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Research Article

Interspecific hybrid confirmation in wide crosses of cotton (*Gossypium spp*) through morphological, cytological and molecular analysis

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Abstract
Interspecific triploid hybrids were developed between tetraploid cultivated species *Gossypium barbadense cv.* Suvin and TCB 37 and diploid wild species *Gossypium armourianum*. The F_1 hybridity was confirmed by morphological, cytological and molecular approaches. The ploidy level of interspecific F_1 hybrid was triploid and completely male sterile. Female parents Suvin and TCB 37 had erect growth habit, yellow petals, palmate leaves, green stem, thick and prominent leaf veins, but Suvin had dense yellow anthers while TCB 37 was with dense creamy anthers whereas male parent has a spreading growth habit, yellow petals, cordate leaves, reddish green stem, medium dense yellow anthers, thin leaf veins, protruded stigma and glabrous plant body. The growth habit, petal colour, leaf shape and size of interspecific F_1 hybrid were intermediate. Plant stem colour, hairyness, leaf pubescence and anther colour of *Gossypium armourianum* were observed to be dominant as hybrid fully resembled *Gossypium armourianum* for these characters. Petal spot was observed in both male and female parents but F_1 hybrids had different intensity of petal spot in their petals. Variable expression of petal spot, anther colour and filament colour was observed in the F_1 hybrids. *Gossypium barbadense cv.* Suvin and TCB 37 had 52 chromosomes, *Gossypium armourianum* had 26 chromosomes and the interspecific F_1 was with 39 chromosomes. Significant differences were observed between pollen size, pollen fertility of parents and their hybrids. The F_1 interspecific hybrid having more than 97% of sterile pollen grains. Out of 6 polymorphic SSR markers between parents, 3 markers unambiguously confirmed the hybrid status of interspecific hybrid. This hybrid may serve as useful genetic resource for the transfer of jassid resistance gene to *Gossypium barbadense* cultivars.

Keywords
***Gossypium*, Wild hybridization, Wild species, Triploid**

INTRODUCTION

Cotton is a dicotyledon plant, belonging to the family-Malvaceae, genus *Gossypium* and is a highly diverse genus. This genus comprises of approximately 50 species, including 45 diploids (2n = 2x = 26) and five allotetraploids (2n = 4x = 52) which are distributed throughout tropical and subtropical regions of the world (Shim *et al*., 2018). Among the four cultivated species *Gossypium hirsutum* and *Gossypium barbadense* are tetraploid, *Gossypium arboreum* and *Gossypium herbaceum* are diploid. Diploid *Gossypium* species fall into eight cytological groups designated A, B, C, D, E, F, G, and K based on their chromosomal pairing relationships and geographical distribution (Wendel and Grover, 2015). *Gossypium hirsutum* also known as American cotton, is the most widely grown cotton species which is responsible for approximately 90% of the total cotton production worldwide followed by *Gossypium barbadense* contribute about 8% of world cotton production. Being cotton a fiber crop, quality of the fiber is very much essential for the textile industries. *Gossypium barbadense (AD)*$_2$, also known as extra-long staple (ELS) cotton, is a species of cotton plant that has been cultivated to have ELS fibres – fibres longer than 35 mm which is useful for manufacturing high quality cotton cloth. *Gossypium armourianum is* one of the 13

wild diploid D-genome species, reportedly possesses resistance to jassids (Pushpam and Raveendran, 2006), pink bollworm (Brazzel and Martin, 1956) and white flies. Wild *Gossypium* species possess massive amount of unexplored genetic diversity that can be exploited to broaden the genetic base of cotton (Shim *et al*., 2018).

In the present study, crosses were effected between *Gossypium barbadense* and *Gossypium armourianum* to transfer the characters for insect resistance due to D_2 smoothness trait is due to a single dominant gene and the caducous bract trait that could reduce and help solve the trash and lint-cleaning problems of mechanically harvested cotton (Meyer, 1957). The F_1 hybrids and the parents were analysed to discriminate true hybrids using molecular marker (SSR) analysis in conjucation with morphological and cytological analysis (Manickam and Prakash 2014).

MATERIALS AND METHODS

The crossing block has been raised during summer 2019 comprises of two female *Gossypium barbadense* parents *viz.,* Suvin and TCB 37 and the male parent wild species *Gossypium armourianum* is maintained in cotton wild species garden at Department of Cotton, Tamil Nadu Agricultural University (TNAU), Coimbatore. Crosses were effected by using Doak's method (Doak, 1934) of hand emasculation and pollination and crossed bolls were obtained and collected for further evaluation.

The two F_1 hybrids along with their female parents *viz.,* Suvin and TCB 37 were raised during winter 2019. The male parent *Gossypium armourianum* is being maintained in cotton wild species garden. F_1 hybrids along with parents was used for recording data on various morphological, biometrical, cytological and molecular analysis.

Totally 19 morphological characters namely plant growth habit, stem colour, stem pubescence, petiole colour, leaf shape, leaf colour, leaf incision, leaf veins, leaf texture, leaf hairiness, bract size, corolla colour, petal size, petal spot, anther colour, anther density, filament colour, position of stigma and nectar glands and 14 biometrical traits studied bracterial teeth number, bracterial length, bracterial breadth, petiole length, leaf length, leaf breadth, leaf area, pedicel length, petal length, petal breadth, pollen size diameter, pollen fertility (%),gossypol gland density and length of pistil were studied to discriminate the parents and F_1 hybrids. Fourth fully matured and expanded leaves from the top of the plant were taken and their maximum length and breadth was recorded. Leaf area was measured from 5 fully expanded, matured leaves of both parents and F_1 hybrids using Leaf Area Meter and an average used for recording observations.
Flowers were collected in the morning on the day of anthesis between 10.00 am to 11.00 am for pollen fertility study. Pollen fertility was recorded by dusting pollen grains in 1% KI solution and viewed under a compound microscope. Only large, darkly stained and circular pollen grains were considered as fertile. In both parents and F_1 hybrids four microscopic fields were taken to find out the pollen fertility percentage and averaged for further evaluation.

In cytological analysis, mitotic metaphase chromosome study was carried out by using root tips to confirm the ploidy level of F_1 hybrids and their parents. Seeds of parents and their F_1 were soaked overnight and then germinated in the germination paper. The roots were collected from the germinated seeds with 2-3 cm length in quick succession between 9.00 am to 10.00 am on bright sunny days and pre-treated with para-dichloro benzene to accumulate metaphase cells. After 2 hours the pre-treated root tips were washed thoroughly in running tap water to remove excess para-dichloro benzene and fixed in the Ethanol: Glacial acetic acid (3:1) fixative. After keeping the fixed material under low temperature (4°C) for a minimum period of four hours, the roots were thoroughly washed in the distilled water and stored in 70% ethanol. The roots were hydrolysed at 60°C for 8 minutes and washed thoroughly in running tap water and then the root tips are treated in a 0.25% pectinase solution for 30 minutes in dark and put it in basic fuchsin stain for 30 minutes in dark. The darkly stained extreme tip portion of the roots were excised out and macerated in a drop of 1% aceto-carmine. After maceration the slide covered with cover slip and heated gently over a sprit lamp. The excess stain was removed by giving gentle press with thumb between two layers of filter paper. The slide was temporarily sealed using wax and observed under the Olympus system microscope @ 1000X magnification. The chromosomes were counted from the metaphase cells and recorded pictorially.

Genomic DNA was isolated from three parents and their respective F_1 hybrids by the procedure suggested by Zhang and Stewart (2000). The isolated DNA was quantified using NanoDrop™ 1000 spectrophotometer. DNA was amplified using 2X PCR master mix (8µL), forward (1.0µL) and reverse (1.0µL) primers, 70ng DNA template (1µL) and deionised water (4µL) in a 15µL reaction mixture. After PCR amplification, amplified products were resolved using 3% agarose gel. The gels were viewed by UV illumination and documented using gel documentation system (Gel Doc™XR + Gel documentation system). The SSR markers with high PIC value were selected and obtained from cotton marker database (CMD) (http://www.cottonmarker.org/) and were commercially synthesized and procured from Sai Scientific Company, Coimbatore, TamilNadu . The list of primers used to confirm the hybridity and to identify the polymorphism between the parents and their respective F_1 hybrid were presented in Table 1.

RESULTS AND DISCUSSION

Morphological characters of parents and their F_1 hybrids are compared and presented in Table 2 and 3. Interspecific F_1 hybrids showed either dominance or intermediate expression for various morphological traits. Growth habit,

Table 1. List of Primers used for hybridity confirmation

S.No	Primer name	Primer sequence (5'- 3')	Annealing Temp. (°C)
1	BNL 1034	F: TTGCTTTCAATGGAAAACCC R: CGTCGCAAAGTTGAGAATCA	55
2	BNL 1161	F: CATCTCCTCTGGAAAGAGCG R: ATGAAGCAGCACATTCCATG	55.5
3	BNL 2443	F: TTTATTGGTCGGTCTTTGCC R: TTAGGGTGTTCTTTGGGCAC	51
4	BNL 3424	F: TGTGCCGTCTCAAAATGAAG R: AAGACCAATCTGTTGCCAGC	51
5	BNL 3482	F: ATTTGCCCCAGGTTTTTTTT R: GCAACACCTTTTCCTCCCTA	48.5
6	BNL 3623	F: TTAATAGAGGGACCAA AAGTGATAT R: TTAGCGTTAATATTGTATGTTCAACTC	47.2
7	BNL 3874	F: CATGTTCTAATCATATATATATGATATATATGTGT R: AAAATAACAAAAGCCAT GGAATAA	48.5
8	BNL 3948	F: GTAATGTTCAACACTTTGCTATTCC R: GTTGGTTGGGTGAGCAGAAT	51
9	BNL 3955	F: AGAGATGCAATGGGATCGAC R: ATGTGATAATGCGGGGAATG	50
10	BNL 4108	F: TCCACCATTCCCGTAAATGT R: TGGCCAAGTCATTAGGCTTT	50.2
11	CIR 407	F: GCACAGAACATCCATACA R: TCTCTCTCTCTTTCACACAC	51
12	CIR 413	F: TTAAAGCTCACACACACA R: CAACAGTAACGAAGAACAAT	46.5
13	CM 029	F: TTCCAAGTTCCAATTTCTTC R: ATCAACCACTTTGACAATGTT	44.5
14	JESPR 127	F: GATTTGGGTAACATTGGCTC R: CTGCAGTGTTGTGTTGGGTAGA	51
15	NAU 874	F: AAATGGCGTGCTTGAAATAC R: TGTGATGAAGAACCCTCTCA	49.5
16	NAU 1369	F: TGGCAGAGATGAATGTAAGC R: GGTAACGGATGGAAAATCAC	49.5

leaf incision, leaf shape and size of interspecific hybrid were found to be intermediate. Suvin and TCB 37 had deep leaf incision and *Gossypium armourianum* had no leaf incision, whereas the F_1 hybrid had shallow leaf incision. Leaf shape of Suvin and TCB 37 was palmate with 5 lobes, whereas *Gossypium armourianum* had cordate shape of leaf. In case of F_1 hybrid, leaves were palmate with 3-4 lobes and reduced in size as compared to *Gossypium barbadense* leaves (Plate 1). The results are in agreement with Pushpam and Raveendran (2006). Kaur *et al*., (2016) reported that intermediate leaf shape and size in hybrids between *Gossypium hirsutum* and *Gossypium armourianum*. Similarly, theintermediate expression of plant growth habit, leaf size and petal colour have reported in other interspecific hybrids such as between *Gossypium davidsonii* x *Gossypium anomalum*, *Gossypium hirsutum* x *Gossypium arboreum* (Ahmad *et al*., 2011; Tahir *et al*., 2011), *Gossypium herbaceum* x *Gossypium australe* (Liu *et al*., 2015) and *Gossypium arboreum* x *Gossypium thurberi* (Kale *et al*., 2007). However, some researchers have reported dominance for some characters. For example, *Gossypium hirsutum* x *Gossypium raimondii* triploid hybrid resembled the paternal parent in growth habit (Saravanan *et al*., 2007), Plant stem colouration and hairiness, leaf pubescence, position of stigma, anther colour of *Gossypium armourianum* were found to be dominant as hybrid fully resembled the male parent for these characters (Plate 2). The study of pollen fertility among parents and hybrids also showed significant differences. The pollen fertility percentage was recorded an average of TCB 37 (97.67%), Suvin (96.67%), *Gossypium armourianum* (95.33%), TCB 37 x *G.armourianum* (1.67%) and Suvin x *G.armourianum* (2.33%). Low percentage of pollen fertility was observed in *Gossypium hirsutum* x *Gossypium armourianum* hybrid (9.04 %) and *Gossypium hirsutum* x *Gossypium raimondii* hybrids (9.67%) by Pushpam and Raveendran (2006) and *Gossypium hirsutum* x *Gossypium armourianum* hybrid (2.19 %) by Kaur *et al*., (2016). Pollen size of F_1 hybrids also showed more variation when compared to parents and significant differences were observed. The pollen size of parents and F_1 hybrid was measured using scope image at 100X magnification. The average pollen size of TCB 37 (41.60µ), Suvin (40.87µ), *Gossypium armourianum* (34.34µ), TCB 37 x *G.armourianum* (22.45µ) and Suvin x *G.armourianum* (23.94 µ) were observed. This study can

be used to identify the dominant and recessive characters through F_1s. This can also be used as morphological markers for the confirmation of true hybrids.

Table 2. Morphological traits of parents and F_1 hybrid of Suvin x *G. armourianum*

S.No.	Characters	Suvin	Suvin x *G.armourianum*	*G.armourianum*
1.	Growth habit	Annual, erect	Perennial, semi spreading	Perennial, semi spreading
2.	Stem colour	Dark green with brown	Brownish purple	Brownish purple
3.	Stem pubescence	Sparsely pubescent	Glabrous	Glabrous
4.	Petiole colour	Greenish brown	Brownish green	Brownish green
5.	Leaf shape	Palmate with 5 lobes	Palmae with 3 lobes	Cordate
6.	Leaf colour	Dark green	Green	Green
7.	Leaf incision	Deep	Shallow	Shallow
8.	Leaf veins	Thick and prominent	Thick and prominent	Thin
9.	Leaf texture	Medium smooth and thick	Smooth	Smooth
10.	Leaf hairiness	Very sparse	Glabrous	Glabrous
11.	Bract size	Large	Small	Caducous bract
12.	Corolla colour	Bright yellow	Light yellow	Yellow
13.	Petal size	Medium	Medium	Medium
14.	Petal spot	Present (dark red)	Present (light to dark)	Present
15.	Anther colour	Creamy	Yellow with red spot	Yellow with red spot
16.	Anther density	Dense	Dense	Dense
17.	Filament colour	White to creamy white	Dark red	Creamy white
18.	Position of stigma	Protruded	Protruded	Protruded
19.	Nectar gland	Present	Absent	Absent

Table 3. Morphological traits of parents and F_1 hybrid of TCB 37 x *G. armourianum*

S.No.	Characters	TCB 37	TCB 37 x *G.armourianum*	*G.armourianum*
1.	Growth habit	Annual, erect	Perennial, semi spreading	Perennial, semi spreading
2.	Stem colour	Dark green with brown	Brownish purple	Brownish purple
3.	Stem pubescence	Sparsely pubescent	Glabrous	Glabrous
4.	Petiole colour	Greenish brown	Brownish green	Brownish green
5.	Leaf shape	Palmate with 5 lobes	Palmate with 3 lobes	Cordate
6.	Leaf colour	Dark green	Green	Green
7.	Leaf incision	Deep	Shallow	Shallow
8.	Leaf veins	Thick and prominent	Thick and prominent	Thin
9.	Leaf texture	Medium smooth and thick	Smooth	Smooth
10.	Leaf hairiness	Very sparse	Glabrous	Glabrous
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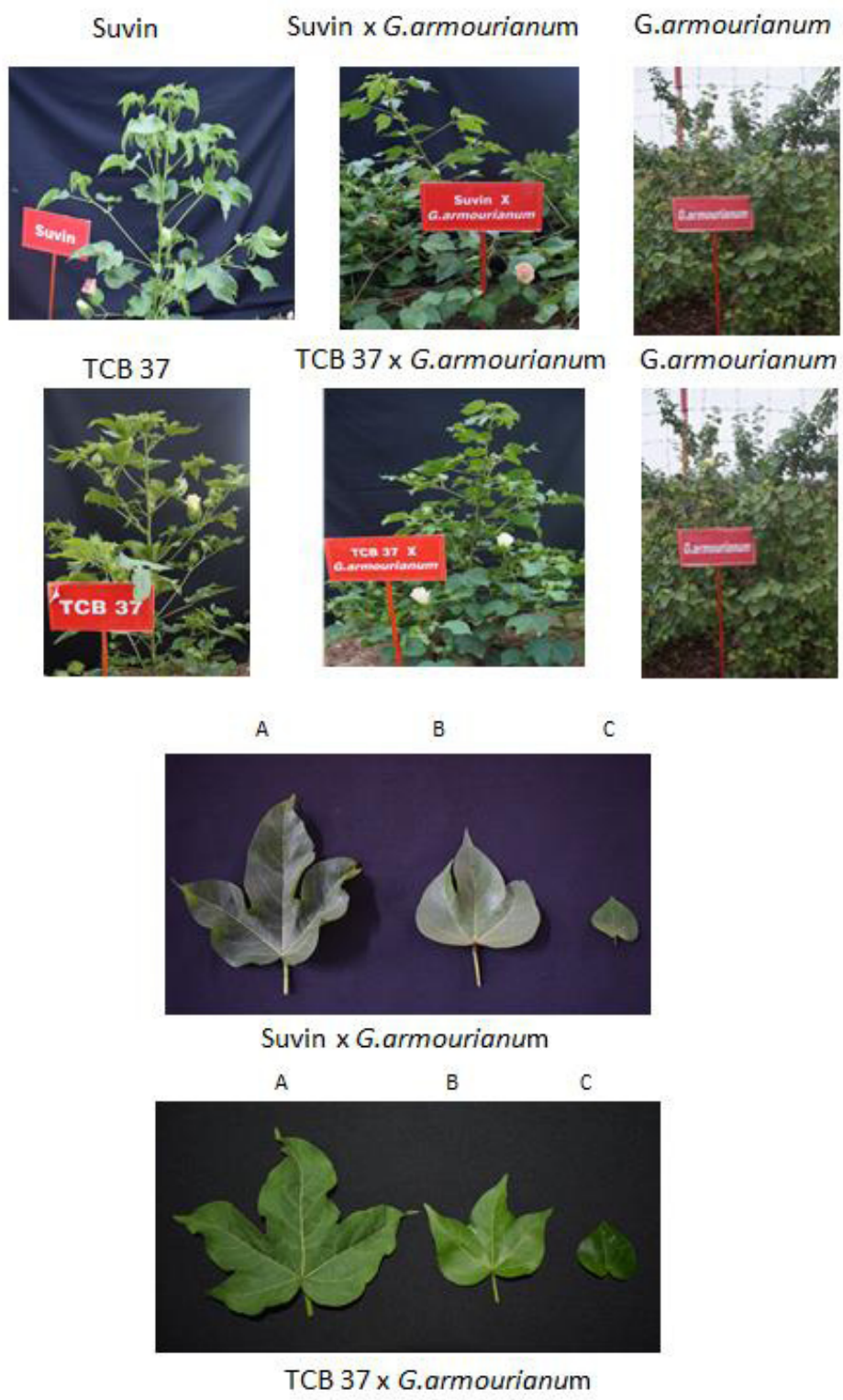
12.	Corolla colour	Bright yellow	Light yellow	Yellow
13.	Petal size	Medium	Medium	Medium
14.	Petal spot	Present (dark red)	Present (light to dark)	Present
15.	Anther colour	Creamy	Yellow with red spot	Yellow with red spot
16.	Anther density	Medium	Dense	Dense
17.	Filament colour	White to creamy white	Dark red	creamy white
18.	Position of stigma	Protruded	Protruded	Protruded
19	Nectar gland	Present	Absent	Absent

Plate 1. Plant and Leaf morphology of parents and F1 interspecific hybrids Suvin x Gossypium armourianum and TCB 37 x Gossypium armourianum



A- Suvin B- Suvin x Gossypium armourianum , TCB 37 x Gossypium armourianum
B- C- TCB 37

Plate 2. Hybridity confirmation through floral morphological features

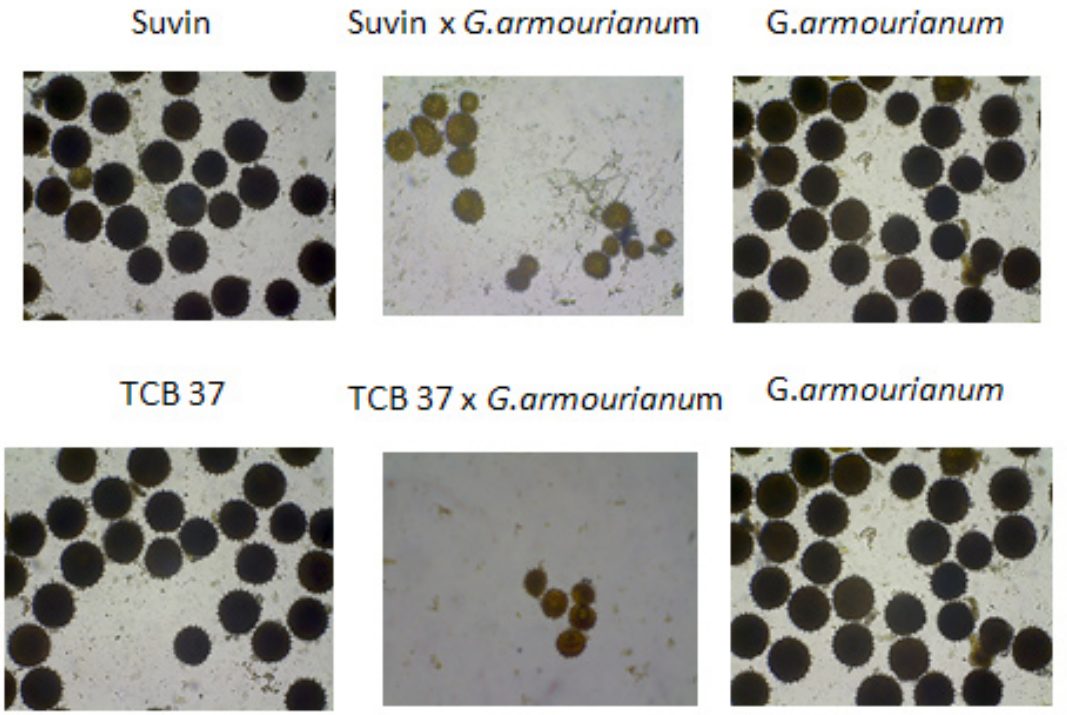


Plate 3. Pollen fertility study for F1 hybridity confirmation

Table 4. Biometrical traits of parents and F_1 hybrid of Suvin x *G. armourianum*

S.No.	CHARACTERS	Suvin	Suvin x G.armourianum	G.armourianum
1.	Bracterial teeth	8.67	8.67	-
2.	Bracterial length(cm)	4.70	2.50	-
3.	Bracterial breadth(cm)	2.17	1.57	-
4.	Petiole length(cm)	7.60	4.47	1.70
5.	Leaf length(cm)	8.97	6.63	3.57
6.	Leaf breadth(cm)	10.77	6.67	4.30
7.	Leaf area(cm^2)	62.87	29.05	9.98
8.	Pedicel length(cm)	2.80	3.43	1.53
9.	Petal length(cm)	5.70	3.47	4.00
10.	Petal breadth(cm)	4.90	2.73	4.17
11.	Pollen size diameter(µ)	40.87	23.94	34.34
12.	Pollen fertility (%)	96.67	2.33	95.33
13.	Gossypol gland density	12.33	8.33	10.33
14.	Length of pistil(cm)	2.73	3.27	3.42

Biometrical characters of parents and F_1 hybrids are compared and presented in Table 4 and 5. Leaf area, leaf length, leaf breadth, bract length, bract breadth, pedicle length and pollen size of interspecific F_1 hybrids were found to be intermediate. TCB 37, Suvin and *Gossypium armourianum* had the leaf area of 56.81 cm^2, 62.87 cm^2 and 9.98 cm^2 respectively whereas the leaf area of F_1 hybrids TCB 37 x *Gossypium armourianum* and Suvin x *Gossypium armourianum* were 25.37 cm^2 and 29.05 cm^2 respectively which are intermediate between both the parents. Petiole length of Suvin and *Gossypium armourianum* was 7.60 cm, 1.70 cm respectively, whereas the F_1 hybrid exhibits the intermediate length of 4.47 cm. Petiole length of TCB 37 and *Gossypium armourianum* was 5.56 cm, 1.70 cm respectively, whereas the F_1 exhibits the intermediate length of 3.73 cm. Thus, the F_1 hybrids are almost intermediate in all the biometrical traits in corresponding to their parents indicate true hybrid. This study is used to increase the yield of the hybrid in subsequent generations.

Table 5. Biometrical traits of parents and F_1 hybrid of TCB 37 x *G. armourianum*

S.No.	CHARACTERS	TCB 37	TCB 37 x G.armourianum	G.armourianum
1.	Bracterial teeth	8.67	8.67	-
2.	Bracterial length(cm)	3.97	2.83	-
3.	Bracterial breadth(cm)	2.97	1.87	-
4.	Petiole length(cm)	5.60	3.73	1.70
5.	Leaf length(cm)	9.10	5.83	3.57
6.	Leaf breadth(cm)	7.93	6.70	4.30
7.	Leaf area(cm^2)	56.81	25.37	9.98
8.	Pedicel length(cm)	1.80	3.20	1.53
9.	Petal length(cm)	3.77	4.30	4.00
10.	Petal breadth(cm)	2.40	3.07	4.17
11.	Pollen size diameter(µ)	41.60	22.45	34.34
12.	Pollen fertility (%)	97.67	1.67	95.33
13.	Gossypol gland density	9.33	9.33	10.33
14.	Length of pistil(cm)	2.77	3.22	3.42

The study of mitotic metaphase counts revealed that the presence of 52 chromosomes in *Gossypium barbadense cv*. Suvin and TCB 37, 26 chromosomes in *Gossypium armourianum*, 39 chromosomes in F_1 hybrids and confirmed the triploid status of the F_1 hybrids developed from cross between Suvin x *Gossypium armourianum* and TCB 37 x *Gossypium armourianum*(Plate 4). The results are in agreement with Manickam and Prakash (2014) were chromosome study showed 39 chromosomes were present in hybrid which is intermediate between *G.hirsutum* (2n = 4x = 52) and *G.armourianum* (2n = 2x = 26). Similarly, Zhang *et al.,* (2014) reported 78 chromosomes (*G. hirsutum* × *G. anomalum* hexaploidy hybrid confirming the amphiploid status of the

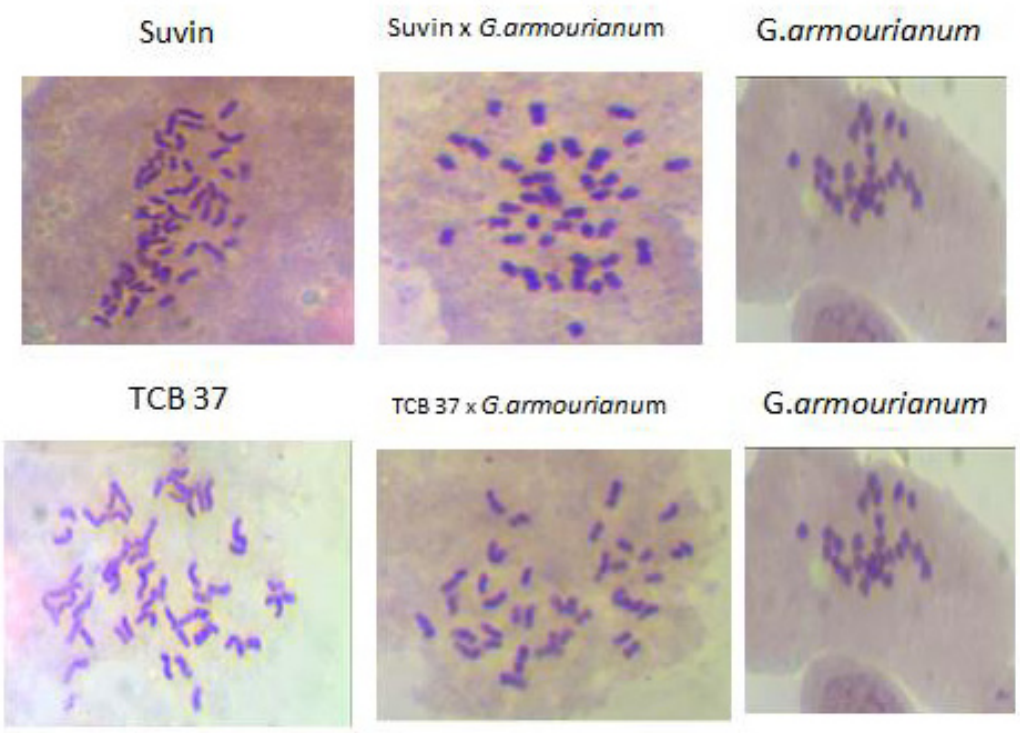


Plate 4. Cytologyical analysis for F1 hybridity confirmation

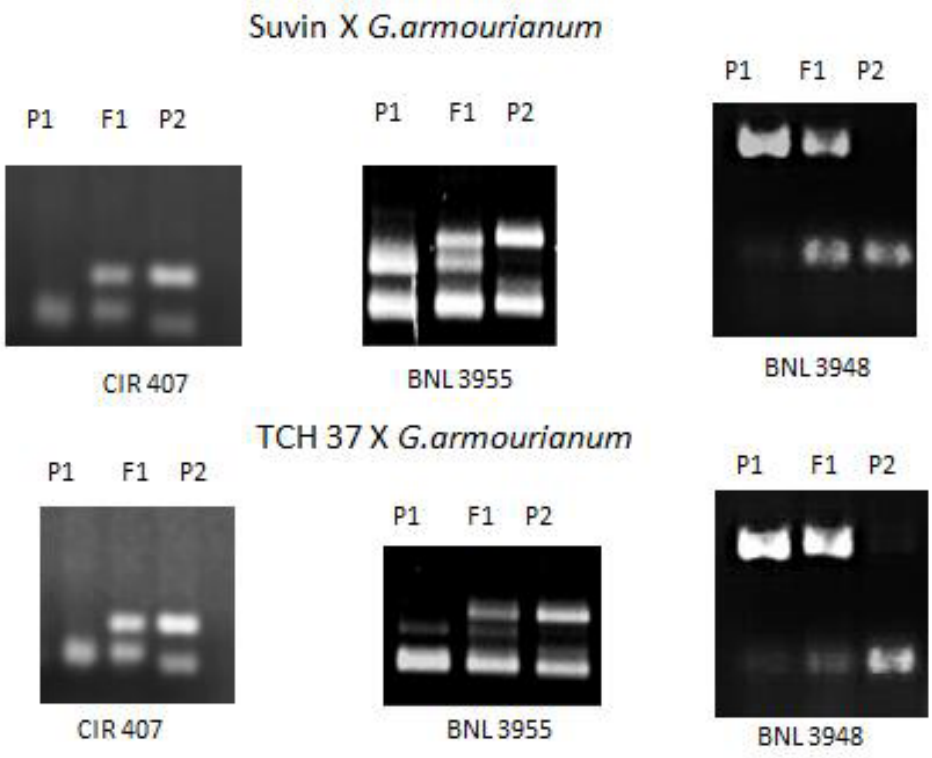


Plate 5. Hybridity confirmation through Molecular marker (SSR) analysis

material because it is in agreement with the number of chromosomes expected for a synthetic hexaploid (2n = 6x = 78) resulting from a cross of *G. hirsutum* (2n =4x =52) and *G. anomalum* (2n=2x = 26). The main purpose of cytogenetic analysis is to confirm the hybrid nature of triploid which can be fairly achieved and to know the pairing behaviour of chromosomes in triploid with generic constitution (Tahir *et al.,* 2011). The interspecific hybrid between Gossypium barbadense cv., Suvin and TCB 37 and *G.armourianum* is an important source for cotton breeders. It has been observed to be resistant to cotton jassid resistance under the field condition. Cotton jassid is a major pest of American as well as Egyptian cotton. Efforts are being made to use this hybrid for the transfer of jassid resistance through crosses with the susceptible Egyptian cotton lines. The interspecific hybrid (ADD) not only provides an opportunity to study chromosomal behaviour during meiosis but also investigating the genic and genomic consequences of allopolyploidy.

For hybridity confirmation female parents (Suvin and TCB 37), male parent (*Gossypium armourianum*) and F_1 hybrids were subjected to polymorphic analysis using 16 cotton specific SSR markers in which 3 SSR markers namely BNL 3948, BNL 3955 and CIR 407 to confirm the hybrid status of interspecific hybrid. BNL 3948 amplified only one locus in female and male parent and two loci in hybrid. BNL 3955 amplified two loci in female and two loci in male parent and four loci were amplified in F_1s. CIR 407 amplified one locus in female and one locus in male parent and two loci in hybrid (Plate 5). The amplification of locus are in accordance with Vij *et al.,* (2016) were they used 55 SSR markers out of which 22 primers showed polymorphisms but only 7 markers confirmed the hybridity status with amplified locus with one and two. This study is used to detect the hybrids in the early stages and to track the flow of genetic material during backcrossing **(**Zhang *et al.,* 2014**)**

The hybrid confirmations for wide crosses are foremost important or further evaluation of segregation generations. The study revealed that the hybridity status of F_1 hybrids developed from cross between Suvin x *Gossypium armourianum* and TCB 37 x *Gossypium armourianum* using morphological, cytological and molecular marker analysis. These F_1 hybrids become an important genetic resources for cotton breeders to develop pest and disease resistant cultivars. These materials can be used as bridges for the transfer of pest and diseases resistant genes from the wild species to cultivated varieties. Even after backcrosses triploid are sterile with no bolls so it should be doubled to hexaploid so that it may be used for further backcrosses with *G.barbadense* to transfer useful traits from *G.armourianum* (Manickam and Prakash ,2014).

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