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# Leaf cuticular wax content is involved in cotton leaf curl virus disease resistance in cotton (*Gossypium hirsutum* L.)

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## Abstract

Cotton leaf curl virus disease (CLCuVD) limits cotton production in many cotton growing countries of the world, including Pakistan. In the past, efforts were made to combat this disease by different approaches. Cuticular wax is reported to confer resistance to plants against various biotic and abiotic stresses. Present study was designed to assess the role of cuticular wax content (WC) to resist CLCuVD infestation. The WC of 42 cotton genotypes, originating from various countries (Pakistan, USA, China, etc.), was quantified during two culture periods (2015 & 2016). Cotton germplasm was also scored for % disease index (%DI), seed cotton yield (SCY), number of bolls/plant (NB), and plant height (PHt) for the same culture periods. Significant negative correlation between WC and %DI was found during the two years of experimentation. WC was found positively correlated with SCY and NB. Six cotton genotypes (A-7233, B-557, A-162, BLANCO-3363, CIM-473, and SLH-2010-11) did not show any signs of CLCuVD infestation during both 2015 and 2016. These cotton genotypes contained relatively higher WC. The results from analysis of variance (ANOVA) demonstrated that there were significant differences among genotypes for %DI, WC, SCY, NB, and PHt. These results indicated that WC was involved in resisting CLCuVD and it also had positive effect on plant growth and yield potential. On the basis of these findings, it was concluded that cuticular wax could be used as an indirect criterion for distinguishing and selecting resistant/susceptible cotton genotypes.

**Additional keywords:** biotic stress; cuticle; disease index; morphological traits; seed cotton yield.

**Abbreviations used:** CLCuVB (*Cotton leaf curl virus* Burewala); CLCuVD (cotton leaf curl virus disease); %DI (percent disease index); EC (emulsifiable concentrate); NB (number of bolls/plant); PHt (plant height); RCBd (randomized complete block disease); SCY (seed cotton yield); WC (wax content).

**Authors' contributions:** Conceived, designed and performed the experiments, contributed reagents/materials/analysis tools: MS, SXL and MR. All authors analyzed the data, wrote and approved the final manuscript.

**Citation:** Saeed, M.; Xianliang, S.; Xuezhen, S.; Riaz, M. (2018). Leaf cuticular wax content is involved in cotton leaf curl virus disease resistance in cotton (*Gossypium hirsutum* L.). Spanish Journal of Agricultural Research, Volume 16, Issue 4, e0705. <https://doi.org/10.5424/sjar/2018164-13085>

**Received:** 22 Feb 2018. **Accepted:** 21 Nov 2018.

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**Funding:** National Key Research and Development Program (2018YFD0100303); Major Projects for Transgenic Breeding of China (2017ZX08005-004-006); System of Modern Agriculture Industrial Technology (SDAIT-03-03/05); Natural Science Foundation (ZR2017MC057); Agricultural Seed Project (cotton variety development, 2014-2017) of Shandong Province; Innovation Projects for improvement of Saline Land (2015); Shandong Agricultural University, China, Open Project (2014KF01).

**Competing interests:** The authors have declared that no competing interests exist.

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## Introduction

Cotton (*Gossypium hirsutum* L.) is an important crop globally for its diverse uses (Zhang *et al.*, 2008). It is important for fiber, oil, biofuel production and also feedstock industry (De-Sousa *et al.*, 2015). It is also called as white gold for its importance as cash and industrial crop (Iqbal *et al.*, 2005). China, India, USA and Pakistan are major cotton producing countries. A large number of people throughout the world are

involved in different aspects of cotton production, cotton products processing, and cotton products trade (Zhang *et al.*, 2008). In Pakistan, cotton accounts for more than 50% of total foreign export earnings (Anonymous, 2017).

Cotton production is faced with a number of biotic and abiotic stresses. Among these production constraints, CLCuVD (cotton leaf curl virus disease) severely affects cotton production world-wide (Farooq *et al.*, 2011; Hashmi *et al.*, 2011). This disease is caused

by a virus known as *Cotton leaf curl virus* (CLCuV). CLCuV belongs to genus *Begomovirus* and family *Geminiviridae*. It was noticed for the first time in Nigeria (Farquharson, 1912), and the first symptoms of CLCuVD were observed in Pakistan during 1967 (Hussain & Ali, 1975). In Pakistan, CLCuVD appeared in epidemic form after 1988 and heavy cotton production losses occurred in 1992 due to vast cotton cropped area affected by CLCuVD infestation. In 1992, world cotton production was also affected badly due to CLCuVD infestation. Cotton breeders started efforts to find CLCuVD resistant germplasm. Conventional breeding efforts were fruitful and soon CLCuVD resistant varieties of *G. hirsutum* were developed.

Rate of evolution in CLCuV genome is relatively high. In 2001, a new strain of this virus was noticed in Burewala, district Vehari, Punjab, Pakistan. This strain was named CLCuVB (*Cotton leaf curl virus* Burewala). All resistant genotypes of upland cotton became susceptible to this new viral strain (Mansoor *et al.*, 2003). Nowadays, there is no report of any available upland cotton cultivar resistant to CLCuVB attack. It has direct social and economic concerns. In Pakistan, approx. 30% yield losses per year are caused due to CLCuVD (Briddon *et al.*, 2000; Rahman *et al.*, 2014; Hassan *et al.*, 2016). In past, this disease was reported in Pakistan and India. But, it has spread to other important cotton growing areas of the world also such as southern China (Mao *et al.*, 2008; Cai *et al.*, 2010).

Plants affected by CLCuVD show a complex of symptoms including downward cupping of younger leaves, veins swelling and thickening, and leaf margins upward or downward curling. Frequently, there is emergence of outgrowths, resembling small leaves, on the lower side of leaves. These outgrowths are known as 'enations' (Akhtar *et al.*, 2008). In severely affected plants, yield is adversely low and poor-quality fiber is produced (Akhtar *et al.*, 2009). Fortunately, CLCuVD is not transmitted through seed, but has the capacity to survive in different hosts (Khan & Ahmad, 2005). Because its vector is whitefly (*Bemisia tabaci* Genn.), an approach to limit the infection is to control the population density of this insect. But, this is not a practically feasible solution (Holt *et al.*, 1999). There may be chances of evolution of pesticide resistance in whiteflies and also environmental concerns about heavy use of pesticides (Palumbo *et al.*, 2001). Search for resistant sources is the most desirable solution to handle a disease stress and it is an environment friendly option (Hogenboom, 1993).

Cuticular wax, an important constituent of plant cuticle, has been found to give resistance to plants against a number of biotic and abiotic stresses (Smith, 1999; Jeffree, 2006; Kosma *et al.*, 2010; Bourdenx *et*

*al.*, 2011; Weidenbach *et al.*, 2014). Cuticular wax is reported to inhibit attack of different pathogens on various plant species (Martin *et al.*, 1957; Johnston & Sproston, 1965; Heather, 1967; Blakeman & Sztejnberg, 1973; Alcerito *et al.*, 2002; Kosma *et al.*, 2010; Yin *et al.*, 2011; Weidenbach *et al.*, 2014). Thinning of wax layer on leaves is found to promote susceptibility to stress conditions (Rawlinson *et al.*, 1978; El-Otmani & Coggins, 1985a,b).

This research project was designed to investigate the role of cuticular wax content (WC) in resisting CLCuVD infestation. The objectives of this study were to estimate, in different genotypes of cotton: a) WC ( $\mu\text{g}/\text{cm}^2$  of leaf area), b) rate of CLCuVD infestation, and c) relationship between WC and CLCuVD infestation.

## Material and methods

### Plant material

Forty two genotypes of cotton were used in this study (Table 1). Twenty two genotypes originated from Pakistan and the rest were exotic, originated from diverse countries of the world. Plant material was sown at Ayub Agricultural Research Institute (AARI), Faisalabad during May 2015 and May 2016, following randomized complete block design (RCBD) with three replications. Standard agricultural practices (hoeing, weeding, fertilizers, insects/pests control, and irrigation) were followed for growing these genotypes. At sowing time, fertilizer di-ammonium phosphate (DAP) at the rate of  $11.5 \text{ g}/\text{m}^2$  was applied. Five irrigations were applied starting after 35 DAS (days after sowing) till 125 DAS. Data for sucking insect pests (jassids, whiteflies and thrips) were scored at weekly intervals. Data were scored by counting number of insect pests from randomly selected 15 leaves from 15 plants of each plot. Leaves were selected as one leaf from upper portion of one plant, second leaf from middle portion of 2<sup>nd</sup> plant, and third leaf from lower portion of 3<sup>rd</sup> plant. This process was repeated, in the same sequence, till the 15<sup>th</sup> plant.

For bollworm pests, data were scored from 5 randomly selected plants of each plot. To calculate % infestation, total number of bolls and squares/flowers; and infested bolls and squares/flowers were counted. During both 2015 and 2016 years, experimental area was sprayed six times for control of insect pests. Special care was taken to control whiteflies, the vector for CLCuV. Pyrethrin 10% EC (20 mL in 10 L of water) was used and sprayed to control whiteflies, when its population crossed the threshold of 5 adults per cotton leaf. This ensured uniform whitefly population

**Table 1.** List of genotypes used in the study.

No.	Genotype	Origin	No.	Genotype	Origin	No.	Genotype	Origin
1	124-F	Local	15	ALDEL-128	Exotic	29	BOSS-111	Exotic
2	199-F	Local	16	ASA(65)-650	Exotic	30	BROWN BWP	Local
3	268-F	Local	17	B-557	Local	31	BRS-37	Exotic
4	281-GL-(443)	Local	18	B-582	Local	32	C2 (37) 1473	Exotic
5	448/4727C	Local	19	BARNT-2-41	Exotic	33	C2 (69) 1485	Exotic
6	FH-207	Local	20	BAYOUSAMI	Exotic	34	CIM-109	Local
7	GLNS	Exotic	21	BH-100	Local	35	CIM-240	Local
8	ACA-285	Exotic	22	BH-118	Local	36	CIM-473	Local
9	ACALA-4-42	Exotic	23	B-69	Local	37	CIM-496	Local
10	ALA	Exotic	24	BH-36	Local	38	CIM-506	Local
11	A-7233	Exotic	25	A-162	Exotic	39	SLH-2010-11	Local
12	ALA-1054-A	Exotic	26	BJAHL	Exotic	40	COKER 1000	Exotic
13	NIAB-2009	Local	27	BLANCO-3363	Exotic	41	IR-NIAB-824	Local
14	ALBACALA	Exotic	28	ALBACALA(70)19	Exotic	42	NIBGE-314	Local

in the experimental field area and each cotton genotype was exposed to a uniform whitefly population pressure. Thus, each cotton genotype had equal chance of exposure to CLCuV infestation.

### Extraction of cuticular wax and quantification of wax content

First of all, 25 days-old leaves from each variety of cotton were collected on September 20, 2015 and September 20, 2016 (on the same day when CLCuVD infestation scoring was done, coinciding with maximum CLCuVD infestation). Cuticular wax was extracted from these leaves following the method of Bondada *et al.* (1996). Leaves were immersed in pre-weighed petri plates having chloroform (10 mL) for 10-15 seconds. Afterwards, leaves were removed and after complete evaporation of chloroform, petri plates were weighed again. WC was calculated by subtracting the initial weight from final weight of petri plates and expressed as  $\mu\text{g}/\text{cm}^2$  of leaf area.

### CLCuVD infestation scoring

All agricultural practices were uniformly applied to the experimental field area so that a constant level of whiteflies (vector) was present to spread the CLCuVD among the cotton genotypes. CLCuVD infestation (% disease index, %DI) was scored following the method described by Akhtar *et al.* (2010). This disease rating scale has been used in different plant species to calculate the % of disease incidence in different plant diseases (Akhtar *et al.*, 2004, 2009, 2010; Saravanakumar *et al.*, 2007; Anand *et al.*, 2010).

### Data recording for morphological and yield-related traits

During the two seasons (2015, 2016), data were also recorded, from every plant of each genotype picked separately at maturity stage, for seed cotton yield per plant (SCY, g/plant), number of bolls per plant (NB), and plant height (PHt, cm, measured with a scale).

### Data analysis

Data were analyzed by statistical software CoStat v. 6.303 for descriptive statistics, Pearson's correlation coefficients, and analysis of variance (ANOVA) estimates. Pearson's correlation coefficients were estimated at probability  $p \leq 0.05$ . ANOVA was performed according to RCBD with two factors (genotypes and years).

## Results

### ANOVA estimates

The mean squares from ANOVA referring to the effect of cotton genotype in %DI, WC, SCY, NB, and PHt for the combined dataset of 2015 and 2016 were significant ( $p \leq 0.05$ ). This showed that the genotypes had significant differences with regard to studied traits (Table 2). For %DI, the differences in values across years and genotype  $\times$  environment were not significant. These results suggested that the behavior of the genotypes was consistent with respect to CLCuVD resistance and susceptibility during 2015 and 2016.

**Table 2.** Mean squares for traits (%DI, disease index; WC, wax content,  $\mu\text{g}/\text{cm}^2$ ; SCY, seed cotton yield/plant, g; NB, number of bolls/plant; PHt, plant height, cm) of cotton genotypes for combined dataset of 2015 and 2016.

SoV	%DI	WC	SCY	NB	PHt
Replication	203 NS	1 NS	764 NS	73 NS	359 NS
Genotypes	170*	6415***	1636***	198***	682***
Year	133 NS	342***	4939**	1289***	4822***
Genotype $\times$ Environment Interaction	102 NS	1093***	1547***	196***	538***
Error	101	4	566	66	183
$R^2$	0.41	1.00	0.60	0.62	0.65
CV (%)	171.1	4.1	32.4	28.4	7.7

SoV= Source of variation;  $R^2$  = Model sum of squares / Total sum of squares; CV = Coefficient of variation. NS, non-significant; \*, \*\*, \*\*\*, significant at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively.

However, great variation for %DI was observed among genotypes and it was reflected in high coefficient of variation (171.1%) for %DI. This indicates that genotypes possess different genetic potential to tolerate CLCuVD. This was also the reason that standard deviation from %DI was higher than mean itself (Table 3). Some genotypes were immune against CLCuVD attack during both 2015 and 2016, so minimum values for %DI were zero during both years (Table 3). For WC, genotypes had significant ( $p \leq 0.001$ ) differences. For each genotype, WC differed by year. Genotype  $\times$  environment interactions for WC were also significant ( $p \leq 0.001$ ). This indicates that WC for genotypes is greatly influenced by environmental conditions. A number of environmental factors (such as drought, temperature, pathogen attack) influence quantity of

cuticular wax in plants (Jeffree, 2006; Kosma *et al.*, 2010; Bourdenx *et al.*, 2011; Mondal, 2011; Yeats & Rose, 2013; Schuster *et al.*, 2016; Xue *et al.*, 2017).

CLCuVD infestation is mainly influenced by whitefly population in the area. As a result of timely application of insecticide for whitefly control, cotton genotypes were exposed to same whitefly population pressure during 2015 and 2016, so Year  $\times$  Genotype interaction with %DI was non-significant. There was significant temperature difference during 2015 and 2016 cotton growing season at the experimental site region (Faisalabad, Pakistan). Temperature influenced WC, so Year  $\times$  Genotype interaction with WC was highly significant. Difference in WC of each individual cotton genotype with temperature fluctuation was proportional to its inherent potential for WC, so each individual

**Table 3.** Statistical parameters for the traits (%DI, disease index; WC, wax content,  $\mu\text{g}/\text{cm}^2$ ; SCY, seed cotton yield/plant, g; NB, number of bolls/plant; PHt, plant height, cm) of cotton genotypes.

Trait	Dataset	Mean	St. Dev	Sum	Min	Max
%DI	Combined	6	11	1481	0	26
	2015	7	10	832	0	23
	2016	5	11	649	0	31
WC	Combined	47	35	11766	5	124
	2015	46	37	5736	2	153
	2016	48	33	6030	3	134
SCY	Combined	74	31	18317	48	118
	2015	78	34	9706	39	160
	2016	69	26	8611	31	122
NB	Combined	29	11	7180	22	45
	2015	31	12	3875	17	60
	2016	26	9	3305	15	45
PH	Combined	176	19	44316	152	200
	2015	172	8	21607	156	184
	2016	180	24	22709	143	220

cotton genotype showed its susceptibility/tolerance to CLCuVD infestation according to its inherent potential of WC.

### Descriptive statistical parameters of genotypes

Descriptive statistical parameters of genotypes showed that extensive variability was present among the genotypes for %DI, WC, SCY, NB, and PHt (Table 3).

Minimum and maximum %DI was found to be 0-23% and 0-31% during 2015 and 2016, respectively (Table 3). Genotypes 199-F and CIM-506 showed the highest %DI during 2015 and 2016, respectively. Minimum and maximum WC during 2015 was found to be 2  $\mu\text{g}/\text{cm}^2$  and 153  $\mu\text{g}/\text{cm}^2$ , respectively. Minimum and maximum WC during 2016 was found to be 3  $\mu\text{g}/\text{cm}^2$  and 134  $\mu\text{g}/\text{cm}^2$ , respectively (Table 3). Comparison of %DI and WC during 2015 and 2016 showed that there was opposite trend between %DI and WC. Genotypes with high WC showed less CLCuVD susceptibility in general. On the other hand, genotypes with less WC showed higher values of %DI.

### Coefficients of correlation

There was a significant negative correlation between %DI and WC during 2015 ( $p \leq 0.001$ ) and 2016 ( $p \leq 0.05$ ) (Table 4). Similarly, %DI had a negative

correlation with SCY, NB, and PHt during both years. WC showed positive correlation with SCY and NB. This correlation was highly significant in combined and 2015 dataset, but it was non-significant in 2016 dataset. WC had also a positive correlation with PHt, but it was non-significant.

### Highly tolerant and less tolerant/susceptible cotton genotypes against CLCuVD infestation

Some cotton genotypes showed consistent behavior with regard to CLCuVD tolerance/susceptibility during 2015 and 2016 seasons (Table 5). Genotypes FH-207, GLNS, ACA-285, A-7233, B-557, BARNT-2-41, BAYOUSAMI, BH-100, A-162, BJAHL, BLANCO-3363, ALBACALA(70)19, CIM-473, and SLH-2010-11 were found to be immune/highly tolerant to CLCuVD infestation during both 2015 and 2016 years. It is important to note that the weight of WC in these cotton genotypes was  $\geq 40 \mu\text{g}/\text{cm}^2$ . Among these genotypes, A-7233, B-557, A-162, BLANCO-3363, CIM-473, and SLH-2010-11 appeared immune and did not show any CLCuVD infestation during both 2015 and 2016 (Table 5). All these CLCuVD immune cotton genotypes possessed relatively higher WC. Among these genotypes, three genotypes (B-557, CIM-473, SLH-2010-11) were approved varieties grown in Pakistan. Three other varieties (A-7233, A-162, BLANCO-3363) were of exotic origin and part of cotton germplasm maintained at Cotton Research Station (CRS),

**Table 4.** Correlation coefficients among traits (%DI, disease index; WC, wax content,  $\mu\text{g}/\text{cm}^2$ ; SCY, seed cotton yield/plant, g; NB, number of bolls/plant; PHt, plant height, cm) of cotton genotypes.

Trait	Dataset	% DI	WC	SCY	NB	PHt (cm)
%DI	Combined	1				
	2015					
	2016					
WC	Combined	-0.30***	1			
	2015	-0.36***				
	2016	-0.23*				
SCY	Combined	-0.37***	0.20**	1		
	2015	-0.43***	0.32***			
	2016	-0.34***	0.05 NS			
NB	Combined	-0.34***	0.23***	0.95***	1	
	2015	-0.45***	0.33***	0.94***		
	2016	-0.28**	0.11 NS	0.95***		
PHt	Combined	-0.38***	0.12 NS	0.15*	0.14*	1
	2015	-0.43***	0.11 NS	0.21*	0.20*	
	2016	-0.40***	0.14 NS	0.25**	0.26**	

NS, non-significant; \*, \*\*, \*\*\*, significant at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$ , respectively.



**Table 5.** List of immune/highly tolerant and less tolerant/susceptible genotypes during 2015 and 2016.

Genotype	WC/SCY	
	2015	2016
<b>Immune</b>		
A-7233	133/47	97/78
B-557	153/94	96/60
A-162	63/81	134/61
BLANCO-3363	86/114	115/92
CIM-473	153/132	93/62
SLH-2010-11	62/66	99/75
<b>Highly tolerant</b>		
FH-207	45/60	50/60
GLNS	43/52	42/74
ACA-285	47/55	41/60
BARNT-2-41	44/103	54/70
BAYOUSAMI	48/95	64/57
BH-100	41/74	59/78
BJAHL	55/57	51/122
ALBACALA(70)19	51/124	44/75
<b>Less tolerant/susceptible</b>		
199-F	7/43	8/36
448/4727C	2/45	9/46
NIAB-2009	8/55	10/36
BH-36	7/47	3/20
BROWN BWP	7/47	4/36
C2 (69) 1485	10/40	6/37
CIM-109	8/57	4/48
CIM-506	7/41	3/23
NIBGE-314	7/51	9/60

WC, wax content,  $\mu\text{g}/\text{cm}^2$ ; SCY, seed cotton yield/plant, g.

Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. BLANCO-3363 is a registered cotton variety in Texas, USA. From the other side, the genotypes 199-F, 448/4727C, NIAB-2009, BH-36, BROWN BWP, C2 (69) 1485, CIM-109, CIM-506, and NIBGE-314, having WC weight lower than  $10 \mu\text{g}/\text{cm}^2$ , showed higher susceptibility to CLCuVD. It indicated that a minimum threshold quantity of WC ( $40 \mu\text{g}/\text{cm}^2$ ) is required to offer resistance to CLCuVD infestation. It was the same for each year. In the absence of this threshold quantity of WC, a genotype was rendered susceptible to CLCuVD.

## Discussion

Cuticle is the first layer of defense for protecting plants from environmental adversities. Cuticle consists

of cutin/cutan matrix to which the cuticular waxes are either embedded (intra-cuticular waxes) or present on the outer layer of this matrix (epi-cuticular waxes) (Yeats & Rose, 2013). These cuticular waxes are reported to provide protection to plants against various biotic and abiotic stresses (Shepherd & Griffiths, 2006; Yeats & Rose, 2013; Schuster *et al.*, 2016; Xue *et al.*, 2017). In this study, a significant negative correlation was found between WC and %DI. This showed that cuticular wax had a role in conferring resistance to cotton plants against CLCuVD infestation. This role may be manifested by the control of density in population of whiteflies, which are the vectors of the virus. The cotton genotypes with more WC were not favored by whiteflies for feeding and thus there was no transmission of CLCuVB to these genotypes, rendering these genotypes resistant to CLCuVD infestation. The findings of our research support a new indirect role of wax for giving tolerance against a viral disease in cotton. Previously, cuticular wax was reported to have a role for giving resistance to another insect, Hessian fly, attacks. Kosma *et al.*, (2010) reported that resistant plants to Hessian fly attack produced more waxes and cutin than susceptible plants. In a similar manner, cuticular wax might checked feeding of whitefly and thus inhibited transmission of CLCuVB to the body of cotton plant. Otherwise, there might be some other mechanism such as cuticular wax components' involvement in signal transduction and activation of defense mechanism against CLCuVB. Lipids, a major constituent of cuticular wax, are reported to mediate defenses against pathogen attack (Okazaki & Saito, 2014). In case of CLCuVB, this signaling role of cuticular wax will open new horizons of research endeavors. In the near future, efforts will be carried out to study the role and expression of these possible mechanisms.

In Pakistan, currently the most prevalent strain of virus, causing CLCuVD, is CLCuVB (Hassan *et al.*, 2016). Six cotton genotypes (A-7233, B-557, A-162, BLANCO-3363, CIM-473, and SLH-2010-11), which did not show any signs of CLCuVD infestation during both 2015 and 2016, possess highest level of tolerance against CLCuVB. In this study, cotton genotypes with  $\leq 10 \mu\text{g}/\text{cm}^2$  WC were found less tolerant or susceptible to CLCuVB attack. It indicated that to resist CLCuVB, a cotton genotype must possess a minimum threshold quantity of WC, otherwise it would be rendered susceptible to CLCuVB attack. On the other hand, a cotton genotype with  $\geq 40 \mu\text{g}/\text{cm}^2$  WC was found tolerant to the attack of CLCuVB. It implied that a judicious quantity of cuticular wax provided required thickness of wax barrier which made hindrance in feeding of whitefly. Even though there was a uniform population of whiteflies in the experimental area,

whiteflies could feed only on leaves of those cotton genotypes with less cuticular wax content and, thus, with thin layer of wax on their leaves. On the basis of these findings, it is suggested that the quantity of WC is critical for resistance to CLCuVB and may be used as a useful indicator for screen tolerance or susceptibility of cotton genotypes.

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