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Research Article

Molecular and Biochemical Characterization of Cotton Epicuticular Wax in Defense Against Cotton Leaf Curl Disease

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Background: *Gossypium arboreum*is resistant to *Cotton leaf curl Burewala virus* and its cognate *Cotton leaf curl Multan beta satellite* (*CLCuBuV* and *CLCuMB*). However, the *G. arboreum* wax deficient mutant (GaWM3) is susceptible to CLCuV. Therefore, epicuticular wax was characterized both quantitatively and qualitatively for its role as physical barrier against whitefly mediated viral transmission and co-related with the titer of each viral component (DNA-A, alphasatellite and betasatellite) in plants.

Objectives: The hypothesis was the *CLCuV* titer in cotton is dependent on the amount of wax laid down on plant surface and the wax composition.

Materials and Methods: The wax was isolated by decoction method and analysed by GC-MS whereas viral particles were quantified by Real-Time PCR. The whitefly feeding assay was performed on plant in Hoagland solution with 1% Nile Blue Dye and color was observed in the gut of whiteflies.

Results: Analysis of the presence of viral genes, namely alphasatellite, betasatellite and DNA-A, via real-time PCR in cotton species indicated that these genes are detectable in *G. hirsutum*, *G. harknessii* and GaWM3, whereas no particle was detected in *G. arboreum*. Quantitative wax analysis revealed that *G. arboreum* contained 183 $\mu g.cm^{-2}$ as compared to GaWM3 with only 95 $\mu g.cm^{-2}$. *G. hirsutum* and *G. harknessii* had 130 $\mu g.cm^{-2}$ and 146 $\mu g.cm^{-2}$, respectively. The GC-MS results depicted that Lanceol, cis was 45% in *G. harknessii*. Heptadecanoic acid was dominant in *G. arboreum* with 25.6%. GaWM3 had 18% 1,2,-Benenedicarboxylic acid. *G. hirsutum* contained 25% diisooctyl ester. The whitefly feeding assay with Nile Blue dye showed no color in whiteflies gut fed on *G. arboreum*. In contrast, color was observed in the rest of whiteflies.

Conclusions: From results, it was concluded that reduced quantity as well as absence of (1) 3-trifluoroacetoxytetradecane, (2) 2-piperidinone,n-|4-bromo-n-butyl|, (3) 4-heptafluorobutyroxypentadecane, (4) Silane, trichlorodocosyl-, (5) 6-Octadecenoic acid, methyl ester, and (6) Heptadecanoicacid,16-methyl-,methyl ester in wax could make plants susceptible to *CLCuV*, infested by whiteflies.

Keywords: Cotton; GaWM3; GC-MS; Leaf curl; Wax mutant; Whitefly

1. Background

Plant viruses are major hindrance in yield improvement and productivity of plant products. Viruses that belong to family *Geminiviridae*, are economically important and transmitted by the members of the phylum Arthropoda (1).

Cotton plants are naturally affected by many stresses from which 75% are biotic (2). Among these pathogens, *Cotton leaf curl virus* (*CLCuV* and its cognate *CLCuBuV* and *CLCuMB*) is a common source of tension for cotton growers especially in Pakistan. *CLCuV* genome consists of a single stranded DNA particle *i.e*. DNA-A along with each of its associated DNA satellites, called alpha satellite and beta satellite (3).

The first and foremost physical barrier in plant pathogen interaction is epicuticular wax (4). This layer not only hinders the bacteria and fungi, but also create a first line of defense against insects (5). For instance, in wax deficient pea mutants the aphid spends more time (6). Wax can be defined as a polyester matrix of hydroxyl- and hydroxyl epoxy fatty acids C_{16} and C_{18} long (cutin) embedded and overlaid with epicuticular wax.

The Asiatic *G. arboreum* is resistant to *CLCuV* (7).

Our hypothesis was to investigate that whether the wax plays a critical barrier in transmission of *CLCuV* by whitefly (*Bemisia tabaci*) in this plant. In 2009, a wax deficient mutant (GaWM3) of Asiatic *G. arboreum* with 50% less wax was produced (8).

2. Objectives

The aims of the present study was (1) to quantify the cuticular waxes and determine the biochemical composition of wax mutant GaWM3 in comparison with *G. arboreum*, *G. hirsutum* and *G. harknessii*, and (2) to determine the *CLCuV* titer and its correlation with quantity and composition of waxes through feeding of whiteflies on plants.

3. Materials and Methods

3.1. Plant Materials

Seeds of *G. hirsutum* less waxy and susceptible to *CLCuV*, *G. arboreum*, “desicotton” resistant to *CLCuV* with more epicuticular wax, *G. harknessii*, more waxy like and susceptible to *CLCuV* were planted along with wax deficient mutant of *G. arboreum* (GaWM3) in pots as well as in field. Upward or downward curling of the leaves, thickened veins and growth of plants was noted in inoculated and non-inoculated plants as indicated by Khan *et al*. (9).

3.2. CLCuV Titer Evaluation

Viruliferous whiteflies (100) were incubated overplants. Field trials have been conducted under natural infection condition with uncharacterized *CLCuV* isolates. However, it was found that *CLCuBV* was more dominant in the field than *CLCuMB* Primers were designed for alpha satellite (FR873751.1), beta satellite (HF567946.1) and DNA-A (X98995.1). The primers and probe (5′Fam and 3′Tamra) were designed from coat protein of DNA-A, C1 region of beta-satelliteand Rep gene of alpha-satellite (Table 1) using “Genscript” website software (https://www.genscript.com/ssl-bin/app/primer). The experiment was performed in 3 replicates. The reaction mixture (25 μL) contained 150 μg of plant DNA, 2.5 μL 10× PCR buffer (Fermentas, USA), 2.5 μL of 2 mM dNTPs, 1.5 μL of $MgCl_2$ (Fermentas, USA) 1 μL of 10 pmol.μL^{-1} each forward and reverse primers (Table 1) and 0.5 μL of 5U *Taq* DNA-polymerase (Fermentas, USA). The PCR was initiated at 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 s, 59ºC for 30 s, and 72ºC for 30 sec with final extension at 72ºC for 10 min. The concentrations of the viral particles were calculated through Real Time-PCR using standard curve through known standards of DNA-A, alpha satellites and beta satellites.

Table 1. Designing of primers and probe to detect DNA-A, alphasatellite and betasatellite

DNA-A: Amplicon size 182 bp Tm: 55°C	
CP-F	5'AAACAACAGGCATGGACAAA'3,
CP-R	5'CCGACACCACGAGTAACATC '3
CP-P	Fam-5'TGGGCCTTCACAACCCTTTGG '3-Tamra
Alphasatellite:Amplicon size 192 bp: 55°C	
Alpha Rep-F	5'GTCTTCCGACGAGTTAAGGC '3
Alpha Rep-R	5'GTCTCTGGCAAAGGTGGATT '3
Alpha Rep-P	Fam-5'AACGGGACCCAGATGACCGC '3-Tamra
Betasatellite: Amplicon size 186 bp: 55°C	
BetaC1-F	5'TTCCTATTCGCATACAACGG '3
BetaC1-R	5'ATGCATTGCTGGTTTGTGTT '3
BetaC1-P	Fam-5'ACGGTTCGATTACATCCATTCCCAA'3-Tamra

3.3. Wax Quantification

The isolation of plant epicuticular wax was performed according to “Decoction” method (10) and leaf surface area was calculated with Adobe Photoshop (11). The total isolated wax from each plant was converted into μg and divided by total leaf surface area (in cm^2).

$$\text{Wax per unit area} = \frac{\text{Weight of wax }(\mu g)}{\text{Total leaf surface area }(cm^2)}$$

3.4. Determination of Biochemical Composition of Epicuticular Wax

Gas chromatograph mass spectrometry: wax samples (1 μg) in 3 replicates were dissolved in hexane and passed through impregnated carbon filter to remove any impurities. Internal standard, tetracosane (10 μg.μL^{-1}) was added to the testing samples prior to analysis. From the wax samples, 2 μL was taken and injected into the column at 50ºC and condition was held for 2 min. The samples were desorbed by increasing the temperature by 40°C.min^{-1} to 200°C, 2 min at 200°C, 3°C.min^{-1} to 310°C, and 30 min at 310°C. The Helium gas was used as the carrier and the gas flow was maintained at 2 mL.min^{-1}. The quantitative composition of the mixtures was studied by capillary GC (Agilent; 30 m HP-1, 0.32-mm i.d. df = 1 μm) and flame ionization detection under the same GC conditions as above but Helium (carrier gas) inlet pressure was programmed for 50 kPa at injection, held for 5 min, raised with 3 kPa.min^{-1} to 150 kPa and held for 40 min at 150 kPa. Single compounds were quantified

against the internal standard by manually integrating peak areas (12). Components were identified by the help of NIST library, 2005 (13).

3.5. Whitefly Feeding Assay

Two week old seedling of plants (*i.e. G. arboreum*, GaWM3, *G. hirsutum*and *G. harknessii*) were placed into Hoagland's solution (14) with 1% Nile Blue (Sigma Aldrich, USA). The whiteflies (*Bemisia tabaci*) were incubated on plants for 3 days and observed under microscope (Zeiss, Imager A1) to observe the color of Nile Blue dye in their gut.

4. Results

4.1. Detection of CLCuV

Symptoms: The plants were exposed to whiteflies in random in field trials and 100 whiteflies per plant were incubated in greenhouse tests. The symptoms of cotton leaf curl disease appeared on *G. hirsutum*, *G. harknessii* and GaWM3 but not on *G. arboreum*. The typical symptoms of upward or downward curling of the leaves and thick enation were appeared on *G. hirsutum* and GaWM3 (Figure 1). The *CLCuV* components (alpha satellite, beta satellite and DNA-A) were quantified by real time PCR. The mean numbers of molecules per microliter of alpha satellite in between-greenhouse and field samples were 5.9×10^8, 4.8×10^7 and 4.6×10^7 for *G. hirsutum*, GaWM3 and *G. harknessii*, respectively. Whereas no alpha satellite was detected in *G. arboreum* (Figure 2A). Beta satellites were determined as 7.2×10^8, 3.6×10^7 and 3.8×10^7 molecules.μL^{-1} in *G. hirsutum*, GaWM3 and *G. harknessii*, respectively. Similarly, beta satellite was not detected in *G. arboreum* (Figure 2B). The copy numbers of DNA-A in *G. hirsutum*, GaWM3 and *G. harknessii* were 8.7×10^8, 6.6×10^7 and 6.3×10^7 molecules.μL^{-1}, respectively. Again, DNA-A was not detected in *G. arboreum* (Figure 2C). In experimental plants, *G. hirsutum*: GaWM3: *G. harknessii*: *G. arboreum*, the ratio of α-satellite was 270:24:23:0 for alpha satellite, for beta satellite was 360:18:19:0, and for DNA-A was 290:22:21:0, respectively.

4.2. Epicuticular Wax per Unit Area

Maximum wax per unit area was obtained from *G.*

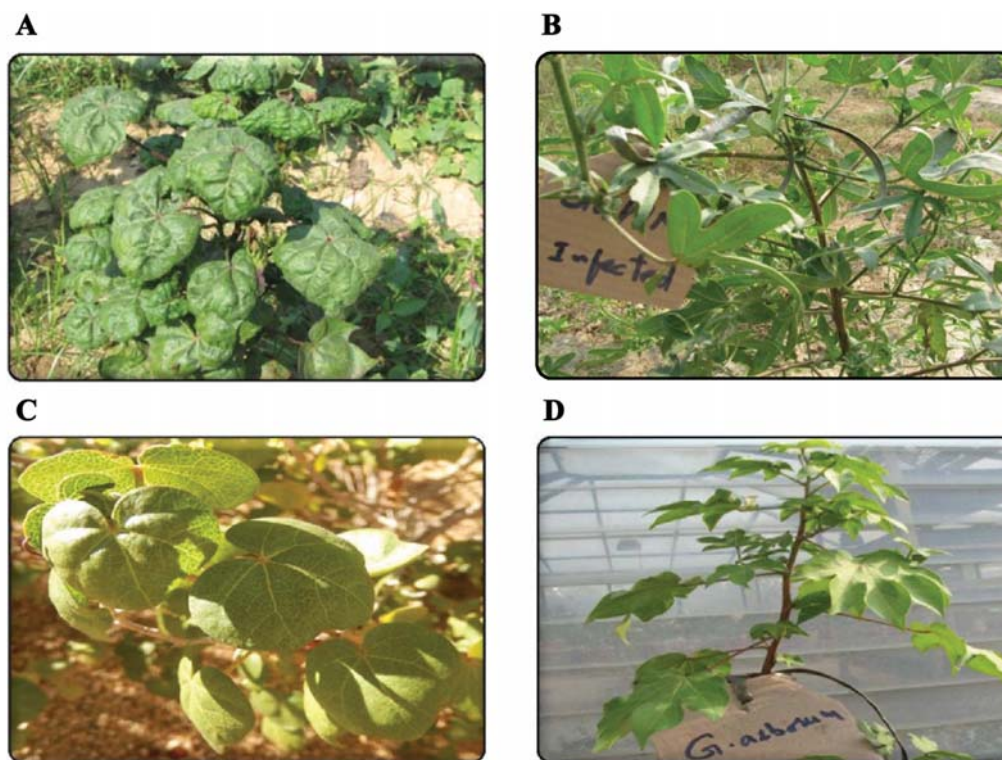

Figure 1. Symptoms on experimental plants after incubation of whiteflies A: severe symptoms were observed in *G. hirsutum* B: GaWM3 showed mild symptoms of upward curling C: mild symptoms of downward curling were also observed in *G. harknessii* D: no symptoms were observed on *G. arboretum* leaves

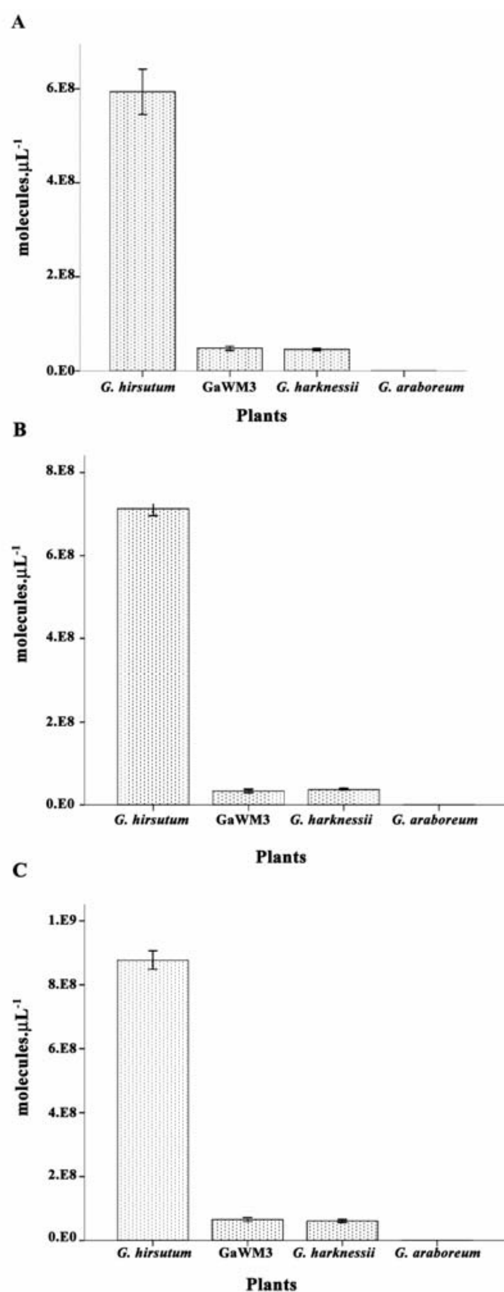

Figure 2. Real Time PCR to determine viral titer A: molecules.µL^{-1} of b-satellite was maximum in *G. hirsutum*, while it was not detectable in *G. arboreum*. B: molecules.µL^{-1} of β-satellite was maximum in *G. hirsutum*, while it was not detectable in *G. arboreum* C: molecules.µL^{-1} of DNA-A was maximum in *G. hirsutum*, while it was not detectable in *G. arboreum* whereas the error bars represent the variation among 3 replicates

arboreum (183 µg.cm^{-2}) as compared to its mutant that had 95 µg.cm^{-2}. In contrast, *G. hirsutum* and *G. harknessii* had130 µg.cm^{-2} and 146 µg.cm^{-2}, respectively.

4.3. Biochemical Composition of Epicuticular Wax

Gas chromatograph mass spectrometry of plants (*G. arboreum*, GaWM3, *G. hirsutum* and *G. harknessii* (Figure 3 A-D, respectively) was carried out to determine the biochemical composition of wax and their quantitative values. The chemical compounds were identified by comparing their retention time in the NIST mass spectra library, 2005 (13).

The top 3 compounds that were dominant in *G. arboreum*are suspected to be (1) 25.6%heptadecanoicacid, 16-methyl-, methyl ester (2) 14.1% phenol, 2,5-bis [1,1- dimethyl] and (3) 10.12% 1,2-benzenedicarboxylic acid, diisooctyl ester. The dominant compounds in wax of GaWM3 were suspepcted to be (1) 18%1,2,- benenedicarboxylic acid, diisooctyl ester (2) 14% octadecane, 1-|2-(hexadecyloxy)ethoxy|- (3) 12%7,9-Di-tet-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2,8-dione and (4) 11% nonadecane having percentage. The three major compounds found in the wax of *G. hirsutum*were (1) 25% 1,2-benzenedicarboxlic acid, diisooctylester (2) 21% nonadecane and (3)14% phenol, 2,5-bis (1,1-dimethyletyhly)- with percentage of, Lanceol, cis- and caryophyllene were the two major wax compounds found in *G. harknessii*, having the percentage of 45% and 36%, respectively. Comparison of wax biochemical composition of experimental plants is shown in (Table 2).

4.4. Whiteflies Feeding Assay

Collected whiteflies on *G. arboreum*, similar to the negative control did not show any gut coloring (Figure 4 A,B), while on the other 3 plants, gut color was observed (Figure 4 C-D).

5. Discussion

Here, a cotton wax mutant (GaWM3) next to 3 other wild type cotton species were analyzed to establish the role of wax in resistance against insects. The plant having less wax is more susceptible to insects, *G. arboreum* wax deficient mutant (GaWM3) was found susceptible to *CLCuV* (Figure 2) as opposed to the wild type (7).

The concentration of the isolated waxes were 183, 146, 130 and 95 µg.cm^{-2} in *G. arboreum*, *G. harknessii*, *G. hirsutum* and GaWM3, respectively. The concentration of the wax was in accordance with the report of Bondada *et al*. (15) *i.e*. from 70 µg.cm^{-2} to 154 µg.cm^{-2} from normal condition to stress conditions in cotton.

The results of virus symptoms appearance were in accordance with (16) and (17). The role of beta satellite

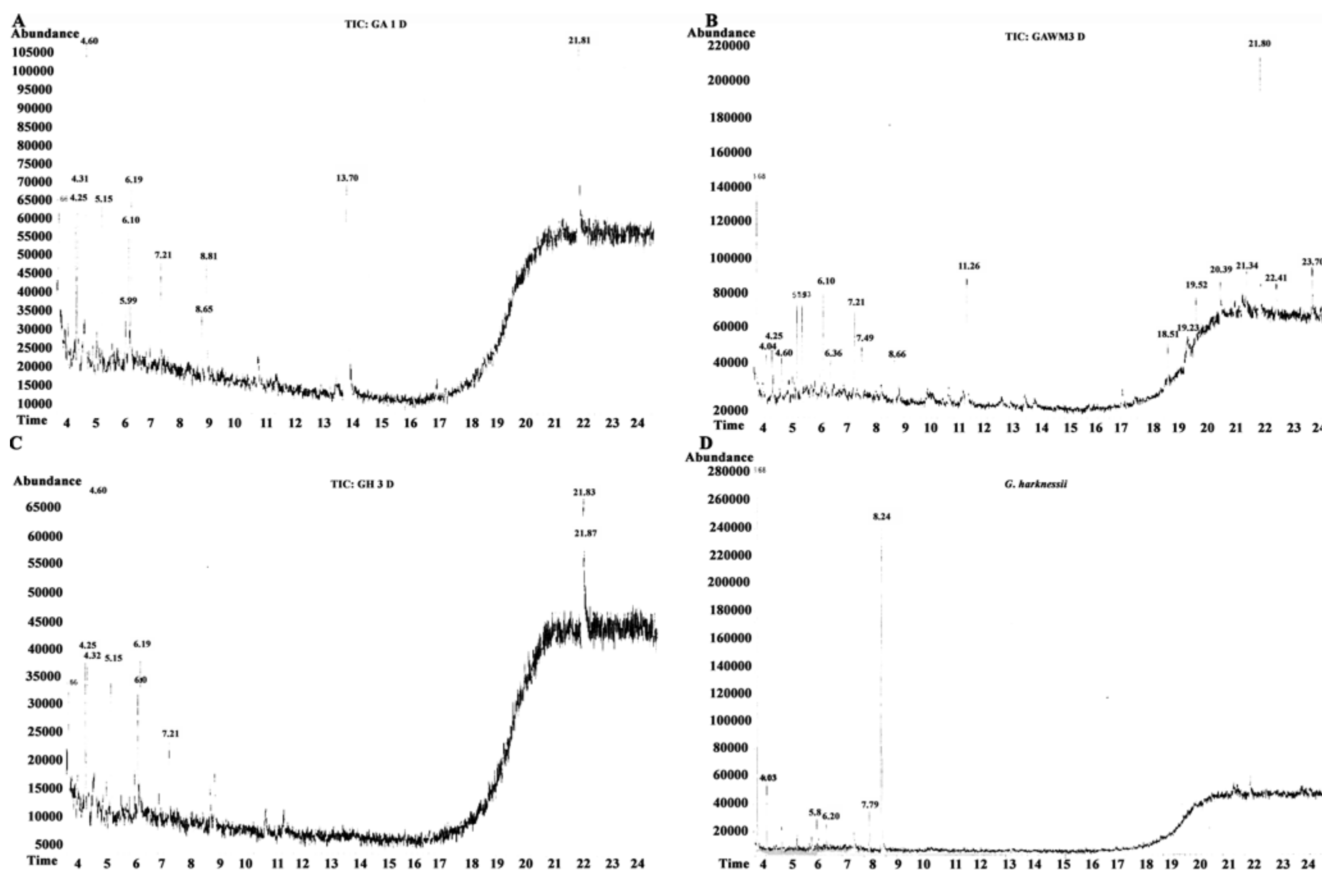

Figure 3. GC-MS TIC (total ion current trace) analysis for the compounds present in isolated wax of A: *G. arboreum*. B: GaWM3 C: *G. hirsutum* D: *G. harknessii*. Compounds were identified on the basis of the retention time through NIST library

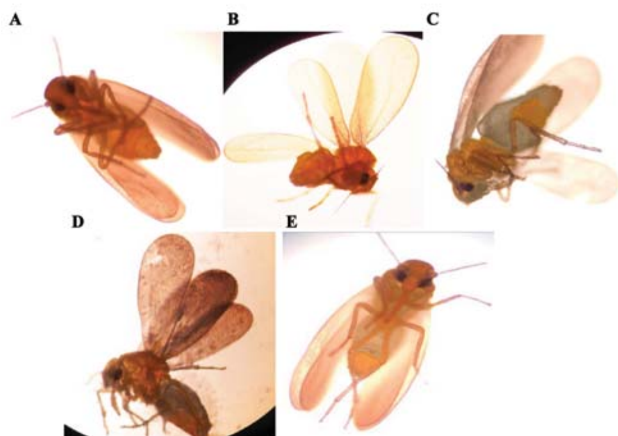

Figure 4. Whiteflies feeding assay. Whiteflies under light microscope when fed on nile blue containing experimental plants A: negative control B: *G. arboreum* C: GaWM3 D: *G. harknessii* E: *G. hirsutum*. Dye was observed in the gut of whiteflies fed on *G. hirsutum*, *G. harknessii* and GaWM3 whereas no color was observed in negative control and the whiteflies fed on *G. arboreum*

is well-defined in suppressing the phyto-immune system that ultimately results in development of severe viral symptoms (18, 19). Our data support this hypothesis that increase in quantity of beta satellite results in increase of symptoms and vice versa. The positive correlation was found in the severity of the symptoms and titer of beta satellite particles along with DNA-A (Figure 1).

The ratio of different organic compounds varies in the epicuticular wax. Hydrocarbons, alcohols and acids were the major compounds found in the wax of red vine (*Brunnichia ovata*) and trumpet creeper plants (*Campsis radicans*) (20). In addition to these classes of compounds, esters, phenols and other aromatic compounds were also found in this study. The most dominant compounds were esters in *G. arboreum*, GaWM3 and *G. hirsutum* (25.6%, 18% and 25%, respectively) and lanceol, cis (45%) was dominant in *G. harknessii*.

The comparison of wax components of GaWM3 and *G. arboreum* clearly demonstrated that the follow-

Table 2. Comparison of chemical compounds present in experimental plants

Sr. No	Biochemical Compounds	*G. arboreum*	GaWM3	*G. hirsutum*	*G. harknessii*
1	2-cyclopentene-1-ol, 1-phenyl-	+	-	+	-
2	Nonadecane	+	+	+	-
3	3-trifluoroacetoxytetradecane	+	-	-	-
4	Phenol, 2,5-bis [1,1-dimethyl]	+	+	+	-
5	Methoxyacetic acid, 2-tridecylester	+	+	-	-
6	2-piperidinone, n-[4-bromo-n-butyl]	+	-	-	-
7	4-heptafluorobutyroxypentadecane	+	-	-	-
8	Tetradecane, 2,6,10-trimethyl-	+	+	+	-
9	Silane, trichlorodocosyl-	+	-	-	-
10	6-Octadecenoic acid, methyl ester	+	-	-	-
11	Heptadecanoic acid, 16-methyl-, methyl ester	+	-	-	-
12	1,2-Benzenedicarboxylic acid, diisooctyl ester	+	+	+	-
13	Caryophyllene	-	+	-	+
14	α-Caryophyllene	-	+	-	+
15	Hexadecane	-	+	-	-
16	Eicosane, 2-methyl-	-	+	-	-
17	Diethyl phthalate	-	+	-	-
18	7,9-Di-tet-butyl-1-oxaspiro[4,5] deca -6, 9-diene-2,8-dione	-	+	-	-
19	Ethanol, 2-[octadecyloxy]	-	+	-	-
20	A-D-Glucopyranoside, methyl-2-[acetylamino]-2-deoxy-3-O-[trimethylsillyl]-,cyclic methyl bronate	-	+	-	-
21	Octadecane, 1-|2-[hexadecyloxy]ethoxy|-	-	+	-	-
22	15,17,19,21- Hexatriacontatetrayne	-	+	-	-
23	|5-[3-methoxymethoxy-10,13-dimethyl-2,3,4,9,10,11,13,14,15,16,17-dodecahydro-1Hcyclopenta|a|[phenanthren-17-yl]-hex-1-ynul|-trime	-	+	-	-
24	2-Trifluoroacetoxyteradecane	-	-	+	-
25	Trichloroacetic acid, hexadecylester	-	-	+	-
26	P-Xylenolpthalein	-	-	+	-
27	Lanceol, cis-	-	-	-	+
28	Napthalene,1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-4methylene-1-[1-methylethyl],[1α,4aβ,8aα]-	-	-	-	+
29	2,6,10-dodecatriene-1-ol,3,7,11-tromethyl-acetate,[E,E]- 2,6,10-dodecatriene-1-ol,3,7,11-tromethyl-acetate,[E,E]-	-	-	-	+
30	2-napthalenemethanol,decahydro-α, α, 4a-trimethyl-8-methylene-,|2R-[2$^{\alpha}$,4aα,8aβ]|-	-	-	-	+

+: present
-: absent

ing six organic compounds were only present *in G. arboreum*: 3-trifluoro acetoxy tetra decane, 2-piperidinone, n-[4-bromo-n-butyl], 4-heptafluorobutyroxypentadecane, silane, trichlorodocosyl-, 6-octadecenoic acid, methyl ester, and heptadecanoic acid, 16-methyl-, methyl ester,may create unique features in its wax and may be involved in its resistance against transmission of *CLCuV* (Table 2). The whitefly feeding assay also suggested that the quantity as well as quality of the wax has its role in feeding of whiteflies (Figure 4).

6. Conclusions

The characterization of cotton epicuticular wax and its role in transmition of CLCuV by whiteflies to plants were demonstrated. It was found that 50% reduction in wax (in leaves of GaWM3) made it possible for the whiteflies to transmit the virus and to develop the relevant symptoms. It is concluded that wax act like barrier in hindering the *CLCuV* transmission in cotton. Moreover, quantities as well as chemical composition of wax had impacts on feeding behavior in whiteflies and transmission of *CLCuV*.

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