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Introgression of cotton leaf curl virus-resistant genes from Asiatic cotton (*Gossypium arboreum*) into upland cotton (*G. hirsutum*)

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ABSTRACT. Cotton is under the constant threat of leaf curl virus, which is a major constraint for successful production of cotton in the Pakistan. A total of 3338 cotton genotypes belonging to different research stations were screened, but none were found to be resistant against the Burewala strain of cotton leaf curl virus (CLCuV). We explored the possibility of transferring virus-resistant genes from *Gossypium arboreum* (2n = 26) into *G. hirsutum* (2n = 52) through conventional breeding techniques. Hybridization was done manually between an artificial autotetraploid of *G. arboreum* and an allotetraploid *G. hirsutum*, under field conditions. Boll shedding was controlled by application of exogenous hormones, 50 mg/L gibberellic acid and 100 mg/L naphthalene acetic acid. Percentage pollen viability in F_1 hybrids was 1.90% in 2(*G. arboreum*) x *G. hirsutum* and 2.38% in *G. hirsutum* x *G. arboreum*. Cytological studies of young buds taken from the F_1

hybrids confirmed that they all were sterile. Resistance against CLCuV in the F_1 hybrids was assessed through grafting, using the hybrid plant as the scion; the stock was a virus susceptible cotton plant, tested under field and greenhouse conditions. All F_1 cotton hybrids showed resistance against CLCuV, indicating that it is possible to transfer resistant genes from the autotetraploid of the diploid donor specie *G. arboreum* into allotetraploid *G. hirsutum* through conventional breeding, and durable resistance against CLCuV can then be deployed in the field.

Key words: Cotton leaf curl virus; Exogenous hormones; *Gossypium arboreum*; *Gossypium hirsutum*; Interspecific crosses

INTRODUCTION

Cotton is infected by several insects, pests and pathogens inducing different diseases. Among them cotton leaf curl virus (CLCuV) is the most damaging disease, causing enormous losses (Khan and Ahmad, 2005). With the passage of time, CLCuV has spread to all provinces of the Pakistan (Tariq, 2005). It has caused a reduction of 9.45 million cotton bales during the last decade. Reduction in yield of tolerant and susceptible varieties is reported to be 50 and 85-90%, respectively (Hussain, 1995; Khan et al., 2001).

Seven species of begomoviruses have been reported so far; five of these have been identified in the Pakistan, one in the India and one in the Sudan (Amin et al., 2006; Sharma and Rishi, 2007). A new recombinant strain of begomovirus derived from cotton leaf curl Multan virus (CLCuMV) and cotton leaf curl Kokhran virus (CLCuKV) has been found to be associated with the breakage of resistance in existing cotton varieties (Akhtar et al., 2010). Recently, sequencing of this virus strain associated with resistance breaking in the Pakistan confirmed the involvement of this recombinant strain of begomovirus (Amin et al., 2006; Amrao et al., 2007). This recombinant strain of virus was named cotton leaf curl Burewala virus (CLCuBV), which is now common in cotton-growing areas of the Pakistan. Breeding-resistant varieties is one of the best ways to combat CLCuV, particularly when all attempts at treatment have been ineffective and when there is high inoculum pressure. Various attempts have been made to transfer resistant genes through interspecific hybridization between wild diploid species and tetraploid cultivated cotton. Blank and Leathers (1963) transferred resistant genes against cotton rust caused by *Puccinia cacabata* from *G. anomalum* and *G. arboreum* into *G. hirsutum* through interspecific hybridization. Genes for resistance against diseases and drought have been transferred between *G. hirsutum* and *G. arboreum* (Amin, 1940). Interspecific introgression has also been done between *G. hirsutum* and *G. arboreum* (Bao-Liang et al., 2003). Similarly, resistant genes against bacterial blight of cotton present in *G. arboreum* have been introgressed into *G. barbadense* (Knight, 1957; Brinkerhoff, 1970). Sacks and Robinson (2009) introgressed resistance against *Rotylenchulus reniformis* from diploid to tetraploid cotton. At present not a single variety of *G. hirsutum* has resistance against Burawala strain of CLCuV and *G. arboreum* is a known source, having resistance against cotton leaf curl virus. In this scenario a project was planned to explore the possibility of transferring virus-resistant genes from Asiatic cotton (*G. arboreum*, 2n = 26) into cultivated upland cotton (*G. hirsutum*, 2n = 52) genotypes through conventional breeding methods.

MATERIAL AND METHODS

Two cotton genotypes *viz*, *G. arboreum* (2n = 26) variety 15-Mollisoni and *G. hirsutum* variety CRSM-38 were used. An artificial autotetraploid of *G. arboreum* (2n = 52) was synthesized by dipping the *G. arboreum* (2n = 26) into 0.05% colchicine solution for 24 h. It was used as the female parent because crosses failed when this autotetraploid was used as a male parent, while *G. hirsutum* (2n = 52) was used both as male and female parents.

Hybridization was done manually; emasculation and pollination were done under field conditions. Emasculation was carried out in the evening and emasculated flowers were pollinated the following morning. In order to control the incidence of boll shedding, a solution of 50 mg/L gibberellic acid and 100 mg/L naphthalene acetic acid was applied at the base of the pedicle after 24 h of pollination for three days. The number of bolls that set was counted at the time of harvest.

Morphological characteristics

The observations on growth habit, stem color, leaf texture, leaf shape and hairiness, bracteole size, corolla color, petal spot, position of the staminal column, anther color, and dehiscence of parents in F_1 generations were recorded.

Pollen viability estimation

To analyze the viability of pollen in F_1 hybrids, flowers were collected in the morning on the day of anthesis. A 2% solution of 2,3,5-triphenyltetrazolium chloride in 60% sucrose was prepared. A drop of the stain was placed on a glass slide with pollens from freshly picked flowers. Excessive pollens on the slide were wiped away using the tip of a very fine needle and pollen grains were agitated with the help of needle for 30 s to obtain uniform immersion in the mount. The prepared slides were stored at room temperature for 24 h. Pollen counts were made after 24 h of staining. The stained pollen grains were considered to be viable.

Cytological studies

Young buds of the parents and F_1 hybrids were collected and fixed in Carnoy's solution from 8 to 9 am. After 24 h these buds were preserved in 70% ethanol. Three to four anthers were squashed on a slide with a drop of 2.5% acetocarmine solution. The chromosome behaviors of parents and their F_1's were examined under a Labomad microscope and photographs were taken.

Screening of F_1 hybrid plants against cotton leaf curl virus

Resistance in F_1 hybrid plants against cotton leaf curl virus under field/greenhouse conditions was assessed through grafting. The scion was made of branches taken from F_1 hybrid, while stock was from virus-susceptible *G. hirsutum* plants.

Development of BC_1 population

BC_1 population of the crosses [2(*G. arboreum*) x *G. hirsutum*] x *G. hirsutum* and (*G. hirsutum* x *G. arboreum*) x *G. hirsutum* was developed and examined for resistance against cotton leaf curl virus.

RESULTS

Crossability studies

The crosses were made between an artificial autotetraploid of *G. arboreum* with *G. hirsutum* and *G. hirsutum* with *G. arboreum* (Table 1). The maximum percentage of boll set was 6.7% for the cross *G. hirsutum* x *G. arboreum* and the minimum was 6.1% for the cross 2(*G. arboreum*) x *G. hirsutum*. Viable seeds were obtained in both combinations. The F_1 hybrids in both combinations were highly sterile, with pollen viability 1.90% in 2(*G. arboreum*) x *G. hirsutum* and 2.38% in *G. hirsutum* x *G. arboreum* (Tables 2 and 3).

The results revealed that there was no boll set on selfing as well as in back crosses with the *G. hirsutum* parent without application of exogenous hormones. However, application of 50 mg/L gibberellic acid and 100 mg/L naphthalene acetic acid, 24 h after pollination, at the base of pedicle provoked boll setting; seeds obtained from these back crosses were used to raise the BC_1 generation (Table 4).

Morphological characteristics

The morphological characteristics of the hybrid plants (F_1) were intermediate between those of the two parents (Tables 2 and 3). The four hybrid plants in the F_1 of the cross between 2(*G. arboreum*) x *G. hirsutum* were vigorous and luxuriant in growth, with profuse branching and foliage, demonstrating heterosis. The stem was brown, with the young portion green and hairy, with more glands compared to the male parent (*G. hirsutum*). Leaves were hairy, intermediate in size and shape between the two parents (Figure 1a). Flowers were light yellow in color, with light pink and purple basal spots (Figure 1b). There were three bracteoles, intermediate in size and shape compared to the parents (Figure 1c). There were five petals also intermediate in size, shape and color (Figure 1d).

The F_1 hybrid of *G. hirsutum* x *G. arboreum* was intermediate for most of the morphological attributes. Leaves were hairy, intermediate in size, shape, lobation and petiole length (Figure 2a). Flowers were medium in size, yellow with pink basal spots on the petal (Figure 2b). Bracteoles were intermediate in size, shape and dentations of both parents (Figure 2c). Petals were also intermediate in size (Figure 2d).

Cytological studies

Meiosis in parents

The course of meiosis was examined in the *G. hirsutum* and *G. arboreum* parents. In these species the reduction division was normal, with regular pairing of chromosomes.

Table 1. Detail of pollination and boll set for development of F_1 hybrids.

Sr. No.	Parentage	No. of pollinations attempted	No. of bolls set	% Age set	No. of seeds obtained	No. of seeds germinated	Germination (% age)
1	2(*G. arboreum*) x *G. hirsutum*	525	32	6.1%	10	4	40.0%
2	*G. hirsutum* x *G. arboreum*	1017	68	6.7%	43	15	34.9%

G. = *Gossypium*.

Table 2. Morphological characteristics of F_1 hybrid 2(*G. arboreum*) x *G. hirsutum* and its parents.

Morphological characteristics	2(*G. arboreum*)	*G. hirsutum*	F_1 hybrid
Stem color	Greenish brown	Green	Brown
Stem hairiness	Profusely hairy	Hairy	Hairy
Black glands	Dense	Sparse	Sparse
Leaf color	Dark green	Green	Light/dark green
Leaf size	Medium (7.3 cm length x 9.9 cm breadth)	Large (10 x 14 cm)	Large (8.8 x 10.7 cm)
Leaf lobation	3-5 narrow, deep lobed	3-5 broad, shallow lobed	3-5 narrow lobed
Leaf texture	Thick, leathery	Herbaceous	Herbaceous
Petiole length	Medium (4.4 cm)	Long (8.8 cm)	Long (7.0 cm)
Leaf hairiness	Profusely hairy	Hairy	Hairy
Bracteole number and size	2-3, large (3.0 x 2.6 cm), united at base	3 large (3.3 x 1.8 cm)	Large (2.9 x 2.2 cm)
Bracteole dentation	Entire	5-11, deep narrow	3-9 medium
Flower size	Medium	Large	Medium
Pedicel size	Long (1.7 cm)	Long (1.2 cm)	Short (0.7 cm)
Calyx	5 sepals forming a cups with wavy margins	5 sepals forming a cup with teeth	5 sepals forming a cup with wavy margins
Corolla color	Light yellow	Creamy	Light yellow
Petal number and size	5, medium (3.0 x 2.6 cm)	5, large (4.6 x 4.5 cm)	5, medium (3.6 x 3.7 cm)
Petal spot	Dark pink	Absent	Light pink
Position of staminal column	Short (0.4 cm)	Long (2.0 cm)	Medium (1.7 cm)
Anther dehiscence	Partial	Normal	Partial
Pollen color	Light yellow	Creamy	Light yellow
Pistil size	Long (2.5 cm)	Long (2.9 cm)	Long (2.7 cm)
Pollen viability (% age)	3.33%	70.10%	1.90%

G. = *Gossypium*.

Table 3. Morphological characteristics of F_1 hybrid *G. hirsutum* x *G. arboreum* and its parents.

Morphological characteristics	*G. hirsutum*	*G. arboreum*	F_1 hybrid
Stem color	Green	Green	Brown young portion green
Stem hairiness	Hairy	Hairy	Hairy
Black glands	Sparse	Sparse/dense	Sparse
Leaf color	Green	Green	Green
Leaf size	Large (10.0 cm length x 14.0 cm breadth)	Small/medium (6.0 x 8.3 cm)	Medium (7.0 cm)
Leaf lobation	3-5 narrow, deep lobed	3-5 broad, shallow lobed	3-5 narrow lobed
Leaf texture	Thick, leathery	Herbaceous	Herbaceous
Petiole length	Medium (4.4 cm)	Long (8.8 cm)	Long (7.0 cm)
Leaf hairiness	Profusely hairy	Hairy	Hairy
Bracteole number and size	2-3, large (3.0 x 2.6 cm), united at base	3, large (3.3 x 1.8 cm)	3, medium (2.8 x 1.8 cm)
Bracteole dentation	5-11, deep, narrow	3-7, superficial	3-7 medium
Flower size	Large	Small	Medium
Pedicel size	Long (1.2cm)	Long (1.2 cm)	Medium (0.8 cm)
Calyx	5 sepals forming a cup with teeth	5 sepals forming a cup with wavy margins	5 sepals forming cups with wavy margins
Corolla color	Creamy	Yellow	Yellow
Petal number and size	5, large (4.6 x 4.5 cm)	5, small (2.6 x 2.5 cm)	5, medium (4.2 x 4.1 cm)
Petal spot	Absent	Light pink	Pink
Position of staminal column	Long (2.0 cm)	Small (1.0 cm)	Medium (1.4 cm)
Anther dehiscence	Normal	Normal	Partial
Pollen color	Creamy	Yellow	Yellow
Pistil size	Long (2.9 cm)	Small (2.1 cm)	Long (3.0 cm)
Pollen viability (% age)	70.10%	96.06%	2.38%

G. = *Gossypium*.

Table 4. Detail of pollination and boll set for development of BC_1 plants.

Sr. No.	Parentage	No. of pollination attempted	No. of bolls set	% Age set	No. of seeds obtained	No. of seeds germinated	Germination (% age)
1	[2(*G. arboreum*) x *G. hirsutum*] x *G. hirsutum*	3929	312	7.9%	27	10	37.0%
2	(*G. hirsutum* x *G. arboreum*) x *G. hirsutum*	1790	125	6.9%	10	7	70.0%

G. = *Gossypium*.

There were 26 bivalents in *G. hirsutum* and 13 bivalents in *G. arboreum* at metaphase-I. The disjunction of the chromosomes was normal at anaphase-I. Meiotic behavior in the artificial autotetraploid of *G. arboreum* parents showed two univalents, 23 bivalents and one quadrivalent (Figure 3a,f).

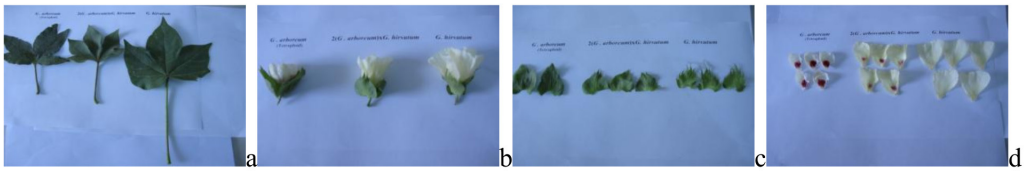

Figure 1. Morphological characteristics of F_1 hybrid 2(*Gossypium arboreum*) x *G. hirsutum* and its parents. **a.** Leaves. **b.** Flowers. **c.** Bracteoles. **d.** Petals.

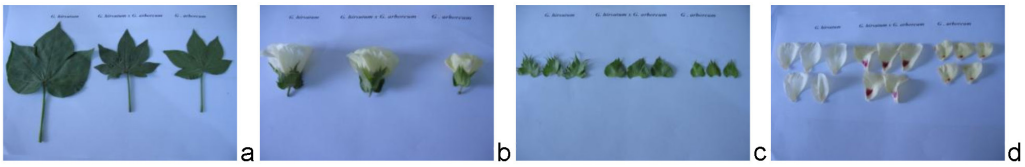

Figure 2. Morphological characteristics of F_1 hybrid *Gossypium hirsutum* x *G. arboreum* and its parents. **a.** Leaves. **b.** Flowers. **c.** Bracteoles. **d.** Petals.

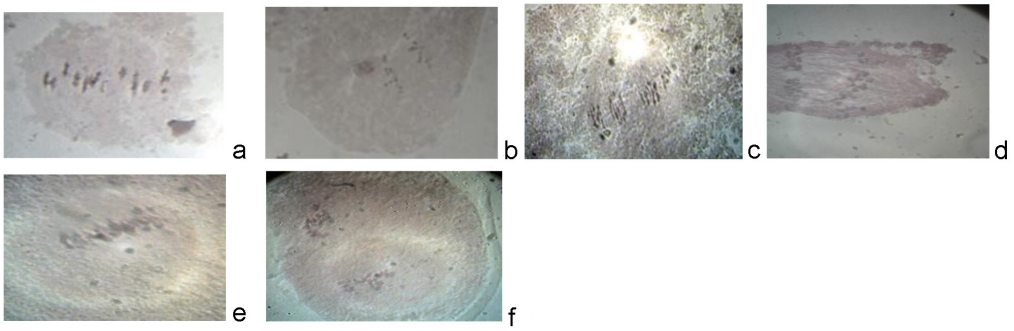

Figure 3. Meiosis in parents. **a.** Metaphase-I of *Gossypium arboreum*. **b.** Anaphase-I of *G. arboreum*. **c.** Metaphase-I of 2(*G. arboreum*). **d.** Anaphase-I of 2(*G. arboreum*). **e.** Metaphase-I of *G. hirsutum*. **f.** Anaphase-I of *G. hirsutum*.

Meiosis in hybrids

[2(*G. arboreum*) x *G. hirsutum*]

Cytological studies revealed that at metaphase-I there were 9-18 Is, 15-18 IIs, 0-1 IIIs, and 1-2 IVs, making a total number of 52 chromosomes. It was also observed that at anaphase-I, chromosomes were distributed unequally. A few lagging chromosomes were also observed at anaphase-I (Figure 4a,b). Due to a large number of univalent and multivalent associations, these plants were highly sterile.

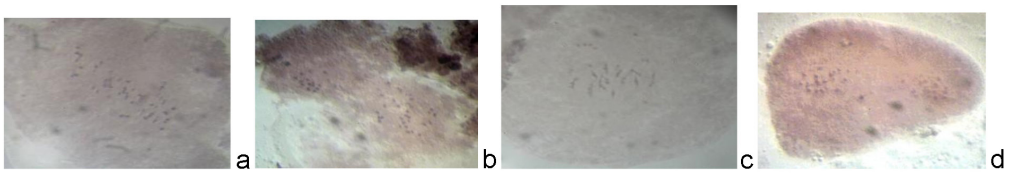

Figure 4. Meiosis in hybrids 2(*Gossypium arboreum*) x *G. hirsutum* (a,b), *G. hirsutum* x *G. arboreum* (c,d). **a.** Metaphase-I of 2(*G. arboreum*) x *G. hirsutum*. **b.** Anaphase-I of 2(*G. arboreum*) x *G. hirsutum*. **c.** Metaphase-I of *G. hirsutum* x *G. arboreum*. **d.** Anaphase-I of *G. hirsutum* x *G. arboreum*.

G. hirsutum x *G. arboreum*

Cytological studies of 15 plants revealed that at metaphase-I there were 8-15 Is, 8-13 IIs, 0-1 IIIs, and 1-2 IVs making a total number of 39 chromosomes. In anaphase-I, chromosomes were distributed unequally. A few lagging chromosomes were also observed at anaphase-I (Figure 4c,d). These plants were also sterile due to the presence of many univalent and multivalent associations.

Screening of F_1 hybrid plants against cotton leaf curl virus

F_1 hybrid plants of 2(*G. arboreum*) x *G. hirsutum* and *G. hirsutum* x *G. arboreum* were screened under natural field/greenhouse conditions through grafting and it was found that all F_1 hybrids were resistant against CLCuV (Table 5).

Table 5. Screening of F_1 hybrid plants.

Sr. No.	F_1 hybrids	Screened No. of plants in		Results
		Field/greenhouse	Grafts	
1	2(*G. arboreum*) x *G. hirsutum*	4	15	These F_1 hybrids showed high level
2	*G. hirsutum* x *G. arboreum*	15	-	of resistance to cotton leaf curl virus

G. = Gossypium.

Development of BC_1 population

Ten BC_1 plants of [2(*G. arboreum*) x *G. hirsutum*] x *G. hirsutum* and seven of (*G. hirsutum* x *G. arboreum*) x *G. hirsutum* were grown and found resistant against cotton leaf curl virus (Table 6).

Table 6. Development of BC_1 population.

Sr. No.	BC_1 population	No. of plants	Results
1	[2(*G. arboreum*) x *G. hirsutum*] x *G. hirsutum*	10	These all BC_1 plants were found
2	(*G. hirsutum* x *G. arboreum*) x *G. hirsutum*	7	resistant against cotton leaf curl virus

DISCUSSION

The primary factor that limits the success of interspecific hybridization in cotton is lack of retention of crossed bolls. Cotton breeders have been trying to obtain hybrids between diploid and tetraploid species for a long time (Gill and Bajaj, 1987), but it is difficult and some times impossible to obtain hybrids under *in situ* conditions because of several incompatibility factors. Weaver Jr. (1957) found ovule failure in the cross *G. hirsutum* x *G. arboreum* due to deranging effect of the hybrid embryo upon endosperm development while in reciprocal crosses (Weaver Jr., 1958) observed main factors of abnormal mitosis of endosperm nuclei and improper embryo differentiation. Many workers have made ovule and embryo cultures of interspecific hybrids through *Gossypium* interspecific hybridization (Stewart and Hsu, 1978; Refaat et al., 1984; Umbeck and Stewart, 1985; Thengane et al., 1986; Gill and Bajaj, 1987; Mirza et al., 1993).

After pollination of flowers, exogenous hormones have been used to promote interspecific hybridization in cotton. A lot of research from China revealed that exogenous hormone application alone can overcome certain crossing barriers between *Gossypium* species (Liang et al., 1978; Liang and Sun, 1982). Gibberellic acid was used as a growth regulator to obtain interspecific hybrids between tetraploid *G. hirsutum* and diploid *G. arboreum* species of cotton (Mofidabadi, 2009).

By conventional breeding methods, crosses between the two species of cotton are rarely successful due to abortion of the embryo after fertilization. The diploid species that cross directly with upland cotton produce sterile triploid F_1 hybrids. Such triploid hybrids have to be treated with colchicine to produce hexaploids (Joshi and Johri, 1972). We synthesized triploid hybrid plants (eight plants) of *G. hirsutum* x *G. arboreum* and treated them with 0.1% colchicine solution for seven days using the cotton swab method, but there was no effect of colchicine because the plants were old.

Our results are similar to those of Altman (1988), who found that in the development of F_1 hybrids and BC_1 plants, application of exogenous hormone was better than *in vitro* methods. The mean number of seeds per boll varied, ranging from immature seed to 1.5 seeds per boll while without application of exogenous hormones, pollinated flowers gave 0.1% harvestable bolls. Similarly, exogenous application of different growth hormones has been used to facilitate interspecific crosses in many crops, including wheat (Sitch and Snap, 1987) and tomato (Gordillo et al., 2003).

Our crossability studies between 2(*G. arboreum*) x *G. hirsutum* and *G. hirsutum* x *G. arboreum* showed that boll set was a maximum 6.7% in the cross *G. hirsutum* with *G. arboreum* and a minimum 6.1% in the cross 2(*G. arboreum*) x *G. hirsutum* (Table 1). Viable seeds were obtained in both combinations. These results are in contrast to those reported by Mofidabadi (2009), who did not obtain viable seeds. This contradiction in the findings may be due to different experimental materials and environments. The F_1 hybrids in both combinations were highly sterile, with pollen viability 1.90% in 2(*G. arboreum*) x *G. hirsutum* and 2.38% in *G. hirsutum* x *G. arboreum* (Tables 2 and 3). The low pollen viability in these hybrids may be due to high temperature during May, when pollen viability percentage was calculated. Induced tetraploid plants of 2(*G. arboreum*) as the female parent facilitated interspecific hybridization. However, interspecific crosses failed when induced tetraploid plants were used as the male parent. Transmission of genes through pollen grains from induced autotetraploids was

very poor, which may be due to slow pollen growth (Coffin and Harney, 1978; Susiacuen and Alvarez, 1997). In general, hybrid plants of both combinations were more vigorous than either parent and were intermediate in several traits between both of the parents (Tables 2 and 3).

In the development of interspecific hybrids for resistance, thorough knowledge about the chromosomal behavior in hybrids and backcross progenies is essential. In our study, in the case of hybrids 2(*G. arboreum*) x *G. hirsutum*, the 'AD' genome of *G. hirsutum* was introgressed into the 'AA' genome of *G. arboreum*, making an 'AAAD' genomic constitution. In hybrids *G. hirsutum* x *G. arboreum* the A genome of *G. arboreum* was introgressed into the 'AD' genome of *G. hirsutum*, making a genomic constitution 'AAD'. In *G. arboreum* and *G. hirsutum* normal orientation, association and disjunction of chromosomes were observed while quadrivalents and low frequency of chromosome associations (bivalents) were observed in the hybrids. Univalents observed in this study can be attributed to asynapsis, because of a lack of homology between the different sets of chromosomes. The presence of laggards demonstrated the occurrence of meiotic disturbances, leading to imbalance in daughter cells.

The F_1 hybrid plants of 2(*G. arboreum*) x *G. hirsutum* (four plants and fifteen grafts) and *G. hirsutum* x *G. arboreum* (fifteen plants) were screened under natural conditions of field/greenhouse through grafting (Table 5). These hybrids were resistant to cotton leaf curl virus.

Ten BC_1 plants of [2(*G. arboreum*) x *G. hirsutum*] x *G. hirsutum* and seven of (*G. hirsutum* x *G. arboreum*) x *G. hirsutum* were grown and found resistant to cotton leaf curl virus (Table 6).

CONCLUSIONS

We demonstrated the introgression of cotton leaf curl virus-resistant genes from diploid (*G. arboreum*) into tetraploid (*G. hirsutum*) through hybridization. These findings further validated the method of using autotetraploids to introgress favorable traits from diploid species into tetraploid upland cotton. In conclusion, using conventional breeding methods cotton breeder can deploy durable resistance against cotton leaf curl virus in elite genetic material.

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