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Production of an Interspecific Triploid Hybrid Between *Gossypium hirsutum* and *G. Arboreum* by Embryo Rescue

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Abstract
Hand pollination of *G. hirsutum* (2*n*= 4*x*= 52, *AA D*$_1$*D*$_1$) with *G. arboreum* (2*n*= 2*x*= 26, *A*$_1$*A*$_1$) produced 18.7% hybrid seeds. None of the hybrid seeds germinated in pots and in all likelihood the embryo (*A*$_1$*AD*$_1$) and the endosperm (*A*$_1$*AAD*$_1$*D*$_1$) were incompatible. *In vitro* culture of ovules 5 days after pollination (DAP), failed to support the growth of viable embryos. Hybrid embryos 50 DAP, excised from field pollinated hybrid seeds, successfully germinated *in vitro*. Triploid hybrid plants were successfully grown to flowering in pots. The frequency of generation of hybrid plants was 2.3% of cultured embryos. Pollen mother cells of the hybrid plant showed the expected A genome ring of 4 and ring of 6 chromosomes. In addition, there were several unpaired chromosomes of the D genome at metaphase I. The unpaired chromosomes grouped randomly at MII and organized spindles around them. The mature pollen grains were sterile due to aneuploidy.

Keywords: Embryo culture; Gossypium; Interspecific hybrid; Triploid

Introduction

Cotton (*Gossypium* L.) the leading fiber crop of the world consists of 49 species, of which 44 are diploid (2*n*= 2*x*= 26) and possess A through G and K genomes. The remaining five species are AD-genome allotetraploids (2*n*= 4*x*= 52, AADD). The A- and D-genome diploid species are distributed in different hemispheres and show high genetic divergence between them. Only two new world tetraploid species, *G. hirsutum* L. (n= 26, *AA D*$_1$*D*$_1$) and *G. barbadense* L. (n= 26, *AA D*$_2$*D*$_2$) and two old world diploid species, *G. arboreum* L. (n= 13, *A*$_2$*A*$_2$) and *G. herbaceum* L. (n= 13, *A*$_1$*A*$_1$) are cultivated. Cotton is susceptible to biotic and abiotic stresses for which genetic variability available in the cultivated tetraploid species is limited. Molecular studies have demonstrated that upland cotton has a narrow genetic base (Wendel *et al*. 1992; Vafaie-Tabar *et al*. 2004), indicating that crop improvement efforts would benefit from the introgression of new genes. *G. arboreum* is a domesticated diploid (AA genomic constitution) Asian species that is a potential source of genes for upland cotton improvement, especially drought tolerance, and resistances to pests and diseases (Kapoor 2003; Patil *et al*. 2003; Gotmare and Singh 2004). However, upland cotton is reproductively isolated from *G. arboreum* L. via post-zygotic breeding barriers (Beasley 1940; Pundir 1972), in addition to the difference in chromosome number. Inter-specific hybridization among tetraploid and diploid *Gossypium* species followed by breeding, thus hold promise in introducing improved agronomic and quality traits into commercial cottons. Hybrids between allotetraploid species and the two diploid A-genome species, *G. herbaceum* L. *(A*$_1$*A*$_1$*)* and *G. arboreum* L. *(A*$_2$*A*$_2$*)* to generate new gene pools while desirable, are notoriously difficult to obtain. Experimental inter-

specific crosses can only be accomplished between a limited number of closely related species and in all cases, F_1 hybrids between genome groups are poorly fertile (Endrizzi *et al.* 1985).

Successful inter-specific hybridization between diploid species and tetraploid cultivars has been limited by the necessity of embryo rescue and the low fertility of the resulting F_1 hybrids. The hybrids fail to grow because of endosperm and embryo abortion. Improvements in the methods of ovule and embryo culture during the last three decades have however made it possible to produce some triploid hybrids. In all the cases hybrid seedlings or young plants were generated by culturing very young ovules 2-3 days after pollination (DAP) following *in vitro* or *in vivo* pollination (Stewart and Hsu 1977; 1978; Liang *et al.* 1978; Stewart 1979; Gill and Bajaj 1987; Liu *et al*. 1992). Chromosome doubling in Asiatic diploid species can be used as a tool to overcome the incompatibility experienced in *G. arboreum* x *G. hirsutum* species which was reported to be difficult in many cases (Mehetre *et al*. 2003). Recently, some workers identified modification of MS that might also be useful for improving the *G. arboreum* x *G. hirsutum* seedlings from cultured ovules (Wu *et al*. 2004; Sacks 2008).

A reliable, repeatable and easier *in vitro* technique for ovule/embryo culture is desirable and will greatly benefit the development of newer hybrid combinations. This paper reports the successful generation and plant establishment of a triploid hybrid (3x= 39, AAD) between tetraploid *G. hirsutum* (4n= 2x= 52, AADD) and diploid *G. arboreum* (2n= 2x= 26, AA) by a simple method of *in vitro* culture of older (50-60 DAP) hybrid embryos excised from field pollinated seeds.

Material and Methods

Plant materials

Plants of 6 Asian diploid cotton *G. arboreum* and 10 American upland tetraploid cotton *G. hirsutum* were sown in IARI[1] fields during 2004 in 6 m rows and row spacing of 75 cm. The accession numbers/names of the cultivars and their source are listed in *G. hirsutum* was used as the female parent and *G. arboreum* as the male. Flower buds on the maternal plant were hand emasculated at appropriate stages of development during late evenings and pollinated the next morning. Naphthalene acetic acid, 100mg/1 and gibberellic acid 50mg/l was sprayed at the base of the pedicle for 3-5 days after pollination to reduce boll shedding.

Ovule and embryo culture

Calyx was removed from bolls 5 days post-anthesis and the bolls surface sterilized by dipping in absolute alcohol and held briefly to a flame for a few seconds. Ovules were excised aseptically and cultured on liquid medium for 15 days followed by transfer to solid medium. The media were supplemented with 2mg/l IAA and 0.25 mg/l kinetin (Stewart and Hsu 1978).

Embryos from seeds, 50 DAP, were excised aseptically and transferred to liquid Stewart and Hsu (1977) medium on a filter paper bridge for germination and growth. Culture tubes were maintained in a well-illuminated culture room at 27 ± 2^{O}C.

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Table 1. List of cotton cultivars used in this study

	Cultivars	Place of release	Pedigree
	G. arboreum		
1	402	Punjab Agricultural University(PAU), Ludhiana	Selection from local germplasm
2	G27	Punjab Agricultural University(PAU), Ludhiana	Selection from local germplasm
3	LD327	Punjab Agricultural University(PAU), Ludhiana	Selection from arboretum cross (G27 x LD124)
4	Shyamli	Bulandshahar	Selection from local germplasm
5	DLSA17	Dharwad	PA140 (*G. arboreum*) x Purnima (*G. hirsutum*)
6	AK235	Punjabrao Krishi VishwaVidyalaya, Akola	H420 x H487
	G. hirsutum		
1	LH1556	PAU, Ludhiana	(LH886 x LH 901) x LH952
2	H1123	Not available	-
3	F846	PAU, Fazil Kot	452 x LH223-481
4	Pusa 953	IARI, New Delhi	Selection from Bikaneri Narma (Rajisthan)
5	BN	Rajasthan	Selection from *hirsutum* mixture
6	GA	Rajasthan	Reselection of RST9
7	L604	Andhra Pradesh	MCU5 x (L389 x SRT1)
8	RS875	Rajasthan	C-1412 x Delta Pine 66-69-7
9	Pusa4515	IARI, New Delhi	Pusa 959B x BJR734
10	Pusa 8-6	IARI, New Delhi	Pusa 959B x BJR734

Raising plants

Three week old seedlings were maintained in distilled water in culture tubes for 4 days and then transferred to pots. The seedlings were maintained in The National Phytotron Facility at I.A.R.I. After two weeks, plants were transferred to a glass house at 30 ± 2^{O}C and covered with a clear plastic bag. After a few days the bags were partly opened and plants were allowed to harden at low relative humidity before transfer to a bigger pot.

Confirmation of hybridity

Two random primers OPA2 and OPA14 (Operon Biotechnologies, Pvt. Ltd) that were polymorphic between *G. hirsutum cv* L604 and *G. arboreum cv* Shyamli were used to amplify DNA from the parents and hybrid plants by PCR. Parents and hybrid plants were also scored for leaf and floral morphology to confirm hybridity.

Cytology

Anthers were fixed in acetic ethanol, squashed in 1% acetocarmine and pollen meiosis observed in a NIKON E600 microscope.

Results

Field pollination

The results reported here are part of a large program to produce interspecific hybrids in cotton and involve 10 tetraploid *G. hirsutum* as seed parents and 6 diploid *G. arboreum* cultivars as pollen parents (Table 1). A total of 1223 field pollinations were attempted among 14 different cross combinations (Table 2). Boll retention and seed set was observed in all crosses 50 DAP. Highest boll retention (29.3%) was observed in the cross Pusa8-6 x LD327 and the least boll retention (1.8%) in the cross H1123 x LD327. Of the 1223 pollinations attempted in the second year, 687 seeds were generated while ~500 seeds were recovered in the first year. Highest seed set was observed in Pusa 4515 x DLSA17 and BN x Shyamli. Cross

combination Pusa8-6 x DLSA17 showed the lowest seed set per 100 pollinations. On the average, 18% of seeds had embryos. Attempts to germinate the hybrid seeds (from first year) in pots failed. Following failure of seed germination, it was attempted to culture young ovules 5 DAP *in vitro*, the following year. Based on the observation that ~10% of the seeds at 50 DAP had well developed embryos, a similar (if not larger) fraction of ovules 5 DAP were expected to house viable embryos capable of *in vitro* development and germination.

Table 2. Seed setting and recovery of embryos in various crosses among tetraploid (*G. hirsutum*) ♀x diploid ♂ (*G. arboreum*) cotton cultivars. The cross that was successfully taken up to a hybrid plant is highlighted.

Cross Combinations (♀x ♂)	Pollinations	No. (%) # of bolls at 50 DAP	No. of seeds (% seed set) #	% seeds with embryos#
RS875 x Shyamli	132	22(16.7)	82(62.1)	8.3
Pusa8-6 x LD327	48	14(29.2)	8(16.7)	0.0
Pusa8-6 x DLSA17	62	2(3.2)	5(8.1)	0.0
L604 x Shyamli	123	13(10.6)	89(72.4)	20.3
H1123 x LD327	110	2(1.8)	15(13.6)	6.4
GA x 402	83	13(15.7)	38(45.8)	3.6
F846 x AK235	79	5(6.3)	22(27.8)	0.0
Pusa 953 x G27	95	15(15.8)	73(76.8)	22.1
RS875 x 402	51	5(9.8)	9(17.6)	11.8
RS875 x DLSA 17	63	5(7.9)	23(36.5)	7.9
Pusa4515 x DLSA17	102	18(17.6)	136(133.3)	11.8
LH1556 x DLSA17	116	21(18.1)	70(60.3)	10.3
BN x Shyamli	79	13(16.5)	87(110.1)	21.5
L604 X DLSA17	70	10(14.3)	30(42.9)	14.3
Total	1223		687(56.1)	18.7

#All % ages are calculated per 100 pollinations.

Ovule culture

A total of 2727 ovules, 5 DAP, from 14 different *G. hirsutum* x *G .arboreum* crosses, were cultured in *vitro* on Stewart and Hsu medium (Table 3). Ovule growth started within 2-3 days and showed variable callusing responses ranging from 15.75 to 100 % across various crosses. Cross combination Pusa8-6 x LD327 showed 100% callusing while 15% callusing was recorded in the cross RS875 x 402. Ovules, which were not sub-cultured every 4 weeks, stopped growth, secreted phenolic compounds and initiated fiber development. Sixty day-old *in vitro* cultured ovules were as large as *in vivo* developed ovules of the selfed tetraploid but none of the 2727 *in vitro* cultured ovules retained any embryos (Figure 1 A-C). All attempts to culture ovules 5 DAP failed to promote growth of viable hybrid embryos. So this followed by attempt to culture older hybrid embryos (50 DAP) generated from field grown hybrid seeds.

Table 3. *In vitro* callusing response of ovules cultured at 5 DAP from *G. hirsutum* X *G. arboreum* crosses.

	Cross combination	Ovules cultured	Ovules with callus (No., %)
1	Pusa 8-6 X LD327	172	172 (100)
2	RS875 x Shyamli	315	221 (70)
3	GA x 402	187	103 (55)
4	F846 x AK235	150	42 (27.7)
5	L604 x Shyamli	294	186 (63.2)
6	Pusa4515 x DLSA17	305	159 (52.2)
7	Pusa8-6 x DLSA17	112	65 (58)
8	BN x Shyamli	186	104 (55.8)
9	Pusa 953 x G27	202	40 (20)
10	H1123 x LD327	230	195 (84.8)
11	LH1556 x DLSA17	108	50 (46.6)
12	RS875 x 402	209	33 (15.8)
13	RS875 x DLSA 17	80	15 (19)
14	L604 X DLSA17	177	44 (25)
Total		2727	1428 (52.4)

Table 4. *In vitro* germination of embryos (50 DAP) of *G.hirsutum* X *G. arboreum* hybrid seeds from field pollinated flowers

Cross Combinations (♀x ♂)	Seeds dissected	Seeds (50 DAP) with embryo	Embryos cultured	Embryos germinated
RS875 x Shyamli	82	11	11	0
Pusa8-6 x LD327	8	0	0	0
Pusa8-6 x DLSA17	5	0	0	0
L604 x Shyamli	89	25	25	3
H1123 x LD327	15	7	7	0
GA x 402	38	3	3	0
F846 x AK235	22	0	0	0
Pusa 953 x G27	73	21	21	0
RS875 x 402	9	6	6	0
RS875 x DLSA 17	23	5	5	0
Pusa4515 x DLSA17	136	12	12	0
LH1556 x DLSA17	70	12	12	0
BN x Shyamli	87	17	17	0
L604 X DLSA17	30	10	10	0

Embryo culture

Embryos excised from the 687 seeds that were generated by field pollination were cultured. The hybrid embryos (129 in number) were poorly developed, shriveled and necrotic and ranged from $1/10^{th}$–$1/3^{rd}$ the size of the tetraploid (Figure 1 E-G). All the 129 embryos (50 DAP) were cultured on liquid Stewart and Hsu (1977) medium for 1-2 weeks. The three largest embryos from cross combination of L604 x Shyamli (that were about 3-fold larger than the smallest embryo) germinated successfully (Figures 2A, B). Germinated seedlings were transferred from the culture medium to water and then to pots in a phytotron till maturity (Figures 2C, D).

Analysis of hybrids

***Morphological characters*:** The hybrid plants were more vigorous than both parents and showed leaf, flower and fruit morphologies intermediate between them. The diploid *G. arboreum* parent (Shyamli) had smaller and deeply fluted narrow

leaves (Figure 2H) while the tetraploid *G. hirsutum* (L604) had larger, broader and less fluted leaves with acute tips (Figure 2J). The leaves of the hybrid (Figure 2I) were narrow and fluted, like the *arboreum* parent, and had acute tips like the *hirsutum* parent. Petal color, petal spot and anther color of *arboreum* (Figure 2E) was dominant in the hybrid flower (Figure 2F).

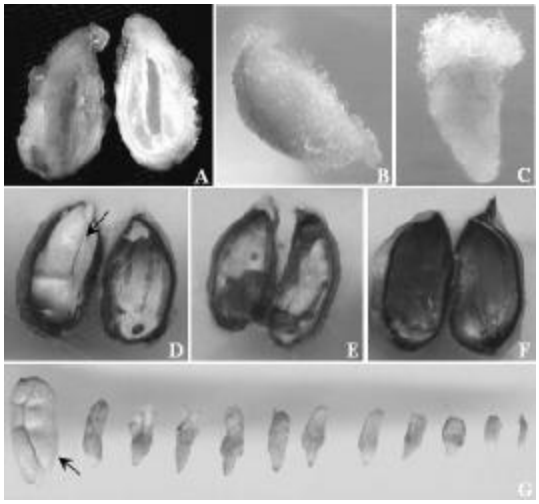

Figure 1. A-C: Ovules of *G. hirsutum* L604 X *G. arboreum* cv Shyamli 5 DAP, 60 days after culture; D: Seeds from *G. hirsutum* selfed controls (female parent) with embryo (arrow); E: Seed harvested from hybrid *G. hirsutum* X *G. arboreum* 50 DAP with embryo inside; F: Empty hybrid seed; G: Embryos excised 50 DAP from *G. hirsutum* control (arrow) and from 11 different *G. hirsutum* X *G. arboreum* hybrid seeds.

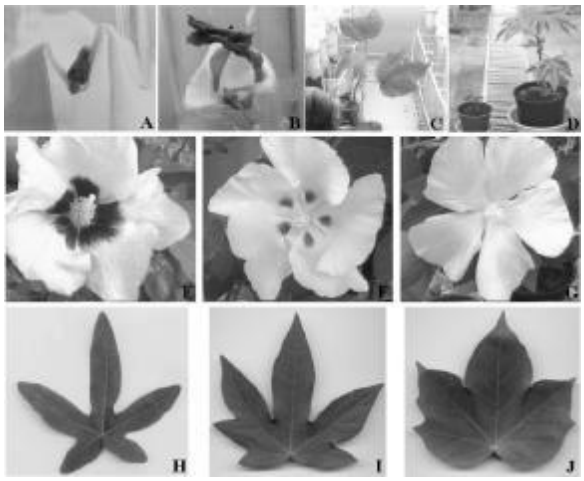

Figure 2. *In vitro* germination and growth of hybrid embryo and obtained plants. A: Hybrid embryo 50 DAP in culture; B: Germinating embryo two weeks after culture; C: Young hybrid seedling transferred to distilled water and to a pot (D); Flowers and leaves of *G. arboreum* (E,H), *G. hirsutum* (G,J) and their hybrid (F,I) .

***Molecular analysis*:** RAPD markers were used to confirm the hybrid status of the putative *G. hirsutum* x *G. arboreum* interspecific hybrid. RAPDs were generated by random primers OPA2 and OPA14 (Figure 3). The RAPD amplicons from the putative hybrid plant generated from each primer pair could be traced to either G. *hirsutum* or *G. arboreum* parents, confirming that it was indeed a hybrid.

Cytological analysis: Pollen Mother Cells (PMCs) of the interspecific F_1 hybrid of *G. hirsutum* X *G. arboreum*, at meiotic metaphase I, showed the expected number of 39 chromosomes (13+13, A, D chromosomes from *G. hirsutum* and 13 A chromosomes from *G. arboreum*) (Figures 4C, D) as against 13 pairs in *G. arboreum* (Figure 4A) and 26 chromosome pairs in *G. hirsutum* (Figure 4B). This confirmed, cytologically, the triploid nature of the hybrid plant. According to theoretical expectations, the PMCs of the triploid ought to have shown 13 (AA) bivalents and 13 (D) univalents. It has been noted by Menzel and Brown (1954) and Endrizzi *et al*. (1985) that the chromosomes of *G. arboreum* and the allotetraploids differ by three interchanges (involving A genome chromosomes) which result in the formation of a ring-of-six and a ring-of-four in their hybrids. Thus a quadrivalent and hexavalent are expected in a *G. hirsutum* X *G. arboreum* hybrid.

In this study, PMCs showed univalents, bivalents, quadri- and hexavalent associations at metaphase I (Figures 4C, D). Mean number of uni-, bi-, tri-, quadri- and hexavalents per PMC was 13.28, 10.94, 0.06, 0.43 and 0.38, respectively (Table 5). The results showed ~13% of PMCs with MI configurations of 13I+8II+ 1IV+1VI. The 13I+13II configuration at MI was most frequent and occurred in 25.8% PMCs, followed by 23% with 13I+10II+1VI. Configurations with 17I and 11II were least prevalent. It was observed that smaller chromosomes mostly appeared in the form of univalents and occasionally associated as bivalents or trivalents at MI. At anaphase I laggards and bridges were often observed. Laggards and bridges at anaphase I resulted in chromosome clusters of two types at metaphase II. Primary clusters consisted of those chromosomes that had reached the spindle poles at anaphase I (Figures 4E, F). In addition there were several secondary clusters consisting of variable chromosome numbers, and were in all likelihood, composed of chromosomes that lagged at anaphase I (Figure 4F).

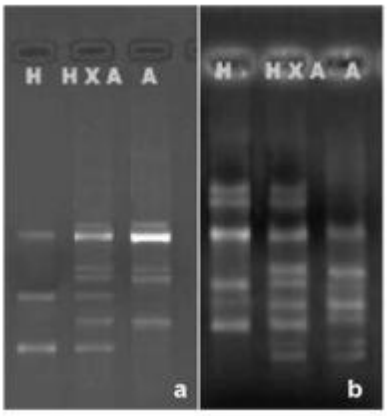

Figure 3. RAPD profiles of *G. hirsutum* (H), *G. arboreum*(A) and their hybrid H × A with random primers OPA2 (a) and OPA14 (b). The H × A lanes (in a and b) shows all the amplicons seen in their respective lanes H and A.

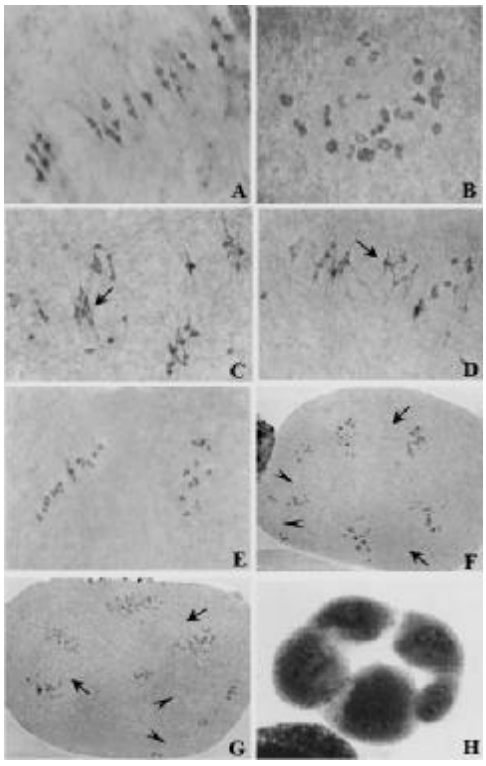

Figure 4. Pollen meiosis in parents and triploid F1 hybrid. A-D: Metaphase I, A: *G. arboreum* AA, 2x= 2n= 26 showing 13 bivalents, B: *G. hirsutum* AADD, 4x= 2n= 54 showing 26 bivalents, C and D: *G. hirsutum* X *G. arboreum* hybrid 3x, 2n= 39, AAD showing mono, bi, tri, tetra (arrow in C) and hexavalent (arrow in D); E: Metaphase II with two primary chromosome clusters in *G. hirsutum* ; F: Metaphase II in triploid F_1 hybrid (*G. hirsutum* X *G. arboreum*) with two primary clusters (arrows) and several secondary clusters (two marked with arrow heads); G: Anaphase II with two primary spindles (arrows) and two supernumerary spindles (arrow heads) in the same PMC; H: Clusters of 5 pollen grains.

At anaphase II, the primary and secondary chromosome clusters entered anaphase II synchronously. While the primary (larger) chromosome clusters formed the two primary spindles, several secondary and smaller clusters of chromosomes (very often with just 1 chromosome) generated spindles around them (Table 6, Figure 4G). Only ~3.2%, of 31 PMCs scored, showed the expected two bipolar spindles. The remaining 97% of PMCs showed multiple

bipolar spindles, often reaching numbers of six spindles in a single PMC. 45.2% of cells showed 3 bipolar spindles, 32.3% of cells showed 4 bipolar spindles and 6.5 % of cells showed 6 bipolar spindles (Table 6, Figure 4G). In general, the secondary spindles had fewer chromosomes than the 2 primary anaphase II spindles. Chromosome numbers in the secondary spindles ranged from as low as 1 to as high as 13 (Table 6). Regardless of their chromosome number, the daughter cells continued the meiotic process and culminated in tetrad, pentad and hexad pollen clusters (Figure 4H). Mature pollen grains were varied in size and were 100% sterile.

Table 5. Chromosome associations at Metaphase I in 209 PMCs of the triploid F_1 derived from *G. hirsutum* and *G. arboreum* (I Univalent, II Bivalent, III Trivalent, IV Quadrivalent, VI Hexavalent)

Combination number	Type of chromosomal associations					Number (%) of PMCs
	I	II	III	IV	VI	
1	13	13				54 (25.8)
2	13	8		1	1	27(12.9)
3	13	10			1	48(23.0)
4	13	11		1		36(17.2)
5	12	12	1			6(2.9)
6	15	10		1		6(2.9)
7	15	12				24(11.5)
8	17	11				1(0.5)
9	12	10	1	1		3(1.4)
10	14	8	1		1	4(1.9)
Average	13.28	10.94	0.06	0.34	0.38	

Table 6. Frequency of bipolar spindles at Anaphase II in PMCs (n=31) of F1 of hybrid *(G. hirsutum* x *G. arboreum).*

	No. of bipolar spindles per PMC				
	2	3	4	5	6
No. of PMCs	1	14	10	4	2
%	3.2	45.2	32.3	12.9	6.5

Discussion

Field pollination between tetraploid *G. hirsutum* and *G. arboreum* generated ~129 hybrid seeds with embryos 50 DAP per 100 pollinations. *G. hirsutum* X *G. arboreum* hybrids are difficult to make and requires the intervention of *in vitro* methods of embryo rescue. Between 1978 and 2002 there have been six reports of attempts to generate hybrids between *G. hirsutum* X *G. arboreum*. All authors resorted to *in vitro* culture of young hybrid ovules 2-5 DAP but a few succeeded in the field establishment of adult hybrid plants up to flowering stages. Stewart and Hsu (1978a,b) and Stewart (1981) cultured 2-4 d hybrid ovules of *G. hirsutum* X *G. arboreum* and reported up to 50% ovules that had embryos of which 10-20% grew to plantlets *in vitro*. Gill and Bajaj (1987) cultured hybrid ovules 3 DAP of which 27.5% of ovules germinated to establish seedlings *in vitro*, but none survived to become adult plants. Liu *et al*. (1992) grew *G. hirsutum* X *G. arboreum* hybrid ovules 2 DAP through five different media to establish

in *vitro* plantlets. Katageri *et al.* (1999) cultured hybrid ovules 3-5 DAP of *G. barbadense* X *G. arboreum* and *G. hirsutum* X *G. herbaceum* and reported less than 1% success in generating plantlets *in vitro*. Surinder Kumar *et al*. (2002) cultured ~3000 *G. hirsutum* X *G. arboreum* hybrid ovules 3 DAP but could not successfully generate hybrid seedlings. Sacks (2008) used different media composition for ovule rescue of interspecific cotton crosses and found a simple modification of MS media (doubling the KNO3) can improve the efficiency of recovering *G. hirsutum* X *G. arboreum* progeny. In the present study direct culture of ovules 2-5 DAP led to extensive callusing and did not support embryonic growth. Ovule culture was abandoned in favor of the culture of older embryos. Embryos 50 DAP successfully germinated *in vitro* and were established as healthy seedlings *in vitro*. Later they were successfully transferred to soil and grown till flowering. The *in vitro* embryo germination method described in this study is a single step method that has advantages over ovule culture through different kinds of media at various growth stages. Secondly, embryos that are 50 DAP are far easier to handle and culture than young ovules 2-5 DAP. Compared to the failure of the previously reported ovule culture attempts, the success rate in the field establishment of healthy flowering hybrid plants was 2.3% (3 plants from 129 cultured embryos).

The *G. hirsutum* X *G. arboreum* hybrid was healthy and flowered normally. Its leaves, flowers and fruit appeared intermediate to the two parents and the hybrid expressed co-dominant RAPD markers unique to each parent. Cytological preparation of meiocytes showed the expected 39 chromosomes ($13AA_1+13D_1$) at metaphase I in 25% of PMCs, confirming cytologically that the hybrid plant was a triploid hybrid between *G. hirsutum* ($2n= 4x= 52$ AAD_1D_1) and *G. arboreum* ($2n= 2x= 26$, A_1A_1). Menzel and Brown (1954) and Endrizzi *et al*. (1985) have shown that the hybrid between *G. hirsutum* and *G. arboreum* has three chromosomal and arrangement differences involving the A genome, which yield a ring of six chromosomes and ring of four. The results showed 13% of the PMCs at MI with one quadrivalent and one hexavalent, confirming the observations made in the past. About 38% PMCs appeared with one hexavalent and 34% PMCs with one quadrivalent. The presence of trivalents in ~6.4% of PMCs and a greater than expected frequency of bivalents in 4% of the PMCs of the hybrid indicates reasonable homology between the A and D genome chromosomes that could permit genetic exchange between them.

The bivalents and multivalents from metaphase I organized into bipolar spindles at metaphase II. Thus there were two “primary” bipolar spindles that were sequestered around the two clusters of bivalent and multivalents from metaphase I. In addition to the two primary spindles that organized around the normally segregated bi and multivalent chromosomes, it was observed that the unpaired univalent chromosomes from metaphase I promoted the nucleation of spindle fibers to organize multiple mini-spindles at metaphase II. Even a single

univalent was sufficient to organize a mini-spindle, and there were several mini secondary spindles that consisted of 2-5 chromosomes. Multiple spindles (as many as 5 per cell) described here were seen in 97% of the PMCs. The primary and secondary spindles were rarely of identical polarity and occupied complementary non-overlapping spaces within the PMC. Despite the large numbers of spindles per cell and high proportion of PMCs with secondary spindle formation, the chromatin cycle appeared synchronous, and meiosis II was completed in all the spindles simultaneously. This clearly suggested that the secondary spindles not only did not interfere with the function of the primary spindles, but also responded to cell cycle cues in synchrony with the primary spindles. The occurrence of multiple spindles very strongly suggests that *Gossypium* does not possess centrosome-like structures that organizes a single bipolar spindle. Microtubule networks (remaining from meiosis I in this case) appear to be spread randomly in the cytoplasm and are capable of forming normal multiple bipolar spindles of diverse polarities when presented with chromatin. The PMCs successfully completed meiosis and developed into mature pollen with different sizes.

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