

Full Length Research Paper

Role of epicuticular waxes in the susceptibility of cotton leaf curl virus (CLCuV)

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Cotton leaf curl virus (CLCuV) is the causal agent of the damaging disease of cotton that is caused by number of begomaviruses and vectored by silver leaf whitefly. In the present study, an attempt was made by infecting *Gossypium arboreum* variety 786, its wax mutant GaWM3 along with *Gossypium hirsutum* MNH-93 with viruliferous whiteflies. The presence of symptoms on leaves and amplification by PCR for virus in *G. hirsutum* MNH-93 and wax mutant GaWM3 but not in *G. arboreum* variety 786 clearly determined the presence of virus in *G. hirsutum* MNH-93 and wax mutant GaWM3 but not in *G. arboreum* variety 786. The results indicate that wax may act as physical barrier and provide hindrance in transfer of virus by whitefly.

Key words: Wax mutant, cotton, cotton leaf curl virus (CLCuV), whitefly, rolling circle amplification (RCA).

INTRODUCTION

Cotton is the most significant fiber crop that shares 60% of the total fiber of the world (Chachral et al., 2008). Cotton plays a critical role in Pakistan's economy. Although, it is a non food crop, it earns significant foreign exchange. It contributes 8.6% of the value added in the agriculture and 1.8% to the GDP of the country. The genus *Gossypium* has almost 50 different species from which four are in agricultural use including *G. hirsutum* L., *G. barbadense* L., *G. arboreum* L., and *G. herbaceum* L. The *G. barbadense* and *G. hirsutum* are tetraploid, while the rest two are diploid (Sakhanokho et al., 2004). *G. hirsutum* is the most widely grown and contribute to 80% of the total cotton production in the Asia.

Plant pathogenic viruses are the major hindrance in increasing yield and productivity of plants in the warmer parts of the world. Most of the viral diseases belong to the viral family *Geminiviridae* and are transmitted by the members of the phylum Arthropoda (Ilyas et al., 2010). *G. hirsutum* is very susceptible to the Geminiviruses - cotton mosaic virus (CotMV), cotton leaf crumple virus (CLCrV)

and cotton leaf curl virus (CLCuV) in natural conditions (Sharma et al., 2004). Between 1992 to 1993 the CLCuV has become a serious threat to cotton and hence a threat to economy of Pakistan. It was the first severe alarming epidemic of CLCuV (Hameed et al., 1994). At plant level, the first and foremost physical barrier in plant pathogen interaction is epicuticular wax (Carver and Gurr, 2006). Epicuticular wax not only hinders the bacteria and fungi but also create a first line of defense against insects (Eigenbrode and Espelie, 1995). Wax may change the wanderer for feed for insects example, in wax deficient pea mutants the aphid spent more time on the pea plants (Chang et al., 2004). Asiatic *G. arboreum* is resistant to CLCuV and it has two fold higher waxes compared to *G. hirsutum* (Zafar et al., 2003).

In present study, a wax deficient mutant GaMW3 from *G. arboreum* were utilized which have 50% less wax than Asiatic *G. arboreum* (Khan, 2010). The idea behind this study was to test these mutant plants for their susceptibility against virus. Rolling circle amplification (RCA) method has been used to detect the CLCuV in cotton plants. This method has the advantage that the prior knowledge of DNA sequence is not required for making the primer instead random hexamer primer are used (Liu et al., 1996). The RCA has authentic sequence

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specificity and allow unambiguous identification of the DNA.

MATERIALS AND METHODS

Sowing of seeds

The wax mutant seeds of GaWM3 were obtained from “Plant Genomic Laboratory” of “Center of Excellence in Molecular Biology (CEMB)” developed by Khan et al. (2010). The mutants have 50% deficiency in epicuticular wax as compared to their ancestors. In this experiment, GaWM3 plants of cotton were chosen as a test plant. MNH-93 was selected as CLCuV positive control, while *G. arboreum* variety 786, was selected as negative control because it has 50% more wax as compared to test plants and is resistant to CLCuV. Five seeds of each of varieties GaWM3, MNH-93 *G. arboreum* variety 786 (parent variety) were sown in each pot. Each set of plants was sown in 3 replications. The germinated plants were thinned and only one plant was kept in each pot on the approach of six leaf stage. The pots were placed in green house at $\pm 30^{\circ}\text{C}$ and at 80% humid condition of green house. Each pot was covered with net cage which has the size $2 \times 2 \times 4$ feet and the mesh size of the net cloth was 32 through which whitefly cannot pass as shown in Figure 1.

Collection of whiteflies and infection on experimental plants

For making the plants infected with CLCuV it was necessary to collect the infected whiteflies. The whiteflies were collected from the cotton field of CEMB. Whiteflies were caught with the help of manmade whitefly respirator. The whiteflies that were caught by respirator were then released to already infected *G. hirsutum* MNH-93 in net cages for 48 h. This step was taken to get the viruliferous whiteflies that contained CLCuV. After 48 h, the viruliferous whiteflies were re-caught from the net cages of *G. hirsutum* MNH-93 plants and were kept at 4°C for 4 h to make them starve. The starved whiteflies were then allowed to feed and infect by releasing them in the cages of GaWM3, MNH-93 and 786 plants.

Rolling circle amplification (RCA)

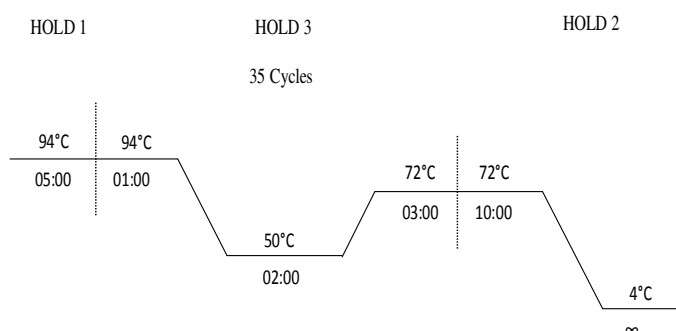
DNA was extracted from whiteflies after three days of incubation according to Lifton buffer method and from plants by CTAB method (Doyle and Doyle, 1990). In rolling circle amplification $4 \mu\text{L}$ of template DNA of whiteflies, *G. arboreum* variety 786, *G. hirsutum* variety MNH-93 and GaWM3 having concentration $50 \text{ ng}/\mu\text{L}$ and 10 mM dNTPs were used. $2 \mu\text{L}$ of hexamer primer (Fermentas Cat # SO142) having concentration $0.2 \mu\text{g}/\mu\text{L}$ were used along with $10 \times \phi 29$ DNA polymerase enzyme (Fermentas Cat # EP 0091) DNA polymerase buffer in a concentration of $2 \times$. The total reaction mixer was planned for $20 \mu\text{L}$, so $8 \mu\text{L}$ water was used for the total volume was $18 \mu\text{L}$. The reaction mixer was centrifuged and it was placed at 94°C for 3 min. After 3 min mixer was gradually cooled to 30°C and the $1 \mu\text{L}$ of pyrophosphatase (Fermentas Cat # EF 0221) having concentration $0.02 \text{ U}/\mu\text{L}$ and $1 \mu\text{L}$ of $\phi 29$ DNA polymerase enzyme was added. The reaction mixer was centrifuged again and then it was placed at 30°C for 16 to 18 h. After 18 h the PCR tubes were placed at 65°C for 10 min and gradually cooled. $2 \mu\text{L}$ of the RCA products was used for agarose gel electrophoresis.

Polymerase chain reaction (PCR) amplification

RCA product was used as a template for amplification of the

CLCuV.

The DNA templates of plants which were RCA positive were diluted in a ratio of 2:28 (RCA product and water) and the DNA templates of plants which gave negative RCA results were not diluted and directly used as template in PCR reaction. In the PCR amplification, $2.5 \mu\text{L}$ of template was used, $2.5 \mu\text{L}$ of $10 \times$ PCR buffer (Fermentas Cat # B34), $2.5 \mu\text{L}$ of 2 mM dNTPs, $1.5 \mu\text{L}$ of MgCl_2 (Fermentas Cat # R0971), $0.5 \mu\text{L}$ of 10 pmol forward primer (5'ACGCGTGCCGTGCTGCTGCCCCATTGTCC3'), $0.5 \mu\text{L}$ of 10 pmol reverse primer (5'ACGCGTATGGGCTGYCGAAGTT SAGAC3') and $0.25 \mu\text{L}$ of 5 U Taq polymerase enzyme (Fermentas Cat # EP0071) were used. $14.75 \mu\text{L}$ deionized distilled water was used to make the final volume of reaction mixture $25 \mu\text{L}$. The following thermocycler condition was used.



The PCR amplification was checked using agarose gel electrophoresis. 0.8% of agarose gel was prepared in $1 \times$ TAE buffer and ethidium bromide with a concentration of $0.5\text{--}1 \mu\text{g}/\text{ml}$ was added. $5 \mu\text{L}$ of PCR product along with $2 \mu\text{L}$ of $6 \times$ loading dye was loaded on gel and run at 80 V for 1 hour.

Restriction analysis of RCA product

The RCA product from cotton plants template were further used to analyze the component it contained. For this purpose, the $5 \mu\text{L}$ of RCA product was used to digest with $0.5 \mu\text{L}$ of Eco R1 (Fermentas Cat # ER0271) enzyme. In this reaction, $1 \mu\text{L}$ of Eco R1 enzyme buffer was also added and finally $3.5 \mu\text{L}$ water was added to make the final volume $10 \mu\text{L}$ of the reaction. The result of the digestion was examined by agarose gel electrophoresis and visualized in the Gel documentation system.

RESULTS

Symptoms on the plants

The most severe attack of virus was observed on *G. hirsutum* MNH-93 plants that were used as positive control in this experiment. The growth was stunted and typical symptoms of CLCuV appeared on the leaves. The leaves were curled upward and bear leaf like enation. On underside of the leaf the veins appear very thick. The wax mutant GaWM3 plants showed almost similar behavior as *G. hirsutum* MNH-93 plants but the symptoms are slightly different than those of *G. hirsutum* MNH-93 as the texture and shape of the leaf is different. Most strikingly, the upward curling of leaves and vein thickening was also observed in these plants. On the

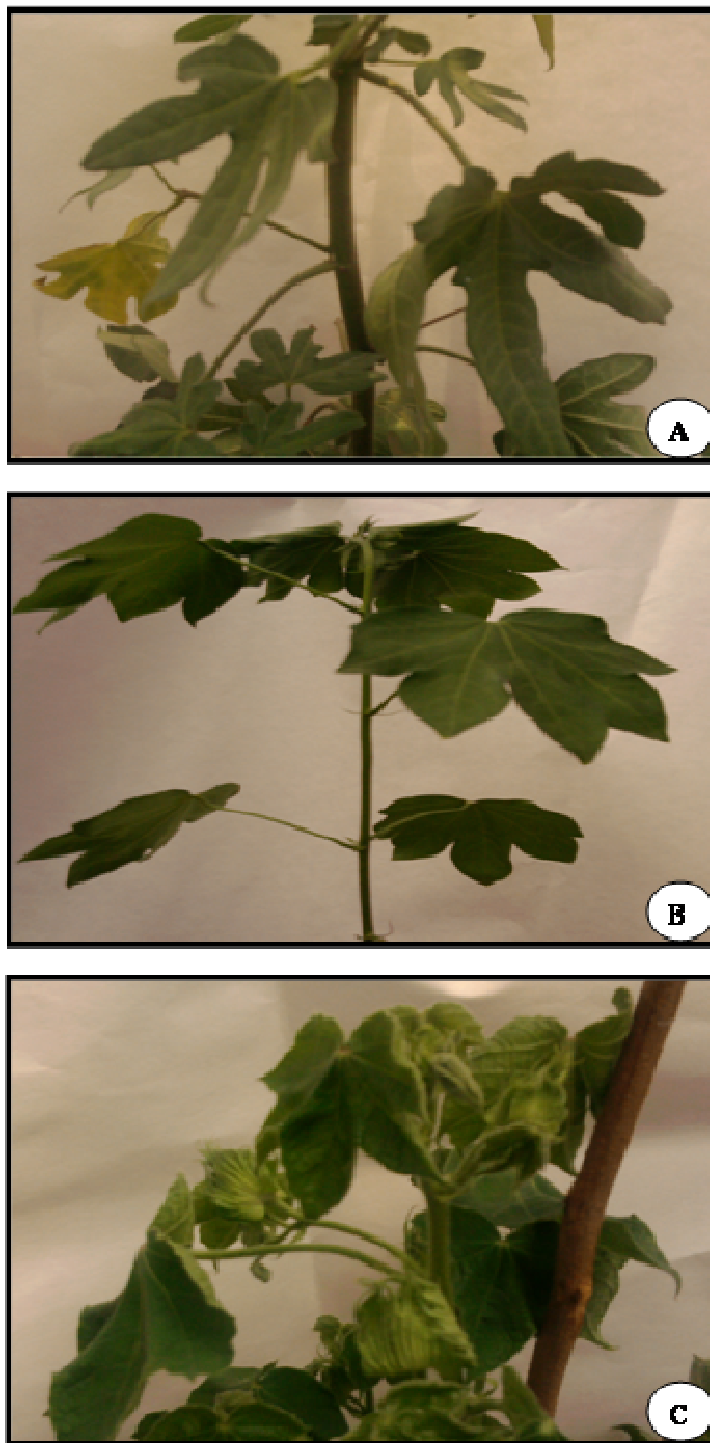


Figure 1. Plants after incubation of whiteflies (A) Wax mutant GaWM3 showing symptoms of upward curling (B) *Gossypium arboreum* - 786 have no symptoms on it while (C) *Gossypium hirsutum* - MNH 93 showing severe leaf curling, enation and thickening of leaf veins.

other hand, the *G. arboreum* variety 786 plants which were used as negative control showed the normal growth and remained asymptomatic. The comparison among the

leaves of wax mutant GaWM3, *G. arboreum* variety 786 and *G. hirsutum* MNH-93 is shown in Figures 1 and 2; the leaves of *G. hirsutum* MNH-93 and wax mutant GaWM3

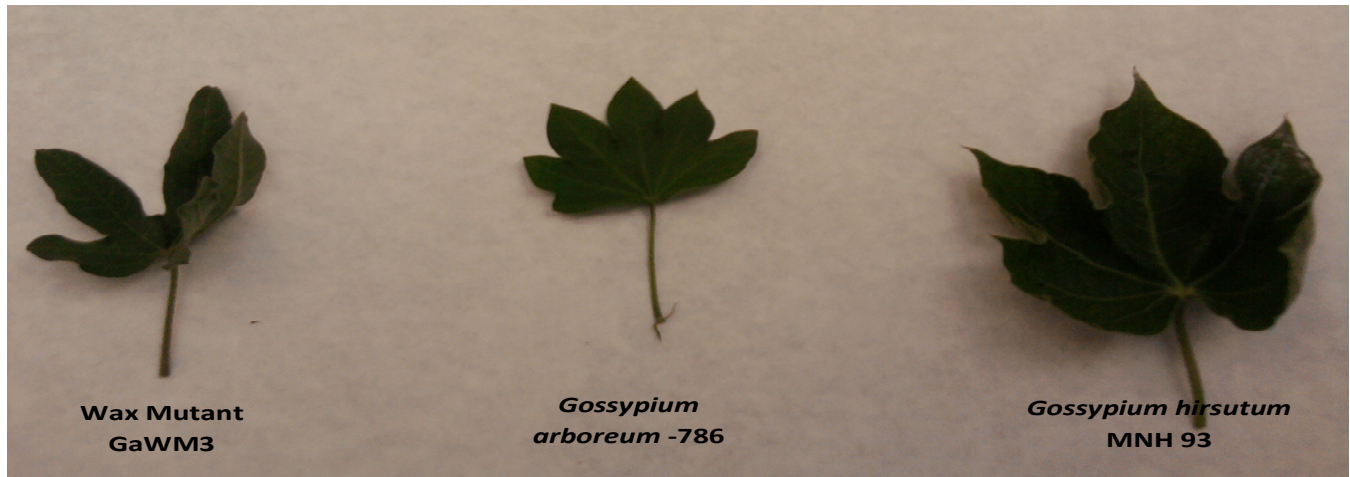


Figure 2. Comparison of leaf symptoms of all three types of experimental plants. Wax mutant GaWM3 and *Gossypium hirsutum* MNH 93 shows upward curling of leaf and thickening of leaf veins, while these symptoms are absent in *Gossypium arboreum* -786.

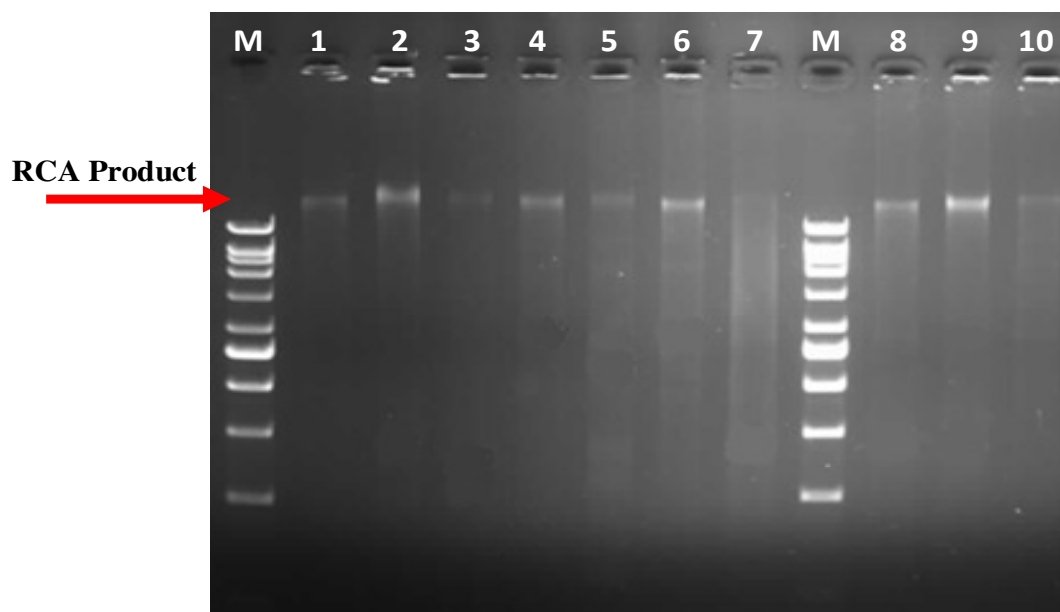


Figure 3. RCA product from whiteflies DNA that incubated on different plants. M is 1Kb DNA Ladder. Lane 1 showing positive control, Lane 2, 3 & 4 showing Wax mutant GaWM3. Lane 5, 6 & 7 showing *Gossypium hirsutum* MNH-93 while lane 8,9,10 showing *Gossypium arboreum* -786 plants.

plant showed upward curling which is absent in the leaf of *G. arboreum* variety 786 plant.

RCA from viral DNA

All replicates of the wax mutant GaWM3 and *G. hirsutum* MNH-93 gave positive results for RCA product. The positive control plants of *G. hirsutum* MNH-93 gave

positive RCA product but no RCA product was found in negative control *G. arboreum* variety 786 plants (Figures 3 and 4).

PCR amplification of RCA product

The amplification of virus genome was done by using viral genome specific primers. The 2.7 kb fragment was

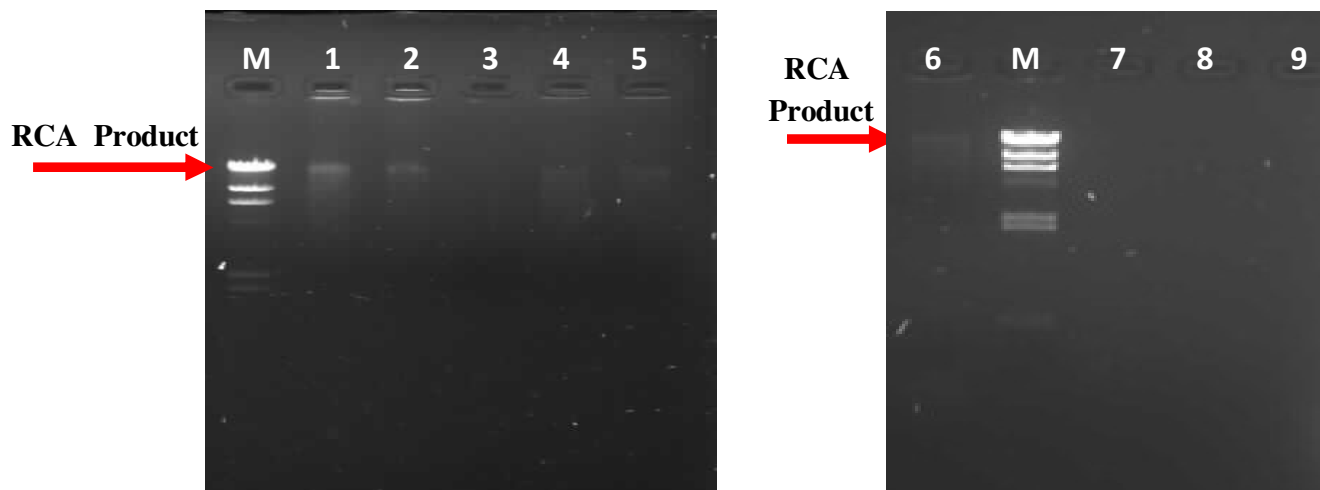


Figure 4. RCA product from different cotton plants. M is Lambda Hindi III DNA Ladder. Lane 1, 2, 3 showing Wax mutant GaWM3. Lane 4, 5, 6, showing *Gossypium hirsutum* MNH-93 while lane 7, 8, 9 showing *Gossypium arboreum* -786 plants.

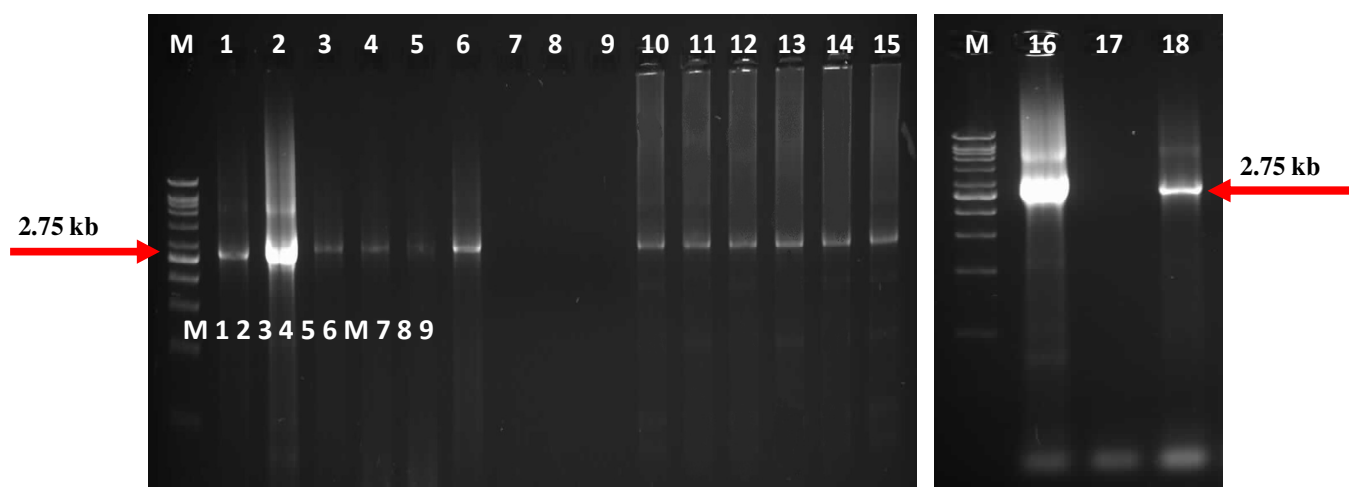


Figure 5. Full length virus amplification from whiteflies DNA incubated on different plant samples. M is 1Kb DNA Ladder. Lanes 1 to 3 shows wax mutant GaWM3 cotton plants; lanes 4, 5 and 6 shows *Gossypium hirsutum* MNH-93, while lanes 7, 8 and 9 shows *Gossypium arboreum* -786 plants. Lanes 10, 11 and 12 shows whiteflies on Wax mutant GaWM3; Lanes 13,14 and 15 shows whiteflies on *Gossypium arboreum* -786 plants, while lane 16,17 and 18 shows whiteflies on *Gossypium hirsutum* MNH-93.

amplified from RCA product of whiteflies, *G. hirsutum* MNH-93 plants and wax mutant GaWM3 plants but no amplification was observed in *G. arboreum* variety 786 as shown in Figure 5.

Digestion of RCA product with ECO R1 enzyme

The RCA products of wax mutant GaWM3 plants and *G. hirsutum* MNH-93 plants were digested with help of Eco R1 enzyme. The digested product was visualized on gel (Figure 6) along with 1 kb DNA ladder “M”. The Figures show the undigested RCA product at top and 2.75 kb full

length viral DNA beneath it in all samples. In sample 1, 2 and 4 there is a band of 1.35 kb β DNA of satellite virus.

DISCUSSION

Insects are the main vectors that transmit the virus in plants (Rubinstein and Czosnek, 1997). The whitefly transmits begomoviruses to many plants including cotton (Nateshan et al., 1996). In cotton, whitefly transmit several viruses including cotton leaf curl virus, cotton leaf crumple virus and cotton mosaic virus (Sharma et al., 2004). The experimental plants, the wax mutant “GaWM3”,

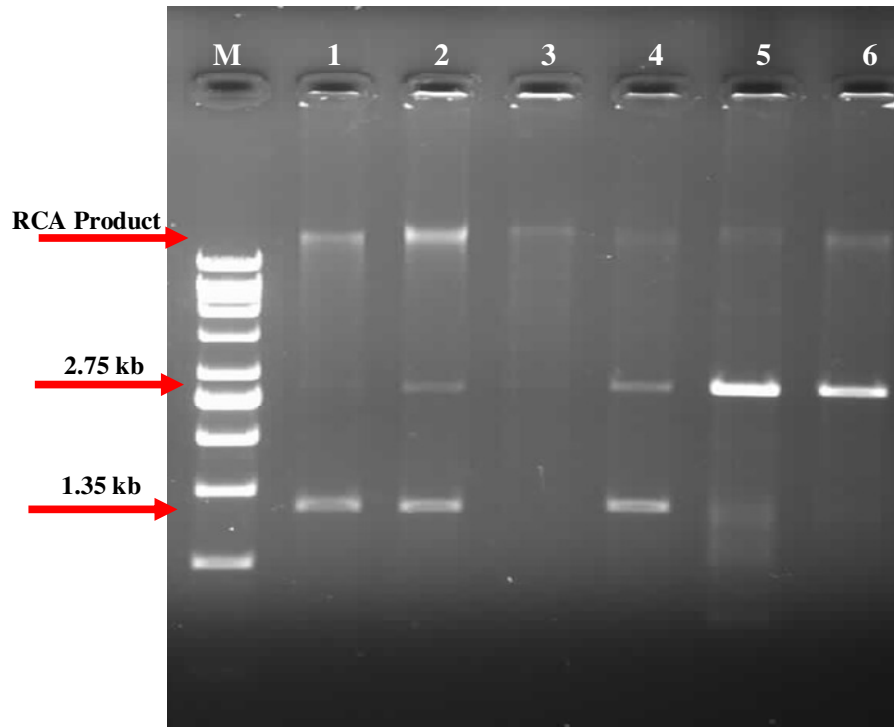


Figure 6. Digestion of RCA product with Eco R1 enzyme. M is the 1 kb ladder. Lanes 1, 2 and 3 shows wax mutant GaWM3, while lanes 4, 5 and 6 shows *Gossypium hirsutum* MNH-93.

G. arboreum and *G. hirsutum*, were incubated with viruliferous whiteflies for 48 h. For making whiteflies viruliferous, they were allowed to acquire virus for 48 h from infected plants of *G. hirsutum* as this is the acquisition time reported by Brown and Nelson (1988). The whiteflies were then incubated with experimental plants as they remain viruliferous for 3 days (Cohen and Berlinger, 1986) for CLCuV transmission.

When the wax mutants GaWM3 were incubated with viruliferous whiteflies, they produced the symptoms of cotton leaf curl disease (CLCuD) having upward curling of leaves and thick enations. These symptoms resembles to the symptoms of CLCuD as reported by Briddon and Markham (2000) on *G. hirsutum* plants. It may be due to deficiency of wax which makes them susceptible against attachment of whiteflies for transmission of virus as suggested by Eigenbrode (2004) that wax crystals contaminate the insects pad surface, thus creating hindrance in contact of insect with plants. In some plants, the major component of wax is triterpenoids which give the wax to its slippery characteristics (Bass and Fidgor, 1978) and cause difficulty for insect to get attached with plants. However, in most of the plants the wax makes the surface rougher and reduces the potential surface area required for adhesion between the insects' pads and plants. Neglecting all other factors if only there is rough surface, there is decrease in attachment of chrysomelid

beetle (*Gastrophysa viridula* Degeer). So, presence or absence of wax plays an important role in insect behavior to plants (Eigenbrode et al., 1999).

The presence of virus as confirmed by amplification of virus DNA both in whiteflies, *G. hirsutum* MNH-93 and mutant confirm the assumption by Eigenbrode and Espelie (1995) who reported that decrease in epicuticular wax increased the insect attack on the plants as decrease in wax percentage ease the insects to feed upon them. For example *Phyllotreta* sp. attacks on the *Brassica oleracea* increased by decreasing the epicuticular wax (Stoner, 1990). Bodnaryk (1992) studied the efficacy of *Phyllotreta* sp. to attack on the same species *Brassica napus* and found that the decrease in wax increase the insect infestation on it as it was in *Brassica oleracea*. Similar results were found when Eigenbrode et al. (2000) found that *Phyllotreta* sp. attack more on *Brassica napus* when there is decrease in epicuticular wax.

Eigenbrode and Kabalo (1999) produced four mutant plant of *B. oleracea* having different percentage of wax and studied the effect of wax of attachment and predation of *Hippodamia convergens*. They found that the *H. convergens* spend more time on mutant plant and less on the normal plants. Epicuticular wax appears to interfere with parasitoid foraging behavior. Same results were reported by Rutledge et al. (2003) in field that the

parasitism of the pea aphid by *Aphidius ervi* is higher on plants having reduced epicuticular wax and by White (1998) in laboratory experiment (Chang et al., 2004).

Conclusion

Virus diseases of cotton have historically been of only sporadic importance to global cotton production. Under changing conditions this pathogen (cotton leaf curl virus) has emerged as a serious problem in Pakistan and India. In the present study, it was found that 50% reduction in wax (in the leaves of GaWM3 plants) made possible for the whiteflies to transmit CLCuV and they produced symptoms of CLCuD. Taking into account that whitefly cannot transmit CLCuV in *G. arboreum* (Zafar et al., 2003), it was concluded that wax act like barrier in hindering the CLCuV transmission in Cotton.

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