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**Sonika**
Department of Genetics and Plant Breeding, CCS HAU Hisar, Haryana, India

**RS Sangwan**
Department of Genetics and Plant Breeding, CCS HAU Hisar, Haryana, India

**Shiwani Mandhania**
Department of Genetics and Plant Breeding, CCS HAU Hisar, Haryana, India

**Correspondence**
**Sonika**
Department of Genetics and Plant Breeding, CCS HAU Hisar, Haryana, India

# Role of biochemical traits for resistance to cotton leaf curl disease (CLCuD) in *Gossypium hirsutum* L.

**Sonika, RS Sangwan and Shiwani Mandhania**

**Abstract**
Cotton leaf curl disease (CLCuD) is a major biotic constraint that can significantly reduce the crop productivity. Development of resistant variety is most effective method to tackle with this and the successful exploitation of source of resistance requires information on the biochemical traits imparting resistance to CLCuD. We examined the role in imparting resistance to CLCuD of different biochemical traits including total sugar, phenols, gossypol, tannin, crude protein contents and enzymatic activities of peroxidase and polyphenoloxidases in resistant and susceptible cotton genotypes and their $F_1$s at two different growth stages by using their standardized methods. Four genetically diverse cotton genotypes *i.e.* two resistant (GCH 3 and H 1353) and two highly susceptible to CLCuD (HS 6 and RST 9) were identified from the germplasm during 2013; four $F_1$ hybrids were developed by crossing these genotypes and raised during *kharif* 2014 and finally evaluated under field conditions in 2015. Two factor analysis depicted that the amount of total phenol, gossypol, tannin, protein and activities of peroxidase & polyphenoloxidase was significantly higher in leaves of resistant genotypes than susceptible genotypes at different growth stages. Also, the significantly higher concentrations of these constituents in resistant genotypes after infection suggested that there is a correlation between increased levels of these constituents and plant resistance. So, these constituents might play an active role in imparting resistance to CLCuD and provide defense mechanism to plants which suggested that these traits could serve as potential biochemical markers and further may be used for early screening of germplasm lines.

**Keywords:** Biochemical constituents, Days after sowing, Percent disease incidence, Resistant, Susceptible genotypes

## Introduction
Cotton is one of the most ancient and important commercial crop next only to food grains. Cotton, although under pressure from synthetic fibers, has made resurgence worldwide and remains as the most improved crop species producing lint plus oil and meal from seed [1]. India was the first country in the world to domesticate cotton for production of cotton fabrics, when members of the Indus Valley Civilization began to grow the fiber in 1750 BC for manufacturing textiles [2]. Cotton is the backbone of our sprawling textile industry contributing about 65% of the raw material for the same. It accounts for 45% of the world fiber and supplies 10% world edible oil [3]. The leading cotton producing countries are China, USA, India and Pakistan [4].

Biotic factors such as viral diseases; alone or in combination with other factors are quite destructive and one of the major factors limiting crop production and commonly leads to substantial and significant loss in yield. Cotton leaf curl virus disease is one of the most common and destructive diseases of the upland cotton in North India mainly in Haryana, Punjab and Rajasthan. Its causative agent has been characterized and disease was found to be caused by a complex of monopartite begomoviruses and a small symptom modulating, single stranded satellite DNA β component transmitted by the whitefly (*Bemisia tabaci*) [5]. The climate of India is favourable for the growth and spread of its vector *i.e.* whitefly that transmits *Gemini virus* which pose serious threat to cotton production. CLCuD-infected plants are usually stunted and younger leaves of infected plants can show either upward or downward curling, swelling and darkening of veins, which frequently develop into cup-shaped leaf-like out-growths called 'enations'.

Use of chemicals to control this disease is not economic and also not so effective. Moreover, it may be hazardous to living beings and environment. Therefore, development and use of resistant varieties is the most effective, long term, economical and safe method to fight against this disease and to enhance and stabilize the productivity of cotton. The successful exploitation of the source of resistance requires information on the biochemical parameters imparting resistance to CLCuD for development of a resistant variety.

Disease resistance in plants is associated with activation of a wide array of defense responses that slow down or halt infection at certain stages of the host-pathogen interaction. Plants have evolved various preexisting physical and chemical barriers, as well as inducible defense responses that interfere with pathogen establishment as reported by Zhao *et al.* [6] and Vanitha *et al.* [7]. The mechanisms of host plant resistance in response to insect infestation and diseases consist of a series of biochemical events, including increased production of phenolics, mediated by the activity of enzymes such as peroxidase and polyphenoloxidase. The primary metabolites include proteins, these primary metabolites also function as precursors of secondary substances, which are major elements of resistance in plants. Age correlated biochemical profiles of host tissues also significantly influence infestation patterns.

Metabolites play a major role in the adaptation of plants to the changing environment and in overcoming stress constraints. Plants have also been known to produce a large number of secondary metabolites such as alkaloids, terpenoids, polyphenols and combined structures of these groups. Micheal [8] reported that secondary metabolites represent adaptive characters that have been subjected to natural selection during evolution and Govindappa *et al.* [9] also reported that secondary metabolites produced in nature belong to heterogeneous group and served as competitive weapons against microorganism. Secondary metabolites can slow down the infection of pathogens and clean the immediate environment of competing microorganism's infections and play a role in defense against herbivores, microbes and viruses. They are important for the plant survival and reproductive fitness and also protect plants from physical stresses like ultraviolet light and heat [10].

Keeping in mind the increasing threat of cotton leaf curl disease in India and its impact on cotton production, it is imperative to investigate the role of various biochemical traits in imparting resistance to cotton leaf curl disease in upland cotton. The objective of this study was to investigate the role of biochemical traits such as total sugar, total phenol, gossypol, tannin, crude protein content, peroxidase (PO) and polyphenol oxidase (PPO) enzymes activity in imparting resistance to CLCuD infection.

## Materials and Methods

### Plant materials

The plant materials comprised four genetically diverse cotton genotypes belonging to *Gossypium hirsutum* L.; two resistant to CLCuD *i.e.* GCH 3 & H 1353 and two highly susceptible to CLCuD *i.e.* HS 6 & RST 9 (Table 1).

**Table 1:** Parental lines, their source and characteristics

| S. No. | Parent | Source | Characteristics |
|---|---|---|---|
| 1 | GCH 3 | CCS HAU, Hisar | High yielding and highly resistant to CLCuD |
| 2 | H 1353 | CCS HAU, Hisar | High yielding, round and smooth boll shape, resistant to CLCuD |
| 3 | RST 9 | ZARS, RAU | Susceptible to CLCuD |
| 4 | HS 6 | CCS HAU, Hisar | High yielding, long maturity duration, round boll shape, highly susceptible to CLCuD |

### Development of breeding materials

The present investigation was conducted at Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar during *Kharif* 2015-16. During *Kharif* 2013, the parents were identified from the germplasm and breeding material to fulfil the objectives. Among these parents GCH 3 and H 1353 were identified as resistant whereas the parents RST 9 and HS 6 showed susceptible reaction to cotton leaf curl disease under field conditions and four $F_1$ crosses between these parents, namely GCH 3, H 1353, RST 9 and HS 6 *i.e*. GCH 3 x HS 6, GCH 3 x RST 9, H 1353 x HS 6 and H 1353 x RST 9 were made. The $F_1$ hybrids and parents were raised during *kharif* 2014. Each $F_1$ was selfed to obtain $F_2$ generation and simultaneously backcrossed to both of its parents to produce backcross generations ($BC_1$ and $BC_2$). Fresh crosses were also made to obtain the $F_1$ seed and all the parents were selfed to get their seeds for the next year. The experimental material comprised of four crosses was grown in a randomized block design (RBD) with three replications during *kharif*, 2015 at Cotton Research Area, CCS Haryana Agricultural University, Hisar. There was a single row of non segregating generations *i.e.* $P_1$, $P_2$ and $F_1$, 8 rows of $F_2$ and 4 rows of each back cross 1 and back cross 2 generations. The length of each row was 6 m with a spacing of 67.5 x 30 cm. In order to build up heavy inoculum pressure one row of highly susceptible line (HS 6) was planted at the periphery of the experimental area. Normal cultural practices were followed except insecticidal spray for control of white fly (*Bemisia tabaci* Genn.) population in the field. Reaction of cotton leaf curl virus disease was recorded on all the plants in all replications and the non segregating generations *i.e.* $P_1$, $P_2$ and $F_1$s of these four crosses were used as experimental material to collect leaf samples for biochemical study. The healthy as well as diseased leaves from two plants (taken at random) from three replications of each of the non segregating generations ($P_1$, $P_2$ and $F_1$) of all the four crosses were taken at two stages of plant growth *i.e.* vegetative stage (60 Days after sowing) and reproductive stage (90 Days after sowing).

### Sample collection

To study the biochemical changes occured due to CLCuD infection in cotton, the fully expanded and fresh leaf samples of uniform age and size were collected from plants of both resistant and susceptible parents to CLCuD *i.e.* $P_1$, $P_2$ and their $F_1$s of four crosses at 60 DAS and 90 DAS. The leaf samples were sun dried for 2-3 days and thereafter oven dried at $60^0$ C till complete loss of moisture. The dried leaves were crushed and finally powdered. The powdered material was used for further study of different biochemical constituents.

Total sugars, total phenols, gossypol, tannin, crude proteins, peroxidase (PO) and polyphenoloxidase (PPO) were analyzed using their standardized methods.

### Total sugar contents

Total sugar was estimated by method of Dubois *et al.* [11]. To 0.5 ml of aliquot, 2 ml of 2 per cent phenol and 5 ml of conc. $H_2SO_4$ was added. The solution was mixed well by vortexing and allowed to cool at room temperature. The color developed was read at 475 nm using UV- Spectrophotometer against reagent blank. Concentration of sugar in samples was determined by standard curve prepared using dextrose (100 mg /ml).

### Total phenol contents

Estimation of phenol was done by using Bray and Thorpe [12] method. From the prepared extract, 0.5 ml aliquot was

pipetted out in another test tube and 1 ml of refluxed Folin-Ciocalteau reagent was added. After three minutes, 2 ml of 20 per cent $Na_2Co_3$ was added to each test tube and shaken thoroughly for colour development. The blue colour developed was read at 650 nm using UV-Spectrophotometer against blank reagent. Concentration of total phenol in each sample was calculated by using catechol (100 mg / ml) as standard.

**Gossypol contents**

Gossypol was estimated by Bell [13] method. To 1 ml of aliquots, 0.5 ml of phlorglucinol reagent was added followed by 1 ml of conc. HCl to each tube. The samples were then incubated for 30 minutes at room temperature and final volume was made to 10 ml with 80% ethanol. Absorbance was read at 550 nm against a reagent blank. Concentration of gossypol was determined by using gossypol acetate (100 µg / ml) as standard.

**Tannin contents**

The estimation of total tannins was done using standard method given by Porter *et al.* [14]. In 1.0 ml of extracted sample, 9.0 ml of acetone was added. Reading was taken at 550 nm using spectrophotometer and tannin concentration was determined by using catechin as standard.

**Determination of Crude Protein**

Analysis of the samples for crude proteins was done by using standard method given by Kjheldahl (A O A C) [15].

*Enzyme Assays*

**Determination of enzymatic activities**

For preparation of the enzyme extract, leaf tissue (500 mg) was washed in ice cold water and hand homogenized in presence of 4 ml 0.1 M Tris buffer (pH 7.6) and 1 ml 0.1% EDTA for peroxidase (PO) and in presence of 4 ml 0.1 M Sodium phosphate buffer (pH 7.0) and 1 ml 0.1% EDTA for polyphenoloxidase (PPO) in a previously chilled pestle and morter placed on crushed ice, by using acid-washed sand as an abrasive. The homogenate was centrifuged at 12,000 rpm for 15 minutes in a refrigerated centrifuge. The supernatant thus obtained was referred as crude extract and stored in deep freezer (-20$^o$C) till the measurement of enzyme activity, which was done on the same day.

**Peroxidase (E. C. 1.11.1.7)**

Peroxidase was assayed according to the modified method of Shannon *et al.* [16]. The reaction mixture contained 3.62 ml of 0.1 M Sodium phosphate buffer (pH 7.0), 200 µl of 0.1 percent o-dianisidine, 80 µl enzyme extract and 100 µl of 0.2 percent $H_2O_2$. The enzymatic reaction was initiated by the addition of $H_2O_2$ and change in absorbance was followed at 430 nm in spectrophotometer. The enzyme activity has been expressed as change in 0.01 absorbance min$^{-1}$.

**Polyphenoloxidase (E. C. 1.10.3.1)**

Polyphenol oxidase activity was assayed following modified method of Taneja and Sachar [17]. The reaction mixture in a final volume of 4 ml contained 1.6 ml of 0.1 M sodium phosphate buffer ( pH 7.0), 2.2 ml of 1 percent catechol solution as substrate and 0.2 ml of enzyme extract. For each sample, a separate blank was run simultaneously and change in absorbance was followed at 420 nm in spectrophotometer. The enzyme activity has been expressed as change in 0.01 absorbance min$^{-1}$.

**Statistical analysis**

The data for all biochemical constituents *i.e.* total sugar, total phenol, gossypol, tannin, crude protein and enzymatic activities of PO and PPO under both healthy and diseased conditions were analyzed following the two factor analysis (CRD). Two observations/ readings for each sample from three replications (24 samples per replication was handled) was taken for each biochemical trait at 60 DAS & 90 DAS and their mean was calculated. The data collected from all experiments was analyzed separately for each parameter and subjected to two-way analysis of variance (ANOVA) using OP-STAT. The means were compared for significance and the values presented are mean of three replications ± standard error (SE). Correlation between different biochemical traits and CLCuD (incidence) grading (disease scoring 0-6) was calculated by subjecting the data obtained from ANOVA to pearson correlation matrix analysis by using OP-STAT separately for 60 DAS and 90 DAS.

**Results and Discussion**

**Disease response**

During the year 2015-16 no variety / strain was observed completely immune to this disease. Even in highly resistant strains few plants showed susceptible reaction. Disease symptoms were mild in the resistant genotypes but were severe in highly susceptible genotypes. Observation on cotton leaf curl virus disease was recorded under field condition in each replication on all the plants of each of the non segregating generations ($P_1$, $P_2$ and $F_1$), backcross generations and the $F_2$ generation. Disease was scored on 0-6 grade proposed by Akhtar *et al.* [18] depending upon the response to the cotton leaf curl virus disease (Table 2).

$$\text{Per cent disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \text{ x } 100$$

**Table 2:** PDI (%) based on CLCuD grading (0-6) of the parents and the different generations of all the four crosses

| parent /generations | PDI % (CR-I) | PDI % (CR-II) | PDI % (CR-III) | PDI % (CR-IV) |
|---|---|---|---|---|
| $P_1$ (R) | 2.23(HR) | 3.22(HR) | 7.95(HR) | 9.49(HR) |
| $P_2$ (S) | 68.62(HS) | 51.16(HS) | 73.71(HS) | 67.37(HS) |
| $F_1$ | 7.89(HR) | 5.26(HR) | 10.13(R) | 13.12(R) |
| $F_2$ (9:7) | 34.15(MS) | 33.53(MS) | 40.88(S) | 34.16(MS) |
| $BC_1$ | 9.52(HR) | 8.02(HR) | 12.74(R) | 11.18(R) |
| $BC_2$ (3:1) | 37.06(MS) | 37.90(MS) | 37.26(MS) | 37.90(MS) |

PDI, percent disease incidence; R, resistant; HR, highly resistant; HS, Highly susceptible; MS, moderately susceptible; S, susceptible

Disease symptoms were observed at 60 and 90 days after sowing in 2015 and susceptible genotypes showed severe symptoms like leaf curling, swelling and darkening of veins and leaf-like out-growths called ‘enations’ at 90 DAS. The

severity of disease in the leaves of plants of resistant genotypes *i.e.* GCH 3 and H 1353 infected with CLCuD was compared with susceptible genotypes *i.e.* HS 6 and RST 9 and it was found lower. The two distinct classes of resistance and susceptibility was thus established confirming findings of Akhtar *et al.* [18] who observed similar trends and ranked them as resistant and susceptible to CLCuD.

The $F_1$s of crosses *viz*., GCH 3 x HS 6, GCH 3 x RST 9, H 1353 x HS 6 and H 1353 x RST 9 had resistance to CLCuD which indicated that resistance is a dominant trait.

**Biochemical Constituents**

The two- way analysis of variance (ANOVA) indicating source of variation, degrees of freedom, sum of square, mean square and F-calculated is presented in Table 3. There was significant variation among parents (genotypes), DAS and DAS x Genotypes interaction for the biochemical traits.

**Table 3:** Analysis of variance for biochemical traits for resistance to CLCuD in cotton. SOV, source of variation; DAS, days after sowing; df, degrees of freedom; MS, mean square; F, F –calculated; PPO, polyphenoloxidase

| SOV | df | Sugar | | Phenol | | Gossypol | | Tannin | | Peroxidase | | PPO | | Protein | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MS | F | MS | F | MS | F | MS | F | MS | F | MS | F | MS | F |
| DAS | 1 | 3.30 | 53.87 | 2.65 | 274.36 | 0.20 | 92.88 | 0.582 | 89.248 | 22.876 | 12.200 | 0.263 | 46.922 | 76.738 | 41.105 |
| Genotypes | 11 | 0.55 | 9.08 | 0.05 | 5.57 | 0.018 | 7.86 | 0.010 | 1.544 | 4.452 | 2.374 | 0.044 | 7.881 | 7.014 | 3.757 |
| DAS x Genotypes | 11 | 0.15 | 2.59 | 0.02 | 2.71 | 0.006 | 2.46 | 0.004 | 0.679 | 0.983 | 0.524 | 0.014 | 2.483 | 1.645 | 0.881 |
| Error | 72 | 0.06 | | 0.01 | | 0.002 | | 0.007 | | 1.875 | | 0.006 | | 1.867 | |

The mean values of total sugar, total phenols, gossypol, tannin, protein content (%) and enzymatic activities of peroxidase and polyphenoloxidase (units) of all the four crosses are presented in Table 4. The sugar content at the different stages of growth *i.e.* 60 DAS and 90 DAS was significantly higher in susceptible parents *i.e*. HS 6 and RST 9 as compared to the resistant parents *i.e.* GCH 3 and H 1353 and it decreased gradually with the advancement of crop age *i.e.* from 60 to 90 DAS in both susceptible parents, both resistant parents and their $F_1$s. The susceptible parent HS 6 in cross III (2.71) recorded significantly higher sugar content and the resistant parent GCH 3 in cross II (1.70) recorded significantly lower content of sugar at 60 DAS. Further at 90 DAS the susceptible parent HS 6 in cross I (2.51) recorded significantly higher content of sugar and the resistant parent H 1353 in cross IV recorded (1.50) significantly lower sugar content at 90 DAS. In case of total phenols, the resistant parent GCH 3 in cross II (0.62) recorded significantly higher and maximum concentration of phenol and the susceptible parent HS 6 in cross I (0.42) recorded minimum content of phenol at 60 DAS. For the gossypol, the resistant parent GCH 3 in cross I (0.36) recorded significantly higher concentration and maximum content and the susceptible parent RST 9 in cross IV (0.18) recorded significantly lower amount of gossypol at 60 DAS. For tannin, the resistant parent H 1353 in cross III (0.33) recorded significantly higher concentration than the susceptible parents HS6 in cross I & III and RST 9 in cross II & IV (0.20 and 021) which recorded significantly lower content of tannin at 60 DAS. For the enzyme peroxidase, the resistant parent H 1353 I cross III (11.38) recorded significantly higher activity of the enzyme and the susceptible parent RST 9 in cross IV (8.57) recorded significantly lower activity of the enzyme at 60 DAS. The resistant parent GCH 3 in cross I (1.42) recorded significantly higher activity of polypenoloxidase and the susceptible parent RST 9 in cross IV (1.05) recorded significantly lower activity of the enzyme at 60 DAS. $F_1$s for these constituents tended towards their respective resistant parents in all the four crosses. Further significant increase was noticed in phenol, gossypol, tannin content and activity of the enzyme peroxidase and similar trend was also observed as the crop advanced at 90 DAS that the resistant parents had (recorded) significantly higher content and activity of the enzymes as compared to the susceptible parents. In crosses I, II, III and IV there was a significant difference between these constituents at 60 DAS and 90 DAS (Table 4). For total phenols, the resistant parent H 1353 in cross IV (1.04) recorded significantly higher content of phenol and the susceptible parent HS 6 in cross I & III (0.71) recorded significantly lower content at 90 DAS. For gossypol, the resistant parent H 1353 in cross III (0.50) recorded significantly higher content and the susceptible parent RST 9 in cross IV (0.23) significantly lower gossypol content at 90 DAS. For tannin, the resistant parent H 1353 in cross III (0.57) recorded significantly higher content and the susceptible parent HS 6 in cross I & III (0.28) recorded significantly lower content of tannin at 90 DAS. For the enzyme peroxidase, the resistant parent GCH 3 in cross II (13.25) recorded significantly higher enzyme activity and susceptible parent RST 9 in cross IV (9.79) recorded significantly lower activity of the enzyme at 90 DAS. $F_1$s tended towards their respective resistant parents for these constituents at 90 DAS. In case of protein both parents (resistant and susceptible) had shown the significant differences in crude protein content at 60 DAS in all the four crosses and gradual reduction was observed in crude protein content with the age of crop *i.e.* from 60 DAS to 90 DAS that the higher crude protein content was observed at 60 DAS and recorded in GCH3 (14.91) in cross II where the susceptible parent HS 6 (11.55) recorded significantly lower content of protein, further reduction in protein content was noticed at 90 DAS which was recorded in RST 9 (10.37) in cross IV which was significantly lower than H 1353 (12.39), but still the similar trend was observed at 90 DAS *i.e.* the protein content in resistant parents was significantly higher than susceptible parents and similar type of pattern was observed in all the four crosses and $F_1$ tended towards their respective resistant parents in all the four crosses. In general, the activity of polyphenoloxidase (PPO) enzyme decreased as the crop advanced yet the resistant parents (GCH 3 and H 1353) had significantly higher activity of the enzyme than the susceptible parents (HS 6 and RST 9) *i.e.* the resistant parent H 1353 in cross III (1.23) recorded significantly higher enzyme activity and susceptible parent RST 9 in cross II (1.00) recorded significantly lower activity of enzyme at 90 DAS. $F_1$ tended towards their respective resistant parents. The similar trend was observed in all of the four crosses at 90 DAS.

**Correlation matrix**

The correlation matrix among different biochemical parameters revealed that Cotton Leaf Curl Disease grading (disease scoring 0-6) (Table 2) was significantly and

positively correlated with sugar content while CLCuD grading depicted significant negative correlation with other biochemical parameters *viz.* phenol, gossypol, tannin, protein and enzymes *i.e.* peroxidase (PO) and polyphenoloxidase (PPO) at 60 DAS and the same trend was observed in all the four crosses at 60 DAS (Table 5). At 90 DAS CLCuD grading (disease scoring 0-6), indicated significant negative correlation with gossypol, tannin and peroxidase (PO) whereas the positive co-relation with sugar and negative correlation with phenol, protein and polyphenoloxidase (PPO) was present but this correlation was non-significant. The same trend was observed in all the four crosses at 90 DAS (Table 6).

Different biochemical parameters *i.e.* sugar content, phenol content, gossypol content, tannin content, peroxidase (PO) activity and polyphenoloxidase (PPO) enzyme activity among parental lines (GCH 3, HS 6, H 1353 and RST 9) in four crosses are given in (Table 7). Resistant parents/ genotypes depicted significant difference among these constituents from susceptible parents/ genotypes in all the four crosses (R x S).

In recent years, it is becoming increasingly evident that several natural and induced defense mechanisms operate in host plants against different diseases. One such defense mechanism is the presence of certain compounds inhibitory to the pathogen. Sometimes, the host plant is induced to synthesize these compounds on infection. Analysis of biochemical constituents and enzymatic activities in selected resistant and susceptible parents to CLCuD and their $F_1$s was carried out at two different growth stages to understand their role in resistance/ susceptibility to CLCuD which will enable plant breeders to develop resistant cotton genotypes having desired resistance against cotton leaf curl disease.

Generally, high levels of total sugars, reducing sugars and non-reducing sugars in the host plants are stated to be responsible for susceptibility. In the present investigation, sugar content was significantly lower in resistant parents (GCH 3 and H 1353) as compared to that of susceptible parents (HS 6 and RST 9). It was opined by Hedin and McCarty [19] that in cotton a high level of gossypol and low sugar contents were reported to have some role in insect, pest and disease resistance which is in support of our findings.

Among all the biochemical components of different hosts, phenols stand out as most important component in imparting resistance to several plant diseases. High concentration causes an instant lethal action by a general tanning effect while, low concentration causes gradual effect on the cellular constituent of the parasite. Phenolic compounds may contribute to enhance the mechanical strength of host cell walls by the synthesis of lignin and suberin that are involved in the formation of physical barriers that can block the spread of pathogens as reported by Ngadze *et al.* [20] and Singh *et al.* [21]. In the present study, significantly lower level of phenol content was observed in susceptible parents at the two stages *i.e.* HS 6 (67.84%) and RST 9 (42.24%) (% here indicates the percent increase or decrease of susceptible parents over the respective resistant parents) as compared to that of resistant parent (GCH 3) in cross I & II and in crosses III & IV, susceptible parent HS 6 had 58.49% and RST 9 had 59.19 per cent lower phenol content than the resistant parent H 1353. These findings infer that rapid accumulation of phenolic compounds occur in incompatible (resistant) host pathogen interaction than the compatible (susceptible). This high level of phenolic content in resistant plants might have been correlated with increased resistance to CLCuD as the accumulation of total phenols is usually found to be higher in resistant genotypes compared to susceptible ones reported by Meena *et al.* [22] and Singh *et al.* [23] which also confirmed our findings. This observation was also in agreement with the earlier findings in case of cotton with Grey mildew reported by Chakrabarthy *et al.* [24] and bacterial blight by Govindappa *et al.* [9] and similar results were also obtained by Rashmi and Vamadevaiah [25] in cotton which also supported the findings of present study. These results are in line with the results reported on other plant-pathogenic fungal bacterial and viral interactions, which showed that certain common phenols and phenolic substances are toxic to the pathogens, which have long been considered as important defense related compounds whose levels are naturally high in the resistant varieties of many crops [26] and accumulates in plants after infection, especially in resistant varieties.

Cotton (*Gossypium hirsutum* L.) produces a number of toxic terpenoid aldehyde (TA) compounds contained in epidermal glands that protect the plant from pests and diseases. One of these compounds is gossypol. In the present study, susceptible parent HS 6 contained lower gossypol content (37.73 per cent, 35.31 per cent) in crosses I and III, RST 9 contained lower content of gossypol (30.00 per cent, 28.64 per cent) in crosses II and IV. Also, the gossypol content in both susceptible and resistant plants, increased significantly from 60 to 90 DAS. The similar results obtained in cotton by Rashmi and Vamadevaiah [25] also support the findings of our investigation. Niles [27], reported that the nature of biochemical resistance against the insect pests ascribed to "high gossypol", increasing gland density in cotton plant appeared to result in increasing concentration of the toxic compounds.

Tannins are astringent, bitter-tasting plant polyphenols that bind and precipitate proteins. Tannins are considered to be the most important secondary plant compound involved in plant defense against insects and disease. Adamczyk *et al.* [28] also reported that tannins along with lignin are considered one of the most important groups of secondary metabolites in the defense of plants, mainly due to their biochemical and molecular properties. In the present investigation, tannin content was significantly higher in resistant plants as compared to susceptible ones. In susceptible parents, concentration of tannin was 40.68, 52.75, 41.79 and 50.74 percent lower than their respective resistant parents in cross I, II, III and IV, respectively. The tannin content under both the situations (healthy and diseased) increased significantly from 60 to 90 DAS. These findings are confirmed by the reports of Beniwal *et al.* [29], Acharya and Singh [30] and Rashmi and Vamadevaiah [25]. Also the phenomenon of increase of condensed tannin content has been reported by many workers attributing various reasons such as it was reported by Singh and Agarwal [31] that the incidence of *A. biguttula biguttula* was negatively correlated with the amount of tannins in the leaves of resistant cotton genotypes; Sharma *et al.* [32] opined that the expression of resistance to pod borer *Helicoverpa armigera* in wild relatives of pigeon pea was also associated with high amounts of tannins; Rao and Panwar [33] studied the influence of tannin content production in groundnut against *S. litura* and *H. armigera* and significant induction in concentration of tannin content soon after mechanical damage in cotton plants was opined by Kranthi and Kranthi [34].

The protein biosynthesis of the host is widely assumed to be significant feature of pathogenesis, particularly during incompatible reaction. Quantitatively the total protein synthesis is much enhanced in the tissues around the infected tissues. Involvement of protein components in plant disease

resistance has been documented in many plant patho-genic interactions by Tornero *et al.* [35]. In the present findings, mean crude protein content was lower in susceptible parents (9.97 per cent, 17.12 per cent, 3.62 per cent and 14.19 per cent) than resistant parents in all of the four crosses. The crude protein content under both the situations (healthy and diseased) decreased significantly from 60 to 90 DAS. The rate of decrease in the protein content in response to the CLCuD infection was more in infected plants as compared to healthy ones. These results are in agreement with the earlier findings in cotton [29] and [30] while contrasting findings was reported [9].

Yoshida *et al.* [36] and Maksimov *et al.* [37] reported that peroxidase is involved in lignin biosynthesis *via* polymerization of polypropane compounds by an oxidative $H_2O_2$ dependent system, suberification and resistance against pathogens in plants. Reports on the enhancement of its activity following infection with potential pathogen are quite common [38]. In the present investigation, peroxidase enzyme activity was 2.63, 9.53, 4.44 and 14.23 units in susceptible parents (HS 6 and RST 9) which was lower than resistant parents (GCH 3 and H 1353) in all of the four crosses respectively. Also, the peroxidase enzyme activity in both susceptible and resistant plants increased significantly from 60 to 90 DAS. The results of the present study indicated that peroxidases contribute towards resistance, as its activity was higher in resistant parents in response to the CLCuD infection at 90 DAS as compared to susceptible parents. Thus, higher activity of peroxidase in resistant genotypes can be responsible for resistance of genotypes to cotton leaf curl virus disease. It has also been reported that the infection with plant pathogens led to an induction in peroxidase activity in plant tissues and increased activity was recorded in resistant plants as compared to the susceptible plants [39]. The present findings are also confirmed by Siddique *et al.* [40], Madhusudhan *et al.* [41], Kiraly *et al.* [42], Dieng *et al.* [43] in cotton (for disease resistance against cotton leaf curl burewala virus), tomato and bell pepper (infected with tobacco mosaic virus and tomato mosaic tobamovirus), tobacco (tobacco mosaic virus infected tobacco plants) and tomato (tomato yellow leaf curl virus infected tomato plants).

It has previously been reported that PPO is important in the initial stage of plant defense where membrane damage causes release of phenols such as chlorogenic acid, PPO catalyzes the oxidation of phenolics to free radicals that can react with biological molecules thus creating an unfavorable environment for pathogen development [44]. In the present investigation, polyphenoloxidase enzyme activity was 8.69, 5.00, 4.23 and 1.94 units in susceptible parents which was lower than resistant parents in all of the four crosses. The higher activity of polyphenoloxidase (PPO) in resistant genotypes indicated the involvement of PPO towards resistance by restricting the infection of disease in them as they produce phenol oxidation products which were more toxic than phenol themselves. This is also supported by the work of Li and Steffens [45]. Many studies have reported that PPO is induced in response to infection by different pathogens [7, 46]. Our findings also suggested that systemic induction of PPO expression in resistant genotypes in response to CLCuD might provide an additional line of defense to protect plants against further attack by pathogen and insects, as previously reported in other plant–pathogen interactions.

These different biochemical parameters *viz*. tannin, phenol, gossypol and enzyme peroxidase and polyphenoloxidase provide defense mechanism to plants so have higher content and activity in resistant plants in response to CLCuD infection while susceptible plants have significantly lower values. In our studies, major biochemical constituents were recorded in the range as for sugar, it was 1.47-2.71 per cent, for phenol 0.42-1.17 per cent, gossypol 0.18-0.50 per cent, tannin 0.20-0.57 per cent, peroxidase enzyme (PO) 8.57-13.25 units and polyphenoloxidase (PPO) 0.97-1.42 units.

The biochemical basis of positive correlation between sugar content and CLCuD infection and negative correlation between total phenols, gossypol, tannin, crude protein contents and activities of enzymes PO and PPO and CLCuD grading suggested that secondary metabolites (total phenol, gossypol, tannin) and enzymes peroxidase and polyphenoloxidase accumulates in the resistant plants in response to the CLCuD infection and might play the defensive role or role in imparting resistance against the cotton leaf curl disease. Correlation matrix in our study also revealed that cotton leaf curl disease grading (disease scoring 0-6) was significantly and positively correlated with sugar content while significantly and negatively correlated with other biochemical parameters *viz.* phenol, gossypol, tannin, protein, peroxidase (PO) and polyphenoloxidase (PPO) at 60 DAS and at 90 DAS (where the positive co-relation with sugar and negative co-realation with phenol and polyphenoloxidase (PPO) was present but this co-relation was non-significant).

**Table 4:** Sugar, Phenol, Gossypol, Tannin, Protein content (%), Peroxidase (PO) and Poyphenoloxidase (PPO) enzyme activity (in units) of four crosses at different stages of growth.

| | | Sugar | | | Phenol | | | Gossypol | | | Tannin | | | Protein | | | PO | | | PPO | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Crosses** | **Genotypes** | **60 DAS** | **90 DAS** | **Mean** | **60 DAS** | **90 DAS** | **Mean** | **60 DAS** | **90 DAS** | **Mean** | **60 DAS** | **90 DAS** | **Mean** | **60 DAS** | **90 DAS** | **Mean** | **60 DAS** | **90 DAS** | **Mean** | **60 DAS** | **90 DAS** | **Mean** |
| CR 1 $P_1$ (R) | GCH 3 | 1.98 | 1.94 | 1.96 | 0.61 | 0.89 | 0.74 | 0.36 | 0.46 | 0.41 | 0.32 | 0.52 | 0.42 | 14.16 | 11.93 | 13.04 | 10.82 | 12.50 | 11.66 | 1.42 | 1.18 | 1.30 |
| $P_2$ (S) | HS 6 | 2.67 | 2.51 | 2.59 | 0.42 | 0.71 | 0.59 | 0.26 | 0.36 | 0.31 | 0.20 | 0.28 | 0.24 | 11.55 | 10.50 | 11.02 | 9.86 | 10.12 | 9.99 | 1.25 | 1.15 | 1.20 |
| $F_1$ | GCH 3 x HS 6 | 2.43 | 2.15 | 2.29 | 0.57 | 0.62 | 0.56 | 0.39 | 0.44 | 0.42 | 0.27 | 0.34 | 0.31 | 12.23 | 12.18 | 12.20 | 11.44 | 11.95 | 11.69 | 1.32 | 1.16 | 1.24 |
| CR 2 $P_1$ (R) | GCH 3 | 1.70 | 1.56 | 1.63 | 0.62 | 0.91 | 0.77 | 0.31 | 0.38 | 0.34 | 0.31 | 0.48 | 0.40 | 14.91 | 12.22 | 13.57 | 11.29 | 13.25 | 12.27 | 1.34 | 1.16 | 1.25 |
| $P_2$ (S) | RST 9 | 2.62 | 2.08 | 2.38 | 0.58 | 0.82 | 0.58 | 0.20 | 0.26 | 0.23 | 0.21 | 0.33 | 0.27 | 13.48 | 11.51 | 12.49 | 9.23 | 10.11 | 10.10 | 1.05 | 1.00 | 1.02 |
| $F_1$ | GCH 3 x RST 9 | 2.52 | 2.48 | 2.50 | 0.61 | 0.86 | 0.81 | 0.35 | 0.38 | 0.36 | 0.20 | 0.38 | 0.29 | 12.63 | 9.66 | 11.14 | 10.13 | 11.73 | 10.93 | 1.30 | 1.29 | 1.30 |
| CR 3 $P_1$ (R) | H 1353 | 1.82 | 1.69 | 1.76 | 0.53 | 0.89 | 0.75 | 0.33 | 0.50 | 0.41 | 0.33 | 0.57 | 0.45 | 13.57 | 12.01 | 12.79 | 11.38 | 11.72 | 11.55 | 1.32 | 1.23 | 1.27 |
| $P_2$ (S) | HS 6 | 2.71 | 2.23 | 2.47 | 0.45 | 0.71 | 0.64 | 0.30 | 0.41 | 0.35 | 0.20 | 0.28 | 0.24 | 12.01 | 11.59 | 11.80 | 9.89 | 10.33 | 10.11 | 1.23 | 1.18 | 1.21 |
| $F_1$ | H 1353 x HS 6 | 2.43 | 2.15 | 2.29 | 0.49 | 0.93 | 0.70 | 0.36 | 0.45 | 0.41 | 0.27 | 0.34 | 0.31 | 12.47 | 9.95 | 11.21 | 10.43 | 10.51 | 10.47 | 1.22 | 1.21 | 1.21 |
| CR 4 $P_1$ (R) | H 1353 | 1.72 | 1.50 | 1.61 | 0.59 | 1.04 | 0.87 | 0.25 | 0.38 | 0.32 | 0.32 | 0.33 | 0.32 | 14.62 | 12.39 | 13.50 | 11.02 | 11.75 | 11.38 | 1.28 | 1.24 | 1.26 |
| $P_2$ (S) | RST 9 | 2.37 | 1.80 | 2.08 | 0.49 | 0.78 | 0.71 | 0.18 | 0.23 | 0.21 | 0.20 | 0.30 | 0.25 | 11.84 | 10.37 | 11.11 | 8.57 | 9.79 | 9.18 | 1.05 | 1.03 | 1.04 |
| $F_1$ | H 1353 x RST 9 | 2.31 | 1.47 | 1.89 | 0.58 | 1.17 | 0.71 | 0.39 | 0.44 | 0.42 | 0.21 | 0.38 | 0.29 | 12.98 | 10.66 | 11.82 | 10.99 | 11.59 | 11.29 | 1.18 | 0.97 | 1.08 |
| Mean | | 2.23 | 1.91 | | 0.54 | 0.86 | | 0.31 | 0.39 | | 0.26 | 0.38 | | 13.04 | 11.25 | | 10.42 | 11.35 | | 1.25 | 1.15 | |
| **Factors** | | **DAS** | **Gen.** | **DAS x Gen.** | **DAS** | **Gen.** | **DAS x Gen.** | **DAS** | **Gen.** | **DAS x Gen.** | **DAS** | **Gen.** | **DAS x Gen.** | **DAS** | **Gen.** | **DAS x Gen.** | **DAS** | **Gen.** | **DAS x Gen.** | **DAS** | **Gen.** | **DAS x Gen.** |
| SE (m) | | 0.030 | 0.075 | 0.106 | 0.014 | 0.035 | 0.049 | 0.006 | 0.015 | 0.022 | 0.006 | 0.015 | 0.022 | 0.197 | 0.483 | 0.683 | 0.184 | 0.450 | 0.636 | 0.010 | 0.025 | 0.036 |
| SE (d) | | 0.043 | 0.106 | 0.149 | 0.020 | 0.049 | 0.070 | 0.009 | 0.022 | 0.031 | 0.009 | 0.022 | 0.031 | 0.279 | 0.683 | 0.966 | 0.260 | 0.636 | 0.899 | 0.015 | 0.036 | 0.050 |
| C.D. at 5% | | 0.086 | 0.211 | 0.298 | 0.040 | 0.099 | 0.139 | 0.018 | 0.043 | 0.061 | 0.018 | 0.043 | 0.061 | 0.557 | 1.365 | N/A | 0.519 | 1.270 | N/A | 0.029 | 0.071 | 0.101 |

(R), resistance; (S), susceptible; PO, peroxidase; PPO, polyphenoloxidase; DAS, days after sowing; Gen., genotypes; DAS x Gen., days after sowing x genotypes; SE, standard error; C.D., critical difference

**Table 5:** Correlation matrix among different biochemical parameters of all the four crosses at 60 DAS

| **4 crosses (60 DAS)** | **PDI** | **Sugar** | **Phenol** | **Gossypol** | **Tannin** | **PO** | **PPO** | **Crude Protein** |
|---|---|---|---|---|---|---|---|---|
| PDI | 1.000 | $0.699^{*}$ | $-0.702^{*}$ | $-0.705^{*}$ | $-0.761^{**}$ | $-0.859^{**}$ | $-0.750^{**}$ | $-0.625^{*}$ |
| Sugar | | 1.000 | $-0.551^{NS}$ | $-0.112^{NS}$ | $-0.876^{**}$ | $-0.625^{*}$ | $0.509^{NS}$ | $-0.846^{**}$ |
| Phenol | | | 1.000 | $0.294^{NS}$ | $0.441^{NS}$ | $0.460^{NS}$ | $0.341^{NS}$ | $0.756^{**}$ |
| Gossypol | | | | 1.000 | $0.287^{NS}$ | $0.742^{**}$ | $0.655^{*}$ | $0.057^{NS}$ |
| Tannin | | | | | 1.000 | $0.734^{**}$ | $0.668^{*}$ | $0.750^{**}$ |
| PO | | | | | | 1.000 | $0.779^{**}$ | $0.527^{NS}$ |
| PPO | | | | | | | 1.000 | $0.411^{NS}$ |
| Crude Protein | | | | | | | | 1.000 |

(*, **) indicates that the value was significant at the 5% and 1% level of significance respectively

**Table 6:** Correlation matrix among different biochemical parameters of all the four crosses at 90 DAS

| 4 crosses (90 DAS) | PDI | Sugar | Phenol | Gossypol | Tannin | PO | PPO | Crude Protein |
|---|---|---|---|---|---|---|---|---|
| PDI | 1.000 | $0.382^{NS}$ | $-0.442^{NS}$ | $-0.685^{*}$ | $-0.701^{*}$ | $-0.880^{**}$ | $-0.473^{NS}$ | $-0.308^{NS}$ |
| Sugar | | 1.000 | $-0.686^{*}$ | $-0.102^{NS}$ | $0.424^{NS}$ | $0.435^{NS}$ | $0.293^{NS}$ | $-0.491^{NS}$ |
| Phenol | | | 1.000 | $0.230^{NS}$ | $0.324^{NS}$ | $0.333^{NS}$ | $-0.139^{NS}$ | $-0.049^{NS}$ |
| Gossypol | | | | 1.000 | $0.552^{NS}$ | $0.541^{NS}$ | $0.492^{NS}$ | $0.238^{NS}$ |
| Tannin | | | | | 1.000 | $0.720^{**}$ | $0.260^{NS}$ | $0.388^{NS}$ |
| PO | | | | | | 1.000 | $0.359^{NS}$ | $0.512^{NS}$ |
| PPO | | | | | | | 1.000 | $0.081^{NS}$ |
| Crude Protein | | | | | | | | 1.000 |

(*, **) indicates that the value was significant at the 5% and 1% level of significance respectively

**Table 7:** Different biochemical parameters among resistant and susceptible parents/ genotypes of all the four crosses at 60 DAS.

| Genotypes | Sugar (%) | | Phenol (%) | | Gossypol (%) | | Tannin (%) | | PO (unit) | | PPO (unit) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GCH 3 (I) | 1.98 | | 0.61 | | 0.36 | | 0.32 | | 10.82 | | 1.42 | |
| HS 6 (I) | 2.67 | | 0.42 | | 0.26 | | 0.20 | | 9.86 | | 1.25 | |
| GCH 3 (II) | 1.70 | | 0.62 | | 0.31 | | 0.31 | | 11.29 | | 1.34 | |
| RST 9 (II) | 2.62 | | 0.58 | | 0.20 | | 0.21 | | 9.23 | | 1.05 | |
| H 1353 (III) | 1.82 | | 0.53 | | 0.33 | | 0.33 | | 11.38 | | 1.32 | |
| HS 6 (III) | 2.71 | | 0.45 | | 0.30 | | 0.20 | | 9.89 | | 1.23 | |
| H 1353 (IV) | 1.72 | | 0.59 | | 0.25 | | 0.32 | | 11.02 | | 1.28 | |
| RST 9 (IV) | 2.37 | | 0.49 | | 0.18 | | 0.20 | | 8.57 | | 1.05 | |
| Factors | S.E.(m) | C.D. at 5% | S.E.(m) | C.D. at 5% | S.E.(m) | C.D. at 5% | S.E.(m) | C.D. at 5% | S.E.(m) | C.D. at 5% | S.E.(m) | C.D. at 5% |
| Genotypes | 0.075 | 0.211 | 0.035 | 0.099 | 0.015 | 0.043 | 0.008 | 0.017 | 0.450 | 1.270 | 0.025 | 0.071 |

PO, peroxidase; PPO, polyphenoloxidase; S.E., standard error; C.D., critical difference

## Conclusions

Phenol, gossypol, tannin, protein, peroxidase and polyphenoloxidase had significantly higher content and activity in resistant plants while susceptible plants had lower values and also these biochemical constituents had significant negative correlation with CLCuD grading (disease scoring 0-6). Thus, it can be argued that there is a correlation between increased levels of total phenols, gossypol, tannin, peroxidase, polyphenoloxidase and the plant resistance. So, these biochemical constituents might play an active role in imparting resistance to cotton leaf curl disease and could be considered as biochemical markers and may be further used for early screening of the germplasm lines/ breeding material.

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