel indicate a significant difference ($p < 0.05$) between genotypes at the same DAI

all genotypes. All genotypes showed a significant difference in H_2O_2 content at all DAI. Maximum content was observed in HS 6 at all DAI (Fig. 1b).

A significantly high concentration of total soluble protein was recorded in resistant genotype (HD 432) compared with other genotypes. Total soluble protein content diminished with the increase in the duration of infestation (Fig. 1f). Total soluble sugar content increased from 0 to 30 DAI, and then it declined. From 0 to 45 DAI, the reduction was significantly higher in GCH 3 (34.87%) as compared with HS 6 and HD 432 (Fig. 1c). Total phenolic content increased significantly, but to a different extent in all genotypes from 0 to 45 DAI. The maximum increase was found in GCH 3

(56.70%) and the least (36.87%) in highly HS 6 from 0 to 45 DAI (Fig. 1d). Gossypol content increased significantly in all genotypes from 0 to 45 DAI (Fig. 1e).

Anti-oxidative and defence enzymes

The biochemical response of cotton plants towards CLCuV infestation was recorded at different DAI. All genotypes demonstrated an increase in activities (significant) of PPO and POX with prolongation of the infection period (Fig. 2a, b). CAT activity was significantly upregulated in all genotypes, with the maximum increase in HD 432 (Fig. 2d). The maximum activity of PAL was recorded in HD 432 at 0 DAI
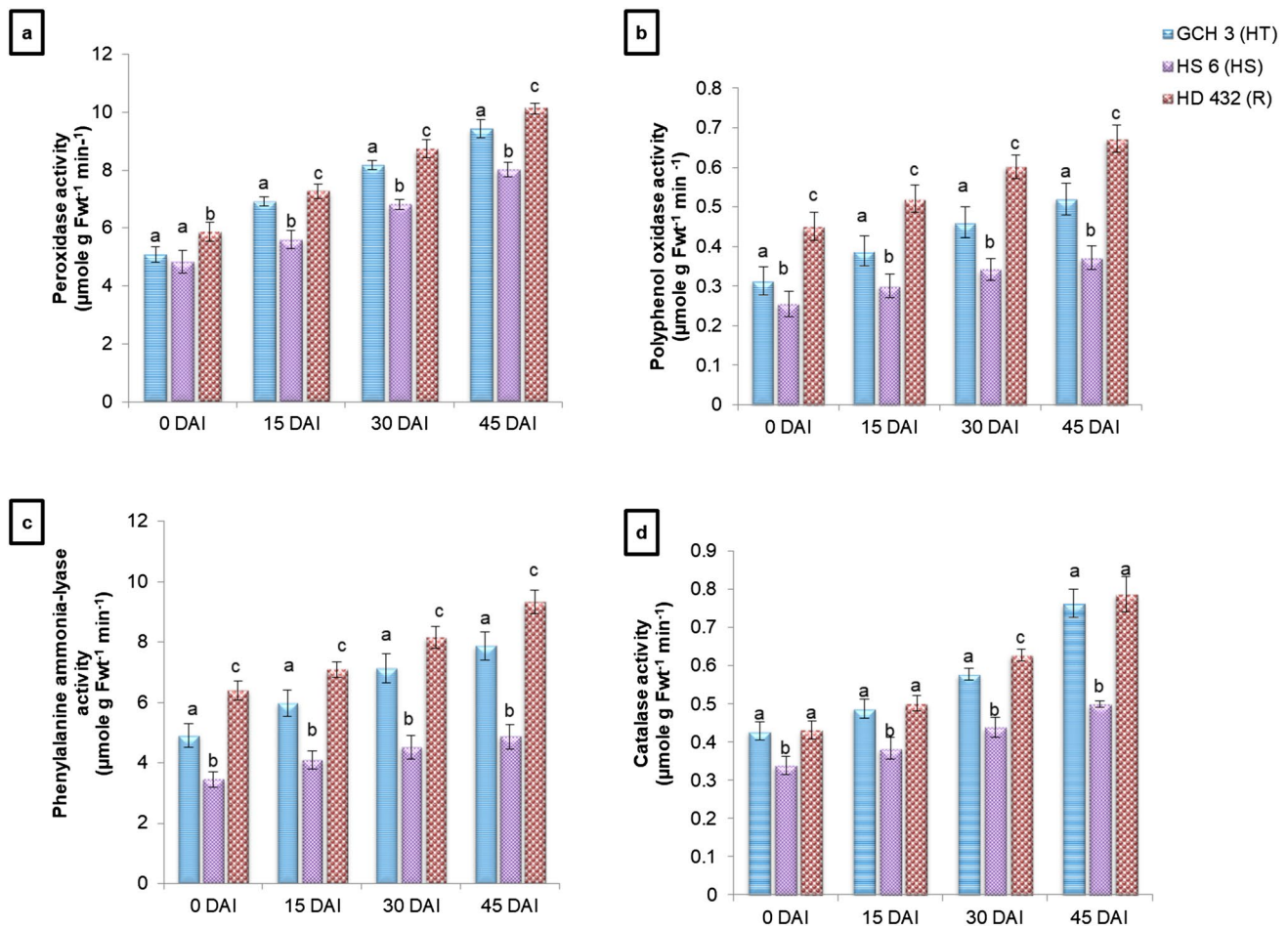
Fig. 2 Effect of CLCuV infestation on: **a** peroxidase activity; **b** polyphenol oxidase activity; **c** phenylalanine ammonia-lyase activity; **d** catalase activity (μmole min^{-1} g^{-1}Fwt) in leaves of cotton genotypes at different days after infestation (DAI). Different small alphabet letters on each panel indicate a significant difference ($p < 0.05$) between genotypes at the same DAI

(Fig. 2c). The rise in the activity of PAL was observed in all genotypes with a maximum increase in GCH 3 with the progression of the DAI. The enzyme activity differed significantly among all the genotypes at all stages of infestation (0, 15, 30 and 45 DAI), respectively. The maximum activity of the anti-oxidative and defence enzymes was reported in HD 432 as compared to other genotypes at all DAI.

The overall comparison of the physiological and biochemical parameters examined across the three genotypes is shown in Table 4. The + symbol denotes a general rise in the genotypes' studied parameters between 0 and 45 DAI. The general decline in the parameters in the genotypes between 0 and 45 DAI is indicated by − symbol. In every genotype, there is a decrease in RWC, total soluble protein content and total soluble sugar content. However, the greatest reduction in these parameters was seen in HS 6 genotype. Conversely, HS 6 experiences the least increase in gossypol, total phenolics contents and activities of CAT, PPO, POX and PAL with maximum increase in EL, MDA and H$_2$O$_2$ contents.

Correlation and PCA

Relationship among 13 traits was uncovered using simple correlation coefficients (Table 5). Analysis between morphological and biochemical characters of CLCuV-infected cotton exhibited that RWC is negatively and significantly associated with EL and MDA, with RWC illustrating 72.59% and 56.85% variation in EL and MDA, respectively. Chl content exhibited a highly significant and positive correlation with total soluble protein, anti-oxidative and defence enzymes such as POX, PPO, CAT and PAL, with secondary metabolites like phenol and gossypol except with H$_2$O$_2$ insignificant negative correlation. At the same time, H$_2$O$_2$ was directly and significantly correlated with MDA, with H$_2$O$_2$ explaining a 55.5% variation in MDA, which accounts for a 65.62% variation in EL. For MDA and H$_2$O$_2$, significant and negative correlation was found with PAL, total phenolic, total soluble protein and Chl content. A statistically significant and positive strong correlation exists between total phenolic

Table 4 Comparison of the physiological and biochemical parameters among three genotypes

Parameters	Genotypes		
	GCH 3 (Highly tolerant)	HS 6 (Highly susceptible)	HD 432 (Resistant)
RWC	– – –	– – – –	–
Chl	+	+	+ + + + + + + + + + + + + + + + + + + +
EL	+ + +	+ + + + + + + + + + + + + + + + + + +	+
MDA	+ + + + + +	+ + + + + + + + +	+ + +
H_2O_2	+ + +	+ + + + + + + + + +	+
Total soluble protein	– – – – –	– – – – – – – –	– – –
Total soluble sugar	– – – – – – – – – –	– – – – – – – – – –	–
Total phenolics	+ + + + + + + + + +	+ + + + + + +	+ + + + + + + +
Gossypol	+ + + + + + + + + + + + +	+ + + + + + + + +	+ + + + + + + + + + + +
CAT	+ + + + + + + + + + + + + +	+ + + + + + + + +	+ + + + + + + + + + + + + + + + + +
PPO	+ + + + + + + + + + + +	+ + + + + + + + +	+ + + + + + + + + +
POX	+ + + + + + + + + + + + + + + + + +	+ + + + + + + + + + + +	+ + + + + + + + + + + + + + + +
PAL	+ + + + + + + + + + +	+ + + + + + + +	+ + + + + + + + +

(The – and + sign shows % decrease and increase in the parameter respectively. One – sign represents approximately a 3% decrease while one + sign represents approximately a 5% increase in the parameter)

content, gossypol content and activity of PAL. PAL is positively correlated with Chl, phenol, POX, PPO, CAT and gossypol and moderately but positively correlated with total soluble protein content.

PCA divides the total variance into different factors. Out of the 13 components in this investigation, three principal components (PCs) have been found with an Eigen value greater than 1. PCA with the variables of this study shows that most of the % of the variability was explained by three components (Fig. 3). It illustrates how, in response to CLCuV infection, these three PCs were responsible for 87.32% of the overall variation among cotton genotypes tested for a variety of physiological and biochemical characteristics. However, only 12.67% of the alterations caused by CLCuD were attributable to the remaining components. The PC I made the biggest contribution to variability (53.561%), followed by the PC II (24.726%) and the PC III (9.042%), respectively. In our experiment, 1st PC was mainly due to variations in components such as Chl, phenol, POX, PPO, gossypol, CAT and PAL. These results agree with the outcome of the correlation analysis. PC 2 was explained by genotype variation with significant positive and negative loadings of RWC, total soluble protein and EL, MDA and H_2O_2 respectively. Similarly, PC 3 was explicated by variation in total soluble sugar content with its considerable favourable factor loading.

Phenolic profiling

The phenolic compound in the highly tolerant genotype (GCH 3) was compared in control and infected leaf samples (Figs. 4 and 5). The standard of 28 phenolic compounds was used to identify and quantify the phenolic compounds in the GCH 3 sample. The control plant's level of taxifolin was significantly higher than that of the infected sample. Vanillin, procyanidin B2 and chlorogenic acid concentrations increased in the infected sample as compared to the control.

The concentrations of rutin hydrate in the infected sample increased by 49.6% as compared to the control one. Among all the phenolic compounds identified, isoquercetin was found in the highest concentration in the infected sample. The highest concentration of taxofolin followed by isoquercetin was found in the control sample (Table 6).

Discussion

CLCuV is undoubtedly among the most pernicious pathogen of cotton crops as it restrains its production in many areas of the world (Mandadi and Scholthof 2013). To understand the mechanism of symptom development, it is crucial to understand the physiology of plant tissues infected with the virus. For its genome to replicate and spread, the virus causes a

Table 5 Pearson's correlation coefficients among different physiological and biochemical parameters in cotton under CLCuV infestation

	RWC	EL	Chl	Total phenolics	Total soluble sugar	POX	PPO	GOSSYPOL	CAT	Total soluble protein	PAL	MDA	H$_2$O$_2$
RWC	1												
EL	-0.852**	1											
Chl	0.174	-0.386**	1										
Total phenolics	0.221	-0.549**	0.701**	1									
Total soluble sugar	0.374**	-0.195	0.304*	-0.237	1								
POX	-0.377**	0.028	0.592**	0.720**	-0.358*	1							
PPO	0.067	-0.368*	0.709**	0.810**	-0.174	0.713**	1						
GOSSYPOL	0.031	-0.376**	0.687**	0.918**	-0.248	0.801**	0.851**	1					
CAT	-0.142	-0.177	0.553**	0808**	-0.346*	0.849**	0.769**	0.868**	1				
Total soluble protein	0.411**	-0.568**	0.555**	0.476**	0.352*	0.085	0.367*	0.361*	0.097	1			
PAL	0.115	-0.433**	0.692**	0.932**	-0.232	0.733**	0.803**	0.927**	0.803**	0.441**	1		
MDA	-0.754**	0.875**	-0.547**	-0.649**	-0.181	-0.075	-0.551**	-0.508**	-0.229	-0.722**	-0.591**	1	
H$_2$O$_2$	-0.510**	0.705**	-0.438**	-0.648**	0.121	-0.209	-0.470**	-0.491**	-0.375*	-0.581**	-0.555**	0.745**	1

(*, **significantly different at the 5% and 1% probability levels, respectively)
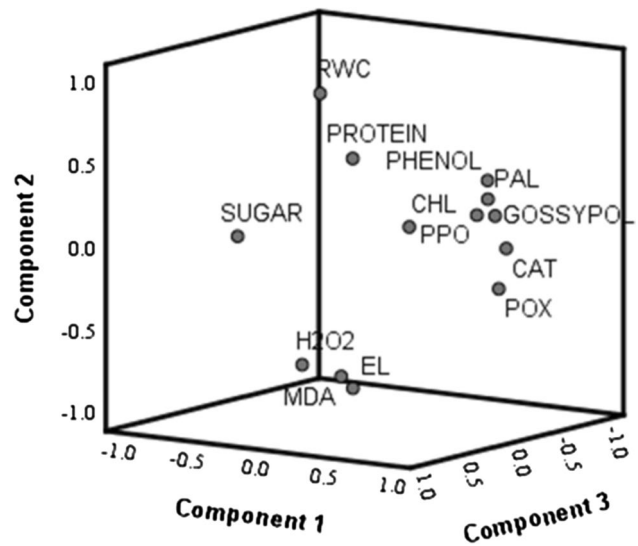
Fig. 3 Principal component analysis of various physiological and biochemical parameters in cotton genotypes

change in leaf pigmentation that leads to symptoms development. Inline, our results showed a decrease in Chl content after 15 DAI with the progression of infestation in HS 6 and GCH 3 while increasing in the resistant genotype. A plausible rationale for the rising concentration in the resistant genotype could be the fact that young leaves may not have matured, but adult leaves did (Kamble et al. 2015; Kumar et al. 2023). The decrease in HS 6 and GCH 3 may be caused by the desiccation or inhibiting chlorophyll synthesis by the growing pathogen within the leaf tissues via upregulating the transcript genes involved in chlorophyll breakdown. Thus, the virus's interference with the plant's molecular machinery may have contributed to the decrease in Chl concentration in infected cotton plants (Liu et al. 2014; Philip 2010). Montasser et al. (2012); Anuradha et al. (2015) observed a significant decrease in photosynthetic pigments in infected plants over healthy ones. RWC is an indicator of tolerance or susceptibility to stress conditions. Increased permeability brought on by the viral infection caused cells to lose water (Sofy et al. 2017). RWC is intended to significantly contribute to plant's ability to resist disease. A decline in RWC from 0 to 45 DAI in susceptible genotypes (1.55–12.58%) is high compared to other genotypes. The decline in RWC might be a consequence of the reduced leaf area of infected plants or its utilization by the proliferating virus. In HD 432, the reduction in RWC might be due to the maturation or emergence of the disease that could not be spread (Montasser et al. 2012). These findings are consistent with a previously documented decline in RWC as reported by Mushtaq et al. (2014) in *Lycopersicon esculentum* and *Nicotiana benthamiana* plants infected with different whiteflies transmitted begomoviruses. Plants respond to the invasion of pathogens
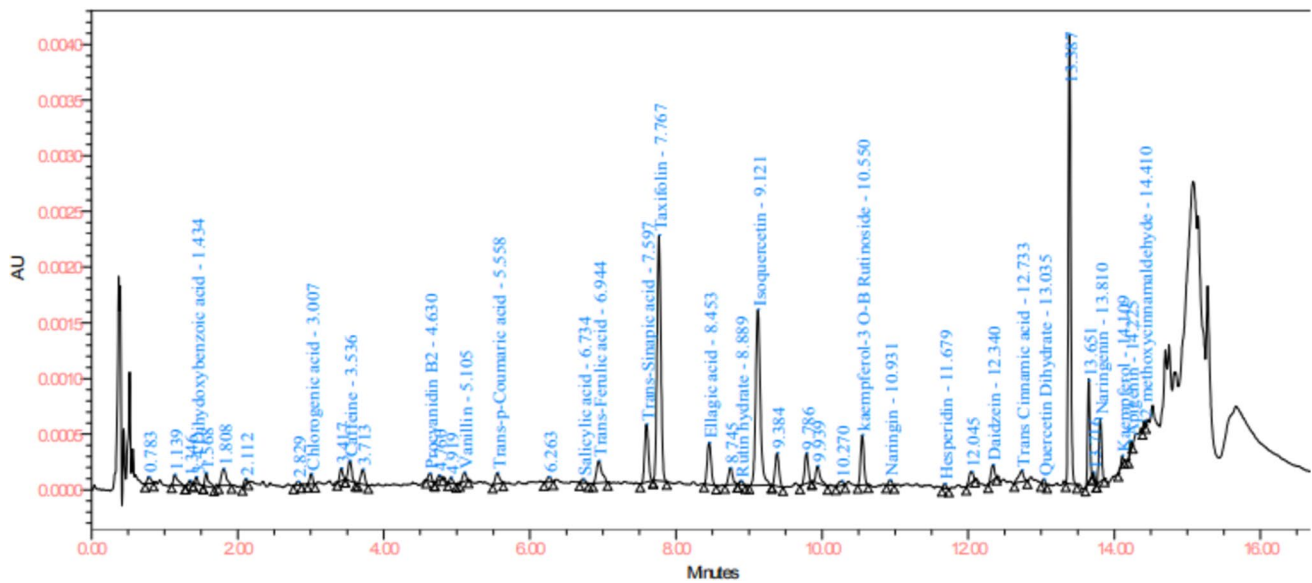
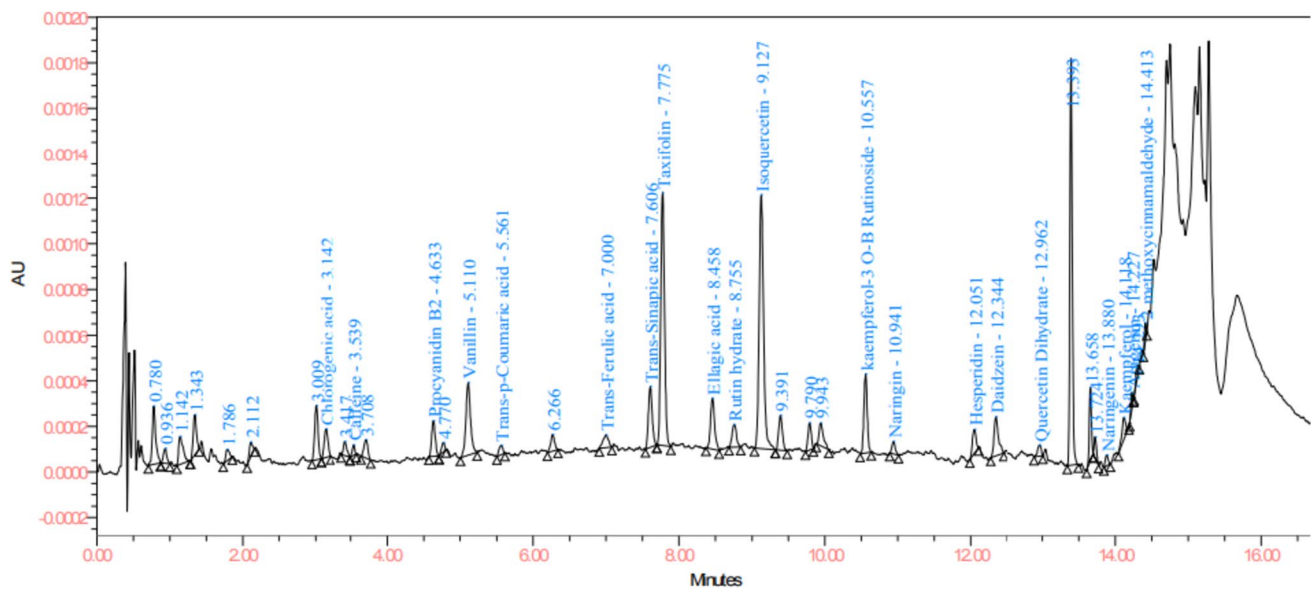**Fig. 4** Chromatogram (at 278 nm) of control GCH 3 leaf sample

Fig. 5 Chromatogram (at 278 nm) of infected GCH 3 leaf sample

through many biochemical changes. EL accompanies plant response to pathogen attack (Demidchik et al. 2014). In this case, the rise in EL became pronounced with the increase in the infestation period. The virus may stimulate the synthesis of ROS molecules, which can damage cell membranes and cause elevated levels of EL in the infected plant cells (Kumar et al. 2023). The observations pertaining to the current experiment were in congruence with the previous results observed by Sofy et al. (2017) in tomato plants, wherein EL rates in infected plants are significantly higher than those of healthy plants.

The stress-induced EL is usually accompanied by ROS accumulation due to the disruption of the equilibrium between the formation and elimination of ROS (Demidchik et al. 2014; Mittler 2002). Prolonged and excessive generation of ROS can cause membrane lipids peroxidation, leading to the formation of MDA (Rady and Osman 2012). In the present instance, a significantly notable enhancement in H_2O_2 level was recorded initially, and then it decreased in all genotypes. H_2O_2 can directly eliminate invasive pathogens or trigger the expression of defence-related genes (Kundu et al. 2013). A simultaneous increase in MDA content in all

Table 6 Amount of different phenols in control and infected sample of GCH 3

S.No	Compound Name	CLCuV Response			
		Control		Disease	
		Amount (ppm)	Standard Error	Amount (ppm)	Standard Error
1	3,4 Dihydoxybenzoic acid	0.483	0.007	0.475	0.000
2	Apigenin	0.505	0.002	0.497	0.001
3	Caffeine	0.528	0.002	0.412	0.000
4	Procyanidin B2	0.714	0.001	0.928	0.007
5	Vanillin	0.428	0.002	0.508	0.091
6	Chlorogenic acid	0.589	0.004	0.711	0.015
7	Salicylic acid	0.891	0.013	–	–
8	Trans-Ferulic acid	0.523	0.095	0.443	0.019
9	Isoquercetin	5.516	0.162	4.065	0.111
10	Taxifolin	2801	66.00	1.687	0.035
11	Ellagic acid	1.170	0.015	0.971	0.008
12	Rutin hydrate	0.482	0.009	0.721	0.031
13	Trans-Sinapic acid	1.280	0.025	0.886	0.015
14	kaempferol-3 O-B Rutinoside	0.869	0.379	1.088	0.029
15	Naringin	0.409	0.010	0.406	0.000
16	Hesperidin	0.404	0.000	0.508	0.003
17	Hesperidin	0.522	0.000	–	–
18	Daidzein	0.476	0.000	0.539	0.003
19	Trans Cinnamic acid	0.403	0.010	0.384	0.000
20	Quercetin Dihydrate	0.563	0.010	0.567	0.001
21	Naringenin	0.785	0.012	0.442	0.002
22	Kaempferol	0.566	0.028	0.587	0.003
23	Trans-p-Coumaric acid	0.436	0.004	0.399	0.003
24	2 methoxycinnamaldehyde	0.411	0.003	0.425	0.000

genotypes was also noticed. However, MDA concentrations were shown to be significantly higher in HS 6 at all DAI compared with GCH 3 and HD 432 which may have resulted from a greater build-up of O^{2-} in conjunction with a smaller rise in the activity of antioxidative enzymes in various cell compartments (Soni et al. 2022). Adi et al. (2012) also found that the intensity of the stain for H_2O_2 was more pronounced in tomato plants that are sensitive compared to ones that are resistant when infected with the Tomato yellow leaf curl virus. A higher accumulation of MDA and polyphenols was noted in plants that are infected with the virus as compared to the control plants (Kumar et al. 2018).

Activities of CAT, PAL, POX and PPO increased in all the genotypes though to a dis-similar extent. The maximum increase was reported in the highly tolerant genotype in the case of POX, PPO and PAL. These findings bolster the notion that observed POX activity is possibly accountable for maintaining the intracellular free radical balance, which has ultimately prevented the disease's severity from developing in the host plant. Additionally, lower levels of MDA have been found in resistant and tolerant genotypes, which may be the result of decreased lipid peroxidation of the membrane

induced by a decrease in intracellular free radicals spurred on by increased POX activity. Furthermore, POX is said to play a role in the polymerization of lignin and the intra- and intermolecular cross-linking of phenolic compounds in plant cell walls, which hardens the cell wall to thwart pathogen ingress (Chakraborty and Basak 2018). The rise in PPO activity seen in the inoculated leaves of both tolerant and resistant genotypes could potentially be attributed to the expression of resistance-coding genes. It is widely known that the presence and production of phenolic compounds in plants in response to infection are related to resistance. Studies have shown that phenolic build-up is caused by an increase in PAL activity, which safeguards against infections (Jayaraj et al. 2010). In contrast, the resistant genotype showed the greatest increase in CAT activity. The smallest rise in activities of all enzymes was associated with a highly susceptible genotype. The present findings agree with Anuradha et al. (2015); Tanuja et al. (2019). They reported significantly higher PPO, POX and CAT activities in banana bunchy top virus-infected leaves over the healthy ones. A similar trend was also observed in mungbean genotypes in which phenol, chlorophyll contents and activities of PPO,

PAL were found to be more prevalent in resistant genotypes than in moderately resistant, moderately susceptible, susceptible and extremely susceptible genotypes (Mantesh et al. 2020). Total phenolics and PPO both contribute in providing resistance against viral pathogens (Li and Steffens 2002; Ngadze et al. 2012). An increase in the host's phenolic compounds after infection by pathogens augments the host cell walls mechanical strength by producing lignin and suberin, which are engaged in building barriers that can physically prevent the expansion of diseases (Ngadze et al. 2012; Singh et al. 2014). In this context, a rise in the amount of gossypol and total phenolic content was recorded due to CLCuV infestation in leaves of all genotypes; however, the percent increase was higher in GCH 3 compared to HS 6 and HD 432. The present study lies in line with the findings of Anuradha et al. (2015). They found that the amount of total phenolic content was significantly higher in virus-infected leaves of two cultivars of banana as compared to healthy ones. A higher level of phenol may be associated with CLCuV resistance in tolerant plants, since phenol accumulation at the infection site was identified as a sign of the beginning of the plant defense system, which may function as a physical barrier to stop pathogen invasion (Chakraborty and Basak 2018). Furthermore, it has been proposed that phenolic compounds limit viral infection in plants by preventing viral replication by simultaneously damaging proteins, nucleic acids and suppressing viral enzyme activities (Kumar and Pandey 2013).

The elevated concentrations of rutin hydrate and chlorogenic acid in the diseased sample were noted in the present study. Because of its antioxidant properties, chlorogenic acid may help boost resistance against pathogens. Similarly, an LC–MS study of infected tomato plants revealed the accumulation of two phenolic metabolites, rutin and chlorogenic acid, involved in pathogen defense response (Sade et al. 2015). Similar to our findings, Yadav et al. (2022) found that the predominant phenolics in highly tolerant genotype that confer resistance against the pathogen are salicylic acid, ellagic acid, ferulic acid, trans-cinnamic acid and sinapic acid. Plant defense against pathogens has been demonstrated to be actively aided by the buildup of phenols, which are brought about by the enhanced expression of certain genes in the infected plant (Sade et al. 2015). The current study's findings demonstrated that as the infection progressed, the gossypol content increased in all genotypes. Sonika et al. (2017) achieved comparable outcomes This result is consistent with the notion that gossypol may enhance the generation of ROS in the leaves, which is followed by an increase in CAT activity and confers resistance to plants against pathogens (Zhang et al. 2017).

Plant pathogenic interactions have revealed the role of protein elements in the resistance to plant diseases (Tornero et al. 2002). With the progression of infection, the total soluble protein content decreased in all the genotypes. The highly susceptible genotype experienced a greater decline, followed by the highly tolerant genotype, which preceded the resistant genotype. The reduction in total soluble protein could probably be due to their utilization by the proliferating virus for its own multiplication (Montasser et al. 2012) alternatively the disease could have resulted in the denaturation of polypeptide chains and bound amino acids (Chatterjee and Ghosh 2008). Mandhania et al. (2018) also reported a higher amount of protein in the healthy plants than the diseased ones. The higher level of these biochemical constituents might aid the plant defence against viral disease (Mandhania et al. 2018; Rai et al. 2010; Siddique et al. 2014). Phenylpropanoids along with phenylpropanoid-acetate pathways are used by plants to produce secondary metabolites such as phenolics and flavonoids, which considerably reduce the negative effects of biotic stressors (Mandhania et al. 2019). The infected area experiences a significant metabolic demand because of the activation of defence responses since energy is needed to power biosynthesis and carbon skeletons are needed to create new molecules (Bolton 2009). Sugars serve as a starting point for the production of phenolics and other secondary metabolites, which are crucial components of plants' defence mechanisms against the invasive pathogen. With the increased age of the crop and the progression of infestation, the total soluble sugar content increased up to 30 DAI, and afterwards, it decreased. The reduction in total soluble sugar in virus-infected plants may have either been due to impairment of the photosynthesis apparatus (which causes decreases in photosynthetic activity) and/or due to increased respiration (Soni et al. 2022). Total soluble sugar content differed significantly among all genotypes at all stages of infestation (15, 30 and 45 DAI) except at 0 DAI, where it differed non-significantly between GCH 3 and HD 432. The resistant genotype differed non-significantly at 0 and 45 DAI. In this line, the virus infection-induced reduction in total sugars of mungbean plants was observed by Sinha and Srivastava (2010) under the effect of the mungbean yellow mosaic virus. For any breeding effort to get off the ground, knowledge of the relationships between distinct traits is crucial. As stated by Ali et al. (2009), it offers the chance to choose desired genotypes with desirable features. In this study, Chl content exhibited a highly significant and positive correlation with protein, anti-oxidative and defence enzymes such as POX, PPO, CAT, and PAL, with secondary metabolites like phenol and gossypol. While MDA and H_2O_2 have significant and negative correlations with PAL, phenol, protein and Chl content. Similar to our results, Sofy et al. (2021) noted a positive significant correlation between the ROS scavenging enzymes and Chl content. In contrast, there was a negative significant correlation between the ROS scavenging enzymes and H_2O_2, MDA. Using the metabolites and other biochemical parameters under investigation, this

study can assist in the screening of the tolerant genotypes. Finding the metabolic markers that contribute to virus resistance should be the aim of breeding programs for pathogen defense. Metabolic markers may be more beneficial than molecular markers since they are more accurate performance indicators for plants. Consequently, it is critical to look into the host plant's natural metabolites to control plant infestation efficiently and sustainably.

Conclusion

The symptoms were less marked in resistant and tolerant genotypes than in susceptible ones. This reaction is because genotypes could cope with the virus by making desirable changes in the biochemical constituents to minimize the damage. The tolerance observed in the current study could result from higher RWC, total chlorophyll content, and decrease in H_2O_2 level under stressful conditions limits the severity of damage to cellular membranes. Furthermore, increased enzyme activities related to defence and increased content of some protective biochemical constituents (phenolic compounds) may play a part in the resistance associated with CLCuV.

Declarations

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