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Comparison of different species of cotton for the transmission of *CLCuV* through whitefly

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Abstract

Most plants have developed some physiological properties that either increase the grip of the insect or inhibit them. In this study, vegetative and floral characteristics of plants, thickness of leaf cross section, number of the mesophyll layer and epicuticular wax of different cotton species namely *Gossypium arboreum*, its wax mutant GaWM3, *G. hirsutum* and *G. harknessii* were characterized, and correlated with detection of *CLCuV* thorough PCR. Viral particles were detected in all experimental plants other than *G. arboreum*. Thickness of cross section of the leaves in *G. arboreum*, its wax mutant 3 (GaWM3) and *G. hirsutum* was ~200-237 µm as compared to *G. harknessii* (~350 µm) as it had 2 layers of mesophyll cells. The thickness of the wax layer was found to be maximum in *G. arboreum* (180 µg cm^{-2}) and least in its wax mutant (95 µg cm^{-2}). From the results it was concluded that the thickness of the leaves and presence of single and double layers of the mesophyll had no role in the transmission of *CLCuV* through whiteflies (*Bemisia tabaci* Gennadius). Moreover, least number of whiteflies were found on the leaves of *G. arboreum* which was resistant to *CLCuV* determined the fact that more epicuticular waxes hindered the attachment and infestation of whiteflies in *G. arboreum* as compared to susceptible plants, i.e., its wax deficient mutant, *G. hirsutum* and *G. harknessii.*

Keywords: *Gossypium*, insect interaction, *leaf curl virus*, leaf thickness, mesophyll layer, mutant.

Introduction

Non-motile nature of plants makes them susceptible to various kinds of stress and diseases. Major losses in yield take place due to biotic and abiotic stresses. Abiotic stresses include temperature, radiations, water, minerals and salt stress while arthropods, nematodes, fungus, bacteria and viruses are some of the major biotic stresses. Among these, plant pathogenic viruses cause considerable losses in yield of crops. The most notorious part is being played by the member of the family *Geminiviridae* which are transmitted by the arthropods (Aftab *et al.*, 2014). Various pathogens attack cotton plant and cause diseases i.e., root rot disease caused by abiotic factor (over-watering) and biotic factor (*Thielviopsis basicola*). Cotton leaf curl disease caused by single stranded DNA (ssDNA) viruses, *Geminiviruses*, is also a major threat to subcontinent cotton (Mansoor *et al.*, 2003). *G. arboreum* show high resistance to root rot disease (Wheeler *et al.*, 1999) and to the *Geminiviridae* viruses (Mansoor *et al.*, 2003). Among 75% naturally occurring diseases of cotton plants are due to pathogens.

In subtropical and tropical region, *G. hirsutum* is vulnerable to *Geminiviruses*, for example, *cotton leaf curl virus* (*CLCuV*), *cotton leaf crumple virus* (*CLCrV*) and c*otton mosaic virus* (*CotMV*) (Sharma *et al.*, 2004). Since 1992, the *CLCuV* has become a serious threat to cotton as well as Pakistan's economy. During these years, the disease was reported to spread over 97,580 hectares which resulted in the loss of 543,394 bales in Punjab. It was the first severe alarming epidemic of *CLCuV* (Mansoor *et al.*, 2003). The *Begomoviruses* are transmitted by the whitefly *Bemisia tabaci* in a persistent circulative manner. (Brown and Czosnek, 2002). Since last thirty years, the whiteflies emerged to be the greatest threat to dicotyledonous plants all across the world (Ribeiro *et al.*, 2003). Proliferation of the diseases caused by Begomoviruses is directly proportional to the increased population of whiteflies. The whiteflies which have developed association with *Geminiviruses* or more specifically *Begomoviruses* have emerged as an important limiting factor for the cultivation of vegetable and fiber crops (Morales and Anderson, 2001). A basic requirement in plant – insect interaction is the attachment of insect with plant surface. If insect are unable to attach themselves, no plant – insect interaction would be possible. Most plants have developed some physiological properties that either increase the grip of the insect or inhibit them (Koch and Barthlott, 2009).

Plant leaf surface are the sites of several important physiological functions in plants. They

also serve as the first layer of defense against insects (Eigenbrode and Espelie, 1995). Epicuticular layer having trichomes-hair and waxes is a pre-existing defense to prevent the infestation of insects. Some of the insects recognize the host surface by perceiving plant surface waxes (Shah, 2005). Physical and chemical composition of wax also gives clues and cues for insect attachment which can determine plant-insect interaction and insect behavior (Müller and Riederer, 2005). The genus *Gossypium* is very diverse not only in the structure, shapes, leaves and flowers but also in the susceptibility of certain insect pests. The *G. arboreum* is resistant to *CLCuV* whereas its wax deficient mutant became susceptible to this virus (Khan *et al.*, 2011). In this study, American *G. hirsutum*, Hawaiian species *G. harknessii*, Asiatic *G. arboreum* and its wax mutant GaWM3 were included. It was hypothesized that the change in leaf morphology may affect the appearance of the symptoms. The study specifically highlights the morphological features of plants, *CLCuV* symptoms and its detection.

Material and Methods

Selection of plant materials

Three cotton species were selected on the basis of susceptibility to *CLCuV* and the amount of the epicuticular wax. In this study, the Asiatic *Gossypium arboreum* along with its wax deficient mutant named as GaWM3 which was developed at Center of Excellence in Molecular Biology (CEMB), University of the Punjab (Khan *et al.*, 2011), upland cotton *G. hirsutum* and cotton from the Hawaiian origin *G. harknessii* were selected. The seeds of these experimental plants were sown in pots as well as in the field of CEMB.

Anatomical studies of plants

In vegetative characters, the colour of the branches, presence or absence of stipules and petioles and the shape of the leaves were recorded. Whereas in the reproductive characters, the characters of pedicels, epicalyx, calyx, corolla and staminal tube were observed.

Slides of the cross section of the leaves of all four groups of cotton plants namely *G. arboreum*, GaWM3, *G. hirsutum* and *G. harknessii* were also taken under consideration to evaluate the role of mesophyll layers of the plant cell in feeding of whiteflies (Jiang *et al.*, 1998). Thin cross sections of the leaves were cut and placed on the slide and observed under the microscope (Zeiss, Imager A1) at 10x eyepiece with 40x objective. The thickness of the mesophyll layer was measured and co-related with quantity of *CLCuV*, quantity of wax and whiteflies number in all experimental plants.

Rolling circular amplification (RCA)

Plant genomic DNA was extracted by the CTAB method and used as a template for rolling circular amplification (RCA) (Khan *et al.*, 2011). The genomic DNA was quantified and normalized to 50 ng μL^{-1}. For the RCA "illustra templiphi" amplification kit (Cat # 25-6400-10) was used. According to the kit manual, the 15 µL of the sample buffer was transferred to 0.2 mL reaction tube and 3 µL of normalized DNA (50 ng μL^{-1}) was dispensed as a template. The tubes were short spun and placed in PCR machine at 95 ^{o}C for 3 min and cooled down to 4 ^{o}C. After cooling, 15 µL of the reaction buffer was added to each tube with 0.6 µL enzyme and tubes were then incubated at 30 ^{o}C for 18 hours. To inactivate the enzyme, tubes were incubated at 65 ^{o}C for 10 min. The integrity of the RCA product was confirmed by running on 0.8% agarose gel that was prepared and run in 1X TAE buffer at 80 V for 60 minutes and visualized in Gel Documentation System (UVP trans-illuminator) using program GrabIT.

Detection of *CLCuV* through PCR

PCR primers were optimized for the reaction. Primers were designed from the CP for helper, Rep for alphasatellite and C1 primer set for betasatellite. The reaction mixture contains 2 µL of RCA product template with a concentration of 10 ng μL^{-1}. 2.5 µL of 10X PCR buffer (Fermentas cat# B34), 2.5 µL of 2 mM dNTPs, 1.5 µL of $MgCl_2$ (Fermentas cat# R0971) 1 µL of each forward primer (CP-R, C1-R and Rep-R) and reverse primer (CP-L, C1-L and Rep-L) having the concentration of 10 pmol μL^{-1} and 0.5 µL of 5u Taq polymerase enzyme (Fermentas cat# EP0071) were used. The final volume of the reaction was adjusted to 25 µL by adding 14 µL water. The PCR reaction was performed as initial denaturation at 95 ^{o}C for 5 minutes, in each cycle denaturation at 95 ^{o}C for 30 sec, annealing at 59 ^{o}C for 30 sec, extension at 72 ^{o}C for 30 sec with the total of 35 cycles and final extension at 72 ^{o}C for 10 minutes. The PCR products were resolved in agarose gel having the concentration of 1.5% in 1X TAE buffer.

Results and Discussion

Vegetative characteristics of the experimental plants

The three species of *Gossypium* namely *G. arboreum, G hirsutum* and *G. harknessii* along with GaWM3 were distinguished on the bases of most important characteristic i.e., leaf morphology, which were palmately 5 lobbed in *G. arboeum* and GaWM3 whereas it is palmately 3 lobbed in *G. hirsutum* and cordate in *G. harknessii*. The leaves in *G. arboreum* and GaWM3 were almost similar but the margin of the leaves was entire in *G. arboreum* whereas it was undulate in GaWM3. Comparison of the other vegetative characteristics is shown in Table 1. From the shapes of the leaves and other vegetative characteristics, it is very clear that the *G. arboreum* and GaWM3 as are very close to each other as GaWM3 is the wax deficient mutant of *G. arboreum* but not of *G. hirsutum* and their leaves are very much different from *G. hirsutum* and *G. harknessii* (Fig. 1).

Floral characteristics of the experimental plants

The reproductive characters, the characters of pedicels, epicalyx, calyx, corolla and staminal tube were observed in all the experimental plants. Two important distinguishing characters, viz., colours of petals and colour of stamens in *G. arboreum*, GaWM3, *G. hirsutum and G. harknessii* were taken into account. The petals in *G. arboreum* and GaWM3 was creamy white to off white with a purplish center, whereas in *G. hirsutum* and *G. harknessii*, petals were yellowish and sulfur yellowish in colour respectively. Purplish center was absent in *G. hirsutum* but present in *G. harknessii*. The colour of the staminal tube was bit golden in *G. arboreum* and GaWM3 but it was yellowish to creamy in *G. hirsutum* and *G. harknessii*. The comparison of the reproductive characteristics is shown in table 2 and also in Fig. 2. The results reflect the close relationship of GaWM3 to *G. arboreum.*

Cross section of the experimental leaves

Morphological adaptations in plants is not the direct defense strategy against virus rather it is a defense strategy against their vectors. The cross sections of the experimental plants were studied in context to the report that the number of mesophyll layers of the plant cell may cause difficulty in feeding of whiteflies and might have a role in the transmission of viruses. To substantiate the hypothesis, the cross sections of the leaves of the experimental plants were studied which showed that *G. arboreum*, GaWM3 and *G. hirsutum* contain a single layer of mesophyll cells, whereas *G. harknessii* contain double layer of mesophyll cells. Even the thickness of the mesophyll layer, vary in each experimental plant (Fig. 3). The thickness of mesophyll layer of *G. arboreum*, GaWM3 and *G. hirsutum* was found to be ~200-237 µm whereas as the thickness of the mesophyll layers in *G. harknessii* was ~350 µm as it has 2 layers of mesophyll cells.

Different results from Jiang *et al.* (1998) were obtained in this study as the number of mesophyll cell layer has no role in the susceptibility or resistance to *CLCuV* through whitefly. *G. arboreum* has a single layer of mesophyll cell and it is resistant to *CLCuV* whereas *G. hirsutum* and GaWM3 also have a single layer of mesophyll cell and they are susceptible to the virus. While *G. harknessii* have double layers of mesophyll cells and was found to be susceptible to *CLCuV* which clear that it's not mesophyll but some other barrier which can have their protective role.

Detection of *CLCuV*

The experimental plants were exposed to whiteflies in the green house and then in the field. As reported (Khan *et al.*, 2011), *G. arboreum* was symptomless, but the symptoms of CLCuD i.e., downward curling of leaves and thickened veins were appeared on GaWM3 and *G. hirsutum.* As the anatomy of the leaves of *G. hirsutum* and GaWM3 are different, so were the symptoms. The symptoms were very clear in *G. hirsutum* with upward curling of leaves and thick veins with the stunted growth. The symptoms in *G. harknessii* were visible as thickened veins and a bit downward curling, but very mild symptoms appeared in GaWM3 in the form of a slight upward curling of the leaves and thickened veins whereas *G. arboreum* was found to be devoid of any symptom i.e., asymptomatic (Fig. 4). No significant change was observed after incubation of *G. arboreum* with viruliferous whiteflies but significant symptoms appeared in its wax deficient mutant. These findings provided solid bases for investigation of the wax role as a mechanical barrier in transmission of *CLCuV* in this study. The results of *CLCuV* symptom appearance were also in accordance with Sattar *et al*. (2013).

The RCA was used as a template and subjected to PCR (Fig. 5). The PCR results confirm the detection of viral particles (alphasatellite, betasatellite and DNA-A) in *G.*

hirsutum, GaWM3 and *G. harknessii* whereas as no viral component was detected in *G. arboreum* (Fig 6).

Epicuticular wax per unit area

The epicuticular wax per unit area was calculated by taking a total wax load on selected leaves of each plant, divided by their respective surface area hence wax per unit area (µg cm^{-2}) was obtained. According to the analysis, the *G. arboreum* was found to have maximum wax per unit area that is 183 µg cm^{-2} as compared to its mutant (GaWM3) have 95 µg cm^{-2} whereas 130 µg cm^{-2} and 146 µg cm^{-2} of wax was calculated in *G. hirsutum* and *G. harknessii* respectively (Fig. 7). The experimental plants were categorized in following pattern on the basis of their wax per unit area: *G. arboreum* > *G. harknessii* > *G. hirsutum* > GaWM3. The concentration of the wax was in accordance with the report of Bondada *et al.* (1996) i.e., from 70 µg cm^{-2} to 154 µg cm^{-2} from normal conditioned to stress conditions in cotton (fig. 7). Similar wax quantitative results were also obtained by Barozai and Husnain (2014) i.e., *G. arboreum* contain 183.7 µg cm^{-2} while its mutant GaWM1, GaWM2 and GaWM3 had 66.79%, 59.69% and 49.02% less wax than *G. arboreum* respectively.

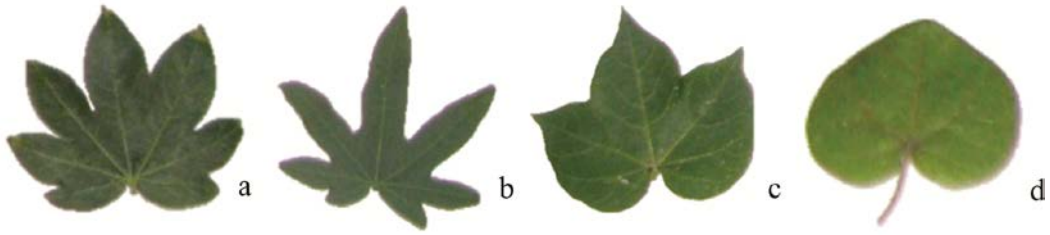

Fig. 1: Leaves of *Gossypium* spp. (a) *G. arboreum* (b) GaWM3 (c) *G. hirsutum* (d) *G. harknessii.* Leaves of the *G. arboreum* and GaWM3 are bit similar to each other and can be clearly differentiated from *G. hirsutum* and *G. harknessii*.

Fig. 2: Flowers of *Gossypium* spp. (a) *G. arboreum* (b) GaWM3 (c) *G. hirsutum* (d) *G. harknessii*. Flowers of the *G. arboreum* and GaWM3 are very similar to each other and can be clearly differentiated with *G. hirsutum*

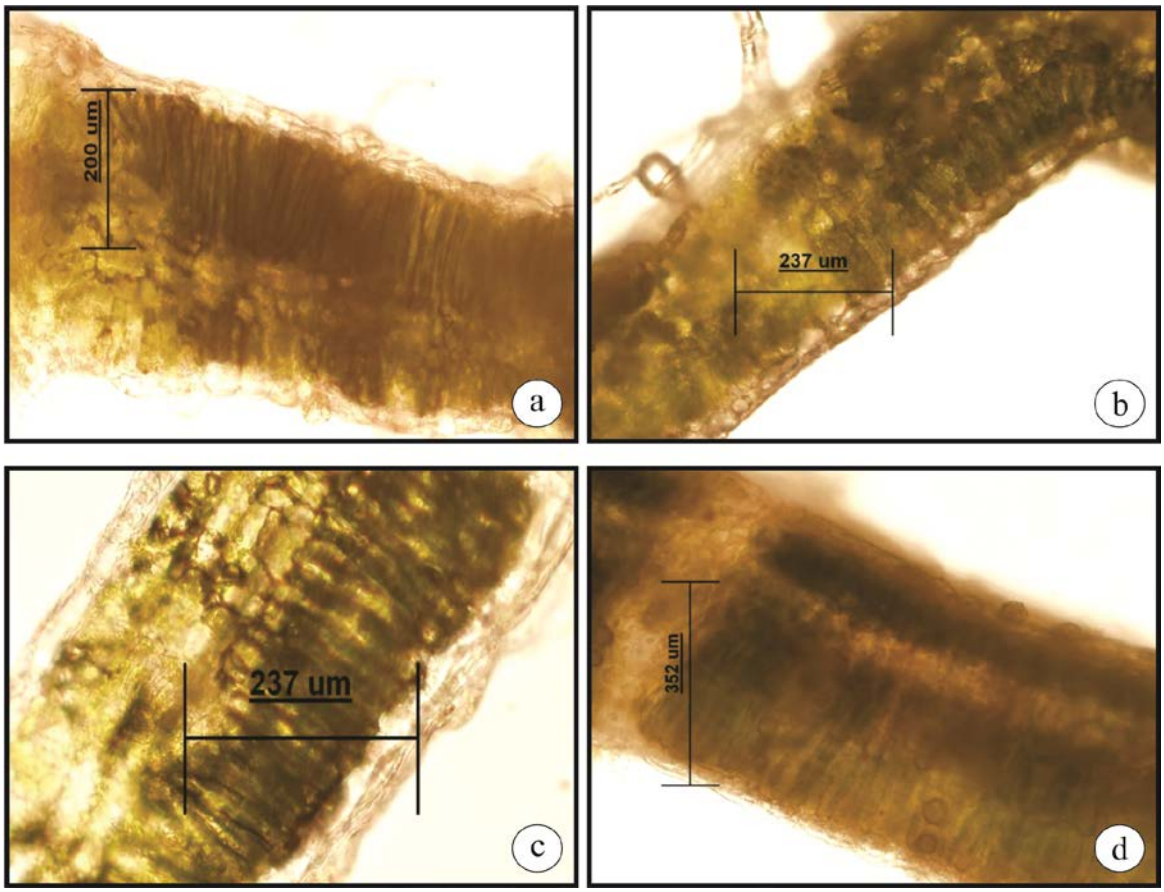

Fig. 3: Cross section of the leaves. (a) *G. arboreum* (b) GaWM3 (c) *G. hirsutum* (d) *G. harknessii.* All experimental plants except plants of *G. harknessii* contain have layer of mesophyll cells. The reference scale in each picture depicts the varied thickness of mesophyll layer in the plants which is maximum in *G. harknessii.*

Number of whiteflies visiting experimental plants

The average number of the whiteflies per leaf that visited the experimental plants were found to be 29 in *G. arboreum*, 42 in GaWM3, 47 in *G. hirsutum* and 27 in *G. harknessii* (Fig. 8) in a single growing season. Maximum whiteflies per leaf was noted for *G. hirsutum* and least for *G. harknessii.* The plant that has more wax offer more hindrance to the attachment of insect to the plant as described by Whitney and Federle (2013). Waxes not only offer hindrance, but also make the movements of the insects difficult even insects were unable to show locomotion after 20 min of feeding on the waxy surface of the plants and flew away from the surface (Gaume *et al.*, 2004).

Conclusion

From the results, it is concluded that the upper surfaces of the leaves have their role in determining the feeding behavior of the whiteflies as minimum number of whiteflies visit the *CLCuV* resistant *G. arboreum* because of high concentration of epicuticular waxes when compared to its wax deficient mutant, *G. hirsutum* and *G. harknessii*. Moreover, the thickness of the leaves and a single or double layer of mesophyll cells have no role in feeding and transmission of *CLCuV* in cotton.

Acknowledgements

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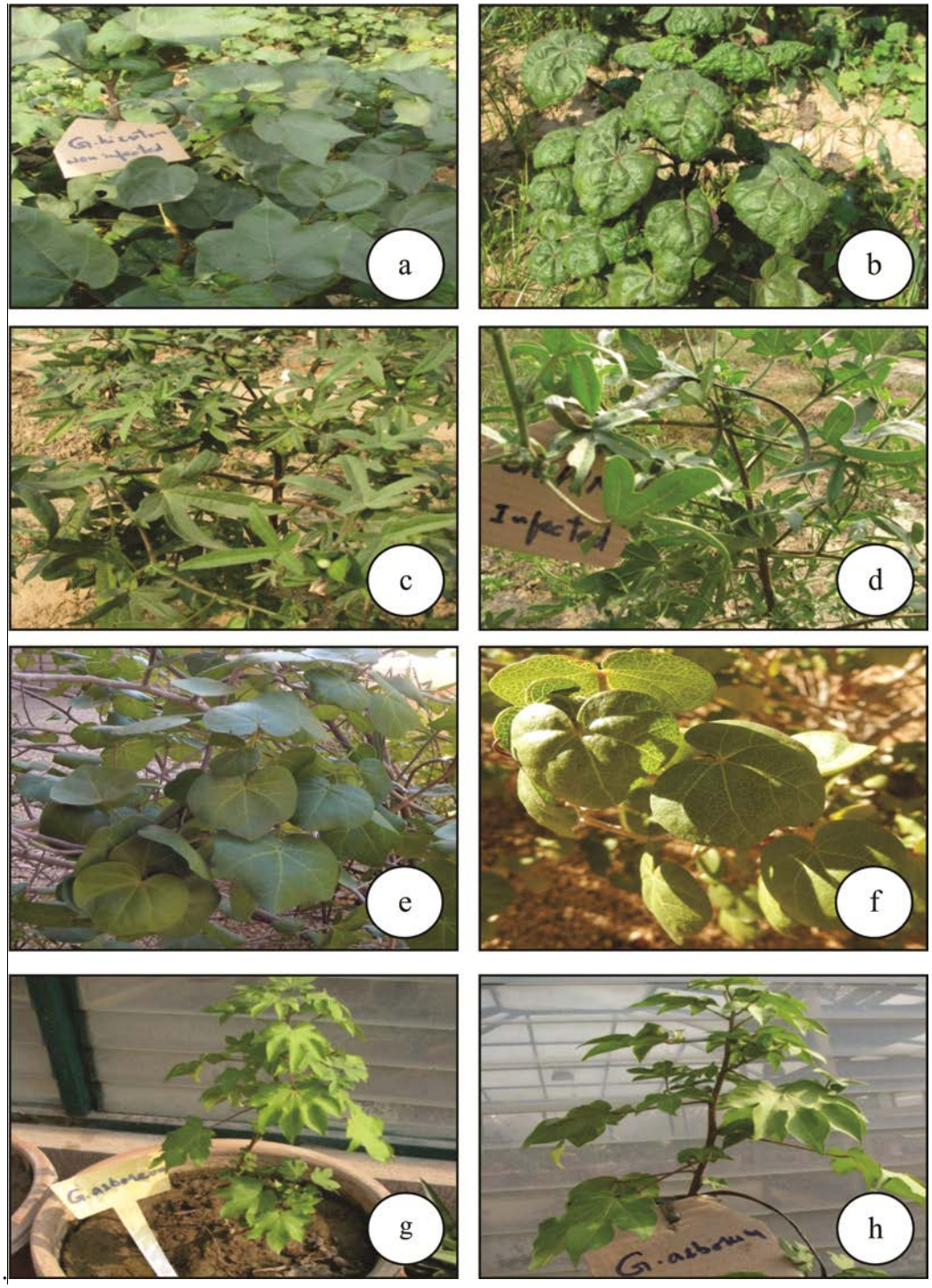

Fig. 4: Symptoms on experimental plants with and without inoculation with whiteflies (a) *G. hirsutum* without inoculation with whiteflies (b) *G. hirsutum* with inoculation with whiteflies (c) GaWM3 without inoculation with whiteflies (d) GaWM3 with inoculation with whiteflies (e) *G. harknessii* without inoculation with whiteflies (f) *G. harknessii* with inoculation with whiteflies (g) *G. arboreum* without inoculation with whiteflies (h) *G. arboreum* with inoculation with whiteflies. Symptoms developed in *G. hirsutum*, *G. harknessii* and GaWM3 after inoculation with whiteflies whereas *G. arboreum* remained healthy.

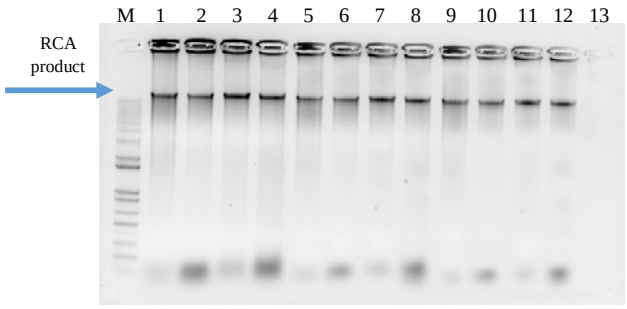

Fig. 5: RCA from the extracted DNA of the experimental plants. Here, M depicts 1 Kb Marker, RCA product of *G. arboreum* is in lane 1-3, GaWM3 in lane 4-6, *G. hirsutum* in lane 7-9, *G. harknessii* in lane 10-12 and negative control is in lane 13.

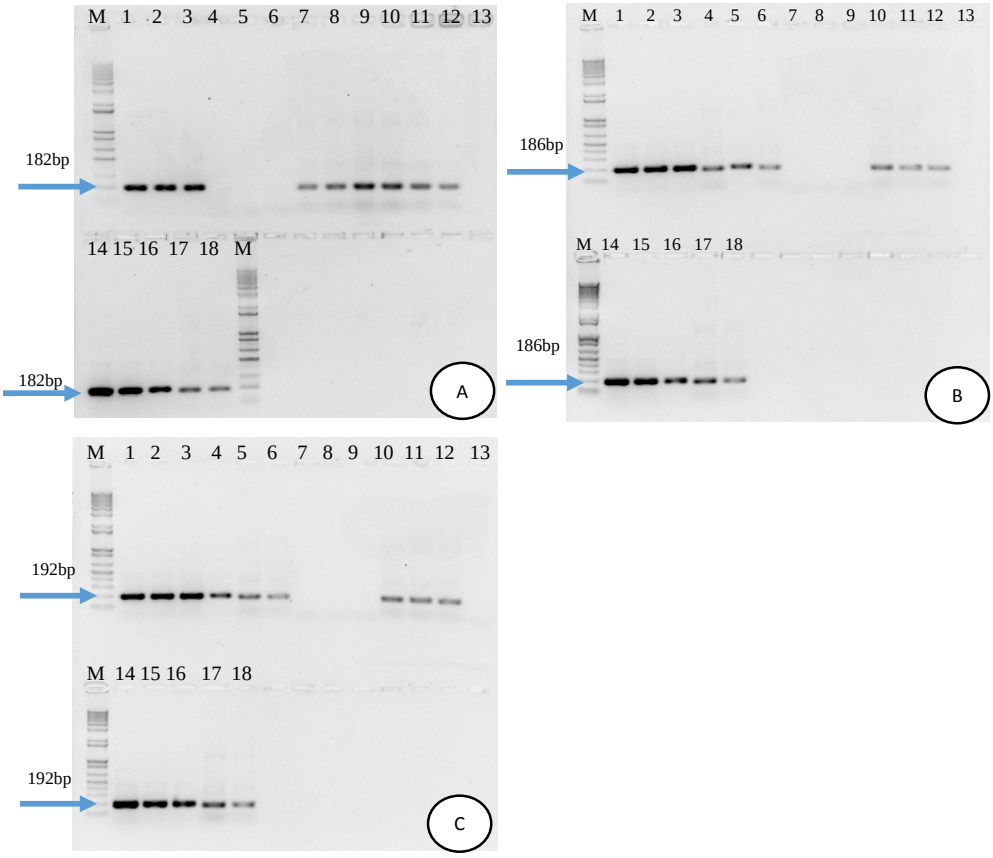

Fig. 6: (A): PCR to detected DNA-A in experimental plants. Here M depicts 1Kb ladder marker, Lane 1-3 represent *G. hirsutum*, lane 4-6 show *G. arboreum*, lane 7-9 GaWM3 and lane 10-12 represent sample of *G. harknessii* whereas lane 13 is negative control and lane 14-18 represent standard positive control 1-5 respectively. (B): PCR to detect betasatellites in experimental plants. Here M depicts 1Kb ladder marker, Lane 1-3 represent *G.hirsutum*, lane 4-6 show GaWM3, lane 7-9 *G. arboreum* and lane 10-12 represent sample of *G. harknessii* whereas lane 13 is negative control and lane 14-18 represent standard positive control 1-5 respectively (C) PCR to detect alphasatellites in experimental plants. Here M depicts 1Kb ladder marker, Lane 1-3 represent *G.hirsutum*, lane 4-6 show GaWM3, lane 7-9 *G. arboreum* and lane 10-12 represent sample of *G. harknessii* whereas lane 13 is negative control and lane 14-18 represent standard positive control 1-5 respectively.

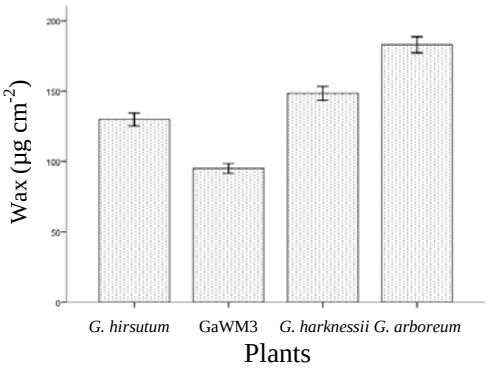

Fig. 7: Mean wax load per unit area (µg cm^{-2}) on the selected leaves of the experimental plants *G. hirsutum*, GaWM3, *G. harknessii* and *G. arboreum.*

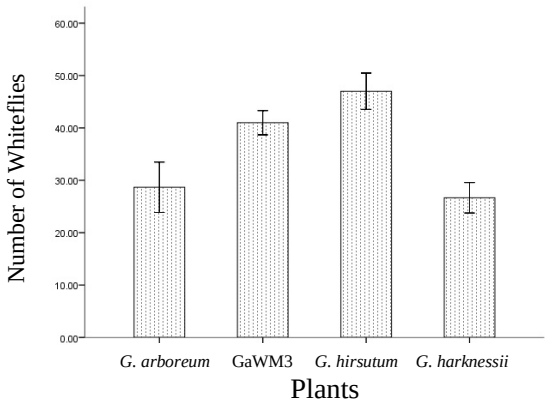

Fig 8: Average number of whiteflies per leaf that visited cotton plants in single growing season.

Table 1 Comparison of vegetative characteristics of experimental plants of different cotton species.

Sr. No.	Vegetative Character	*G. arboreum*	GaWM3	*G. hirsutum*	*G. harknessii*
1	Plant type	Shrub	Shrub	Shrub	Shrub
2	Branches	Purple	Purple	Dark brown spotted with black dots	Dark brown
3	Leaves arrangement	Spiral	Spiral	Spiral	Alternate
4	Leaves shape	Palmately 5-7 lobbed, lobes Ovate to narrowly lanceolate	Palmately 5-7 lobbed, lobes Ovate to narrowly lanceolate	Palmately 3 lobbed, lower leaves may be 5 lobbed, rarely palmatifid	Classic cordate somewhat lobbed
5	Leaves margin	Entire	Undulate	Entire	Crenate or dentate
6	Apex	Acute or acuminate	Acute or acuminate	Acute to acuminate	Acute to acuminate
7	Venation	5–9-veined	5–9-veined	5-7 veined	5-7 veined
8	Stipule	Linear to lanceolate	Linear to lanceolate or obtuse	Ovate to lanceolate	lanceolate
9	Petiole	Present	Present	Present	Present

Table 2 Comparison of reproductive characteristics of experimental plants of different cotton species.

Sr. No.	Floral Characteristics	*G. arboreum*	GaWM3	*G. hirsutum*	*G. harknessii*
1	Pedicels	0.5- 6cm in length, not articulated	0.5- 4cm in length, not articulated	1-4cm long, not articulated	1-4cm long, not articulated
2	Epicalyx	Bracteoles, 3 segments, 1-3.5 cm in length	Bracteoles, 3 segments, 1-3 cm in length	Bracteols, 3 lobbed	Bracteols, 3 lobbed 1- 2.5cm long
3	Calyx	Truncated sepals, cupular, 5-12 cm long , truncate to inconspicuously 5-dentate	Truncated sepals , cupular 4-9 cm in length, truncate to inconspicuously 5-dentate	Cup shaped with 5 lobes and lobes are triangular in shape	Truncated calyx 5
4	Corolla	5 - Creamy to yellow, turn into red to purple, with a purplish center	5 - creamy to yellow, turn into red to purple, with a purplish center	5- yellowish, faded to red or purple, without a purplish center	5- Sulphur yellowish, with a purple dots
5	Androecium	Stamen numerous, in a column, 1.5-2 cm long, golden in colour	Stamen numerous, in a column, 1-2 cm long, golden in colour	Stamen numerous, in a column, 1-2 cm long, yellowish in colour	Column is short numerous stamen, yellowish in colour
6	Gynoecium	3-5 pistils, style short rod shaped, stigma calvate 5 grooved, Ovary superior	3-5 pistils, style short rod shaped, stigma calvate 5 grooved, Ovary superior	3-5 pistils, style short rod shaped, stigma calvate 5 grooved, Ovary superior	3 pistils, style long, stigma joint to top. calvate 5 grooved, Ovary superior

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