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Assessment of gene flow between *Gossypium hirsutum* and *G. herbaceum*: evidence of unreduced gametes in the diploid progenitor.

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Running title

Unreduced gametes in diploid cotton

Key words

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Abstract

In the framework of a gene flow assessment, we investigated the natural hybridization rate between *Gossypium hirsutum* (AADD genome) and *G. herbaceum* (AA genome). The latter species, a diploid progenitor of *G. hirsutum,* is spontaneously present in South Africa. Reciprocal crosses were performed without emasculation between *G. herbaceum* and *G. hirsutum*. Neither examination of the morphological characteristics nor flow cytometry analysis of the 335 plants resulting from the *G. hirsutum* x *G. herbaceum* cross showed any hybrid features. Of the 148 plants produced from the *G. herbaceum* x *G. hirsutum* cross, three showed a hybrid phenotype, and their hybrid status was confirmed by SSR markers. Analysis of DNA content by flow cytometry and morphological traits clearly showed that two of these plants were triploid (AAD). The third plant had a flow cytometry DNA content slightly higher than *G. hirsutum*. In addition, its morphological characteristics (plant architecture, presence and size of petal spots, leaf shape) led us to conclude that this plant was AAAD thus resulting from fertilization with an unreduced AA gamete of the female *G. herbaceum* parent. Fluorescent *In Situ* Hybridization (FISH) and meiotic behavior confirmed this hypothesis. To the best of our knowledge, this is the first description of such gametes in *G. herbaceum*, and it opens new avenues in breeding programs. Furthermore, this plant material could provide a useful tool for studying the expression of genes duplicated in the A and D cotton genome.

INTRODUCTION

Assessment of gene flow from a crop to a wild relative is particularly relevant in the case of allopolyploid species, which are formed by hybridization between related species, when one of its progenitors is present in cultivated areas. In certain geographical areas, this may be the case for the cultivated tetraploid cotton (*Gossypium hirsutum*, AADD, 2n=4x=52) and a wild diploid species (*Gossypium herbaceum*, AA, 2n=2x=26). Cultivated cotton, the most important source of natural fibre, has been the long-standing subject of a number of taxonomical studies (Watt, 1907; Hutchinson *et al*. 1947; Saunders, 1961; cited in Wendel *et al*. 2010; Fryxell, 1979, 1992). The cotton genus (*Gossypium* L.) includes 50 diploid species (2*n*=2*x*=26) that can be differentiated cytogenetically into eight genome groups (A-G & K), and seven allotetraploid species (2*n*=4*x*=52) (Fryxell, 1979; Fryxell, 1992; Stewart 1995, Wendel and Croon, 2003, Grover *et al*. 2015, Gallagher *et al*. 2017). Remarkably, among the four domesticated species, there are two allotetraploids (the New World tetraploids *G. hirsutum* and *G. barbadense*) and two diploids (the Old World diploids *G. arboreum* and *G. herbaceum)*. The tetraploid species originated from a single polyploidization event between an A-genome diploid species and a D-genome diploid species (Wendel and Cronn, 2003). The two African-Asian A-genome cottons, *G. arboreum* and *G. herbaceum*, are the equivalent of the maternal genome donor to polyploid cotton (Wendel, 1989), although some evidence suggests that *G. herbaceum* may more closely resemble the A-genome donor than *G. arboreum* (Wendel *et al*. 2010). Recent results have suggested an independent domestication of these two Old World cotton species (Renny-Byfield *et al*. 2016). Grover *et al* (2015) demonstrated that this combination of a D-genome progenitor, resembling modern *G. raimondii*, and an A-genome progenitor, much like modern *G. arboreum* and *G. herbaceum*, occurred as a single hybridization event (genomic merger and doubling) that gave rise to all polyploid cottons. Interspecific hybridizations have been used extensively to improve yield,

fibre quality or other agronomic traits, and numerous studies have focused on the introgression of traits of interest from wild diploid cotton into cultivated cotton *G. hirsutum* (Meyer, 1974; Stewart, 1995; Mergeai, 2004, Chee *et al*. 2016). Colchicine doubling is an essential step in producing an improved cultivar through interspecific crossing, as the recovered triploid hybrids are sterile. Unreduced gametes have proven useful in enabling crosses between plants of different ploidy levels which otherwise often fail due to unbalanced parental contributions in the developing seed (Barcaccia *et al*. 2003; Carputo *et al*. 2003). If the plant of the lower ploidy level can be induced to produce unreduced gametes, such limitations can be overcome. This strategy has been successfully used in various species such as potato, alfalfa and manioc (reviewed in Brownfield and Kohler, 2011; Dewitte *et al*. 2012).

The large-scale development of transgenic insect-resistant and/or herbicide-tolerant cotton has promoted studies about the ability of native diploid A or D species to produce fertile hybrids with tetraploid cotton (Stewart, 1995; Brubaker *et al*. 1999). In South Africa, and particularly in the KwaZulu Natal Province, where the commercialization of transgenic Bt cotton began in 1998, a wild species (*G. herbaceum)*, one of the progenitors of *G. hirsutum*, is found in areas neighbouring the cultivated cotton fields. Consequently, we have undertaken a study to evaluate the likelihood of gene flow between the allotetraploid cultivated cotton (*G. hirsutum*) and its wild diploid relative (*G. herbaceum*). Transgene escape depends on the generation of fertile hybrids, and the possible selective advantage that would result in the development of weedy derivatives. In this paper, we present our analysis of natural cotton gene flow in South Africa, which furthermore provides evidence for unreduced gametes in the species *G. herbaceum*.

MATERIALS AND METHODS

Plant material

Reciprocal crosses were performed without emasculation between *G. herbaceum* Indian accession Boumi Aria (A_1A_1, 2n=26) and *G. hirsutum* ($A_tA_tD_tD_t$, 2n=52; lower case letter "t" referring to the "tetraploid" genome) Delta Opal RR from Deltapine, South Africa. Forty plants from each accession were maintained in individual pots in a greenhouse at Pretoria University and divided into two batches: donor plants for pollen harvest and recipient plants for hybrid production. To simulate both pollination occurring in the field and regular visits by pollinators to plants, pollen harvested from newly opened flowers was spread on the recipient flowers without emasculation two or three times between 9 and 11 am. All the subsequently produced seeds were sown (one seed per pot) in greenhouses at the Versailles Centre of the French National Institute of Agricultural Research (INRA) to screen for hybrids.

Seeds with a thick seed coat harvested from *G. herbaceum* were scraped with sandpaper to facilitate germination, and then placed on wet paper at 24°C. Germinating seeds were planted in soil in a greenhouse following radicle emergence.

Morphological examinations

Morphological examinations were made both at the vegetative stage (six leaves) and at the reproductive stage. Details of flower morphology were recorded. In plants that exhibited hybrid characteristics, particular attention was paid to flower size and color, and especially to the presence and intensity of red petal spots, as *G. hirsutum* has no spot and *G. herbaceum* shows large, bright red spots.

Pollen viability

Pollen viability was assessed after Alexander dye staining (Alexander, 1969) with a 3-hour incubation period at 50°C. Several samples were taken in the greenhouse between 10 and 11 a.m. on several distinct days.

Estimation of nuclear DNA amount by flow cytometry

Flow cytometry analyses (Partec ploidy analyser, Munster, Germany) were performed for all progeny on leaf tissue to assess their nuclear DNA amount, as described by Eber *et al*. (1997). Barley leaves served as the internal reference with set value of 100. Preliminary experiments confirmed that the measure was not affected neither by leaf age or conservation delay (5 to 20 minutes) of shredded leaves in the buffer (Kit Partec, Munster, Germany). Three measurements with independent leaf samples were performed for each control and potentially hybrid plant. Twenty plants were analysed for the *G. hirsutum* and *G. herbaceum* parents. The two-fold size difference between the two genomes A and D was taken into account to determine chromosome numbers.

Meiotic behavior and chromosome counting

Young squares (flower buds) were sampled in the greenhouse from the two parental lines and for deemed hybrid plants according to flow cytometric analysis and morphological observations. They were fixed in Carnoy's solution (ethanol–chloroform–acetic acid, 6: 3: 1) for 24 hours and preserved in 70% ethanol until characterized. For the observation of pollen mother cells (PMCs), anthers were squashed in 1% acetocarmin and cells inspected with a light microscope. PMCs were observed at metaphase I to establish the meiotic behavior of the hybrids.

SSR marker analysis

Microsatellite (SSR) markers were used to verify the hybrid status of progenies obtained from the different hybridizations. Six SSRs (BNL 3264, BNL 3563, BNL 409, BNL 4049, NAU

0915, NAU 1246) that demonstrated clear polymorphisms between *G. hirsutum* and *G. herbaceum* were selected, based on prior screens of diploid and tetraploid accessions (unpublished data). Their primer sequences are available from CottonGen (https://www.cottongen.org/). Sixty-six DNAs were analyzed, including 64 progenies from the 2 reciprocal crosses (46 for *G hirsutum* x *G herbaceum*, and 18 for *G. herbaceum* x *G. hirsutum*), and from control DNAs of each parental species. Polymorphic markers between the two species were kept for hybrid characterization. DNA extraction, PCR amplification and electrophoresis-based allele separation were performed according to Lacape *et al* (2007). The progenies were assigned an allelic "hybrid" coding when alleles of both *G. hirsutum* (could be 1 or 2 amplicons) and *G. herbaceum* (an additional unique amplicon) were detected.

Fluorescence *in situ* hybridization (FISH)

Mitotic chromosomal squashes were prepared with standard techniques. FISH experiments were carried out with two DNA probes: the 45S rDNA probe (pTa 71) is a cloned 9-kb *Eco*RI fragment of a rDNA repeat unit (18S-5.8S-26S genes and spacers) isolated from *Triticum aestivum* (Gerlach and Bedbrook 1979) and the pTa 794 probe is a 410 bp *Bam*HI fragment of the 5S rDNA from wheat (Gerlach and Dyer 1980). The two probes were labelled by random priming with Alexa-488 dUTP (Invitrogen, Life Technologies) and Alexa 594 (Invitrogen, Life Technologies) respectively. Genomic *in situ* hybridization (GISH) was performed with probe DNAs from the progenitors of the allotetraploids. Total genomic DNA from *G. herbaceum* (A-genome) was labeled by nick translation with biotin-16-dUTP (Roche Diagnostics, Indianapolis, IN, USA). Total genomic DNAs from *G. raimondi* (D-genome) were autoclaved to yield fragments of 100-300 bp and used as a blocking DNA. A series of test assessments determined the amount of blocking DNA that was required to discriminate between the two genomes. Optimum results were obtained with a ratio of 1:50 for A genome probe and D genome blocking DNA in the hybridization mixture. Biotinylated probes were

immunodetected by Texas-red-conjugated with avidin antibodies (Vector Laboratories, Burlingame, CA). The chromosomes were mounted and counterstained in Vectashield (Vector Laboratories) containing 2.5μg/mL 4,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured using a CoolSnap HQ camera (Photometrics, Tucson, Arizona) on an Axioplan 2 microscope (Zeiss, Oberkochen, Germany) and analyzed with the MetaVue^{TM} software (Universal Imaging Corporation, Downington, PA).

RESULTS

Interspecific F1 hybrid Production

G. hirsutum **($A_tA_tD_tD_t$) x** ***G. herbaceum*** **(A_1A_1)**

After pollination, moderate boll shedding was observed, as 60 mature bolls were obtained from 68 pollinated flowers. A total of 335 plants were obtained from 504 sown seeds. Flow cytometry analysis revealed that all of them were identical to the female allotetraploid parent, *G. hirsutum*. This observation was confirmed by their morphological characteristics including plant architecture, leaf shape as well as flower size, shape and color. No interspecific hybrid was obtained.

G. herbaceum **(A_1A_1) x** ***G. hirsutum*** **($A_tA_tD_tD_t$)**

Significant boll abscission was observed from the hybridizations involving *G. herbaceum* as the female (29 mature bolls out of 146 pollinated flowers). Similarly, a large number of aborted seeds were harvested (163 aborted seeds out of a total 498 harvested). Out of 335 seeds sown, 148 developed into plants: among these, three interspecific hybrids were detected. Flow cytometric analysis showed that two of them (namely H1 and H2) had an intermediate value possibly corresponding to AAD hybrids. The third hybrid (H3) exhibited a slightly higher DNA content than *G. hirsutum*.

Our results indicate a hybridization rate of 0.02 hybrid seed per pollinated flower, compared to ~30 seeds per boll observed after selfing of *G. hirsutum*. Among the 148 plants observed, the interspecific hybridization rate detected was 0.02.

Characterization of interspecific F1 hybrids

Morphological traits

Among the 148 plants obtained with *G. herbaceum* as the female parent, observations 20 days after sowing (at the three-leaf stage) led to the morphological identification of the three hybrids. Already at a young stage (three-leaf, 20 days after sowing), three plants exhibited general morphological traits similar to *G. hirsutum*. Several weeks later, two types of plants could be distinguished. Among the three hybrids, H1 and H2 displayed identical morphological characteristics and exhibited the phenotype typical of a triploid hybrid (AAD), with traits intermediate between the two parents (Fig 1). For the third hybrid (H3), the overall plant phenotype was intermediate between *G. hirsutum* and the triploid (Fig 1), with individual plant parts varying between the triploid and *G. herbaceum*. Hybrid H3 leaves had less marked serrations but more lobes and were intermediate between *G. herbaceum* and the triploid (Fig 2). In its first stages of development, the hybrid H3 displayed density of stem hairs and gossypol glands similar to those of *G. herbaceum*. The two types of hybrid phenotypes were also differentiated by flower characteristics: corolla size, petal red spot intensity and size and stamen color and size (Fig 3). The corollas of the H1 and H2 hybrid flowers had the same size as *G. hirsutum*, but they exhibited light red spots (Fig 3). H3 had larger, brighter red spots, intermediate between AAD and *G. herbaceum* (Fig 3), and its corolla size was slightly smaller than those of H1 and H2. These observations can support the hypothesis that the H3 hybrid carries more genome A than the *G. herbaceum* parent. Moreover it has been reported that spot presence is controlled by a single gene carried by the A genome (Stephens, 1974).

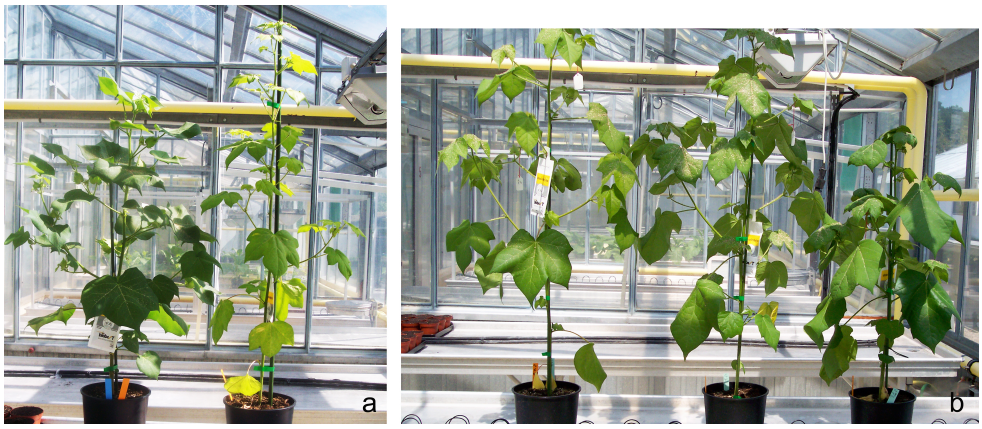

Fig 1: Plants at 70 days of culture. a: left to right *G. hirsutum* , *G herbaceum*, b: left to right, hybrid H1, hybrid H2, hybrid H3.

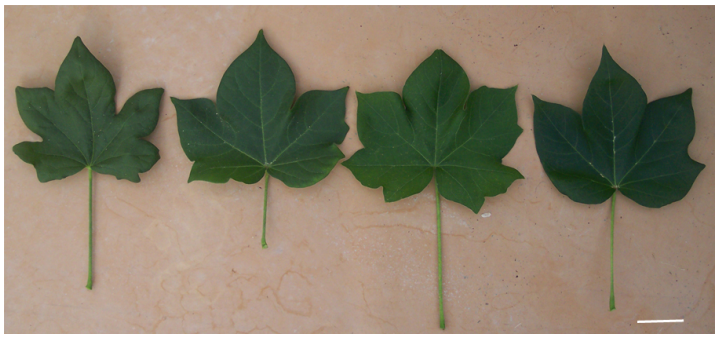
Fig 2. Leaf shapes. Left to right *G. herbaceum*, hybrid H1, hybrid H3, *G. hirsutum*. Bar=1cm


Fig 3. Flower characteristics: Left to right: *G. herbaceum*, hybrid H1, hybrid H3, *G. hirsutum*. Bar=1cm

Microsatellite-based allelic status of the interspecific hybrids

One of the six SSRs tested was not exploited because of ambiguous allele assignment. For the five remaining SSRs (BNL3563, BNL409, BNL4049, NAU0915, and NAU1246), the hybrid status of hybrids H1, H2 and H3 was confirmed (Fig 4). The three hybrids exhibited the alleles from *G. hirsutum* as well as from *G. herbaceum*. However, the amplification profiles did not reveal any quantitative difference between these hybrids.

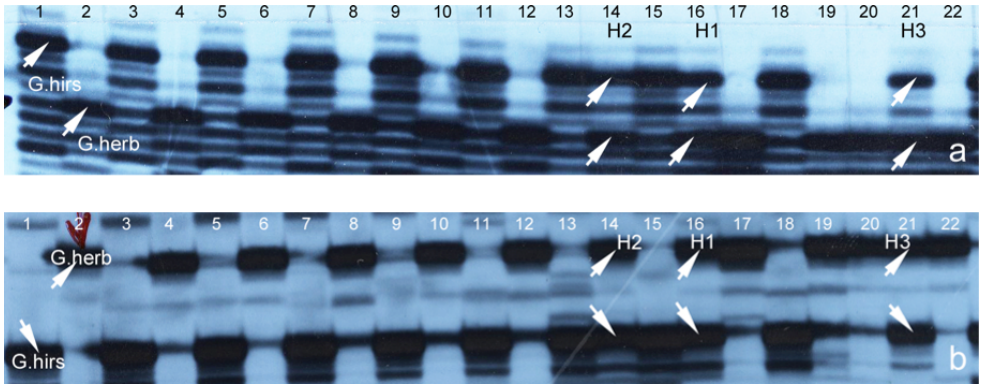

Fig 4: Molecular profiles of parents *G. hirsutum, G. herbaceum* and 20 progenies showing the hybrid profile of H1, H2 and H3. a :marker BNL409, b : marker NAU1246

Identification of the genomic structure of hybrids

Results of the FISH analysis at mitosis showed that *G. hirsutum* ($A_tA_tD_tD_t$) carried six 45S loci with 2 loci on A_t genome (Fig 5 a-b), in accordance with previous reports (Hanson *et al* 1996; Gan *et al*. 2013). However, six 45S loci were also observed on the A genome of *G. herbaceum* (A_1A_1) (Fig 5c). The structure of H1 and H2 hybrids was confirmed with 39 chromosomes, and we found the expected number of 45S loci for an $A_1A_tD_t$ genomic structure, i.e. six in total with three from *G. herbaceum* and three from *G. hirsutum* (Fig 5d). Analysis of H3 resulted in a count of 52 chromosomes carrying nine 45S loci six from *G. herbaceum* plus three from *G. hirsutum* thus compatible with the genome structure $A_1A_1A_tD_t$ (Fig 5e).

Therefore, this hybrid was formed from a female unreduced gamete of *G. herbaceum* and a reduced gamete of *G. hirsutum* (Table 1). According to the genomic composition ($A_1A_tD_t$) of H1 and H2 plants, the expected meiotic behavior is 13 univalents corresponding to the D genome and 13 bivalents between the homologous chromosomes of the A genomes. Thirty-five percent of the cells of 26 analyzed PMCs matched this configuration, but 57.7% revealed the presence of multivalents, trivalents or quadrivalents (Fig 6a, Table 2). In the third H3 hybrid, all the 15 PMCs observed indicated the presence of multivalents likely due to the presence of three homologous A genomes (Fig 6b, Table 2). Tetrads harbouring 3 to 7 microspores have been observed from young flowers of the triploid hybrids H1 and H2.

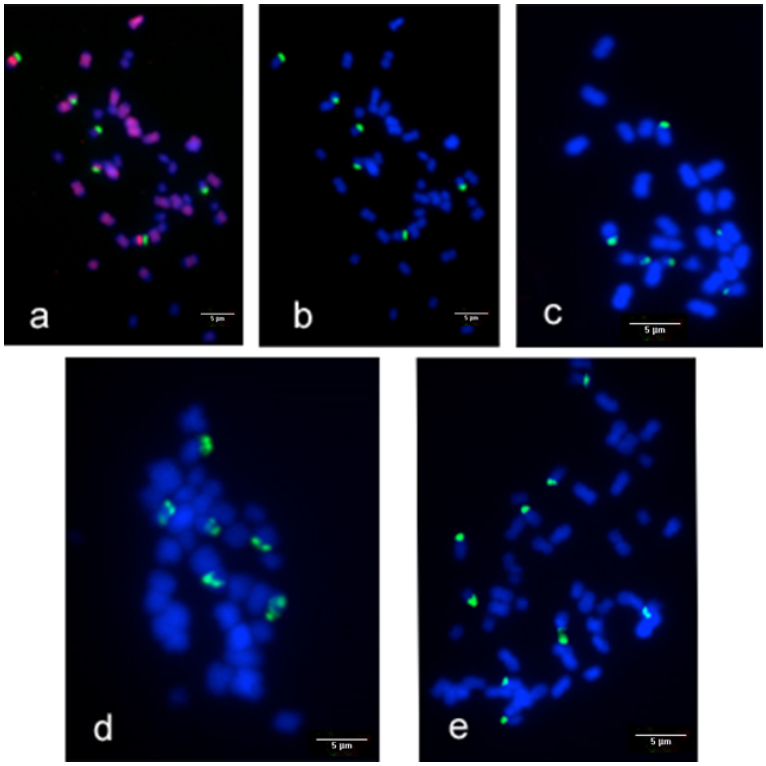

Fig 5: *In situ* hybridization analysis. (**a**) The GISH probe hybridized specifically to all A genome chromosomes (pink) in *G. hirsutum* (AADD); 5SrDNA in red, (**b-e**) FISH with 45S rDNA in green. FISH analyses of somatic metaphase chromosomes from *G. hirsutum* (**b**), *G. herbaceum* (**c**), hybrid H2 (**d**) and hybrid H3 (**e**). Chromosomes were counterstained with DAPI (blue).

	CHROMOSOMES NUMBERS	45SrDNA SIGNAL NUMBERS	GENOME STRUCTURE
G. HIRSUTUM	52	6 : 2 (A_t A_t),4 (D_t D_t)	A_t A_t D_t D_t
HYBRID H1	39	6 : 1 A_t, 3 A_1, 2 D	A_1 A_t D
HYBRID H2	39	6 : 1 A_t, 3 A_1, 2 D	A_1 A_t D
HYBRID H3	52	9 : 3 A_1, 3 A_1, 1 A_t, 2 D	A_1 A_1 A_t D

Table 1: 45S rDNA signal numbers and genome structures

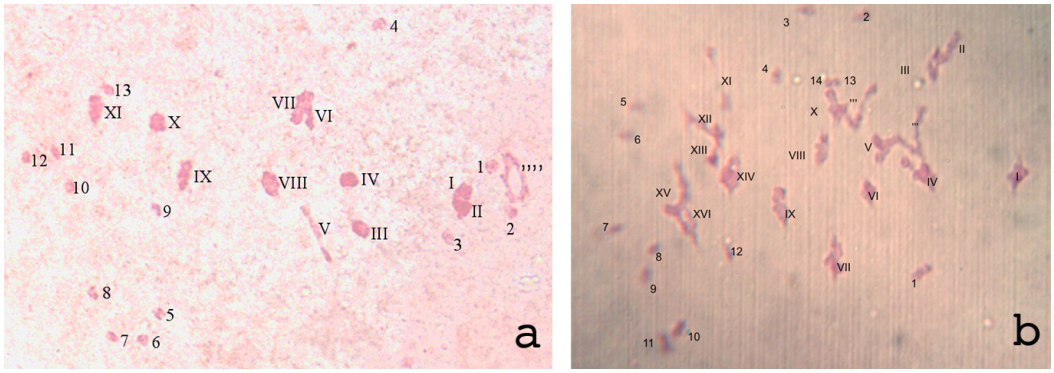

Fig 6: PMC in metaphase I. **a** from AAD hybrid H1, 13 univalents (1-13), 11 bivalents (I-XI) and 1 quadrivalent; **b** from AAAD hybrid H3: 14 univalents (1-14), 16 bivalents (I-XVI) and 2 trivalents.

	2n	PMC	UNIV.	BIVAL.	TRIVAL.	QUADRIVAL.
HYBRID H1 – H2	39	26	12.61* (9-14)**	11.81 (7-14)	0.31 (0-2)	0.17 (0-2)
HYBRID H3	52	15	14.67 (12-17	16.8 (14-20	1.07 (0-4)	0.13 (0-1)

Table 2: Meiotic behavior of hybrids H1, H2 and H3. *: average per cell, ** range

Fertility of interspecific hybrids

As expected, pollen grains from the parental lines had a high viability (Fig 7). In contrast, very few pollen grains (< 5%) from hybrids H1 and H2 were stained by Alexander dye (Fig 7). Pollen grains from the hybrid H3 exhibited a range of viability, up to 20%. Sampling was performed on all the plants at the same time, and the fertility of *G. herbaceum* was observed to be lower than that of *G. hirsutum*. This difference in the viability of the pollen grains from *G. hirsutum* and *G. herbaceum* could be explained by the time of the sampling. Flowers were collected at the same time in the morning, which might not have been optimal for the sampling of *G. herbaceum* pollen.

None of the three hybrids produced any seed, either through open or self-pollination. In order to save the hybrid materials, the plants were reproduced through cutting when they became too large.

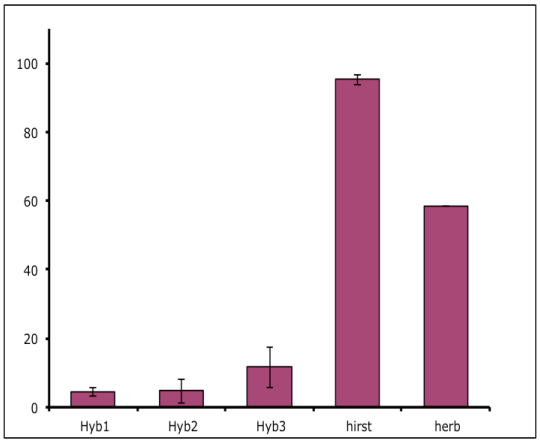

Fig 7: Pollen viability (percentage). Seven repetitions (40 to more than 1000 pollen grains per sampling) for *G. hirsutum* and H1, H2, and H3. One observation on 120 pollen grains for *G. herbaceum*.

DISCUSSION

The results showed a relatively high hybridization rate (three hybrids among 148 progeny) between *G. herbaceum* and *G. hirsutum* without emasculation of the female parent and when the plant with the lower ploidy level was the female. This result is similar to those observed with *Brassica napus*, which is also an allotetraploid species; when one of its progenitors, *B. rapa* was used as female, the spontaneous interspecific hybridization ranged from 3 to 8% (Warwick *et al*. 2003). When the diploid parent *G. herbaceum* was used as male, no hybrids were obtained. These results are not in accordance with those of Brubacker *et al*. (1999), working on the improvement of cultivated cotton by crosses with diploid Australian species (genomes C, G and K). They found that it was more difficult to obtain triploid hybrids when the female parent was diploid. Similarly, Beasley (1940) concluded that the likelihood of successful hybridization is higher if the female parent has the higher ploidy level, reflecting the traditional recommendations concerning the critical role of ploidy ratio of endosperm and zygote for successful embryo development (Beasley, 1942; Stephens, 1942 cited in Ahoton *et al*. 2003). Similarly, it has been observed among *Brassicaceae* species that the production of interspecific hybrids using *B. napus* as female or male with different diploid species depends on the species combination (Chèvre *et al*. 2004). Our combined analyses based on cytometry, fluorescent *in-situ* hybridization and chromosome counting indicated that the three hybrids obtained were of two types. Two, H1 and H2, were triploids (AAD) and, remarkably, the third one (H3) was a tetraploid carrying three A genomes and a single D genome. The meiotic behavior of triploid hybrids is close to what can be expected with a majority of pollen mother cells with 13 bivalents corresponding to homologous pairing of the 13 A chromosomes, whereas the D chromosomes remained at univalent stage with 13 univalents. However, it is important to note that several cells showed the presence of multivalents, indicating the possibility of homoeologous pairing between A and D genomes, even if these exchanges were

difficult to detect after a long evolution in *G. hirsutum* (Page *et al*. 2016). In the third hybrid, the presence of more than two A genomes was shown by the morphological characteristics (leaf shape, flower size and red spots on the corolla). Its genomic structure, $A_1A_1A_tD_t$, was confirmed by observation of metaphase in mitosis, revealing the presence of the nine 45S loci expected for this genomic structure. Additionally, the high frequency of multivalents was in agreement with the presence o f three A genomes.

Our primary objective was to estimate the risk of cross-pollination between allotetraploid cultivated cotton (*G. hirsutum*) and wild diploid cotton (*G. herbaceum*), since in the Makhathini flats area of South Africa the diploid progenitor grows in proximity to the cultivated transgenic cotton fields. Most of published studies were concerned either with gene flow between transgenic and non-transgenic cultivated cotton in various areas of the world (Zhang *et al*. 2005; Van Deynze 2005; Llewellyn *et al*. 2007; Heuberger *et al*. 2010; Loureiro *et al*. 2016), or between wild and cultivated tetraploid cotton (Wegier *et al*. 2011; Pereira *et al*. 2012). Our study on the hybridization rate between diploid and tetraploid cotton, showed that the three recovered hybrids produced no seeds and that the viability of their pollen was very low. The single seed was produced after crossing a triploid hybrid and *G. hirsutum* developed after hand-pollination on an emasculated flower of the triploid (data not shown). It is reasonable to conclude from the present study that the probability of obtaining offspring is very low. Moreover, only the species *G. herbaceum* var. *africanum* is endemic in South Africa (van Wyk and van Wyk, 1997). Entomological investigations have shown that the beetle, *Astylus atromaculatus* could be an unexpected but efficient pollinator on *G. hirsutum* in South Africa (Pierre and Hofs, 2010). However, *A. atromaculatus* and domestic bees are not present on *G. herbaceum* (JL Hofs personal communication). In contrast, Anthophoridae are common on both *G. hirsutum* and *G. herbaceum* and have a great capacity for carrying pollen and may thus be a potential vector for pollen transfer between the two species (JL Hofs

personal communication). However, in the case of trials involving interspecific hybridization, reverting to a more fertile form required several generations of backcrosses with the cultivated species (Kammacher, 1966; Mergeai, 2004).

Our results have demonstrated the possibility of the formation of unreduced gametes. To our knowledge, this phenomenon as been poorly documented in cotton, while it is prevalent in diverse plant species and considered to be an important mechanism for polyploidization (reviewed in Mason and Pires, 2015). The formation of unreduced gametes in *Gossypium* interspecific hybrids has been reported in crosses between *G. hirsutum* and Australian K-genome diploids *G. exiguum* or *G. nobile* (Brubaker *et al*. 1999). Observations of meiosis in these hybrids suggested that some may have derived from unreduced gametes arising from dysfunctions during M2, hence the duplication of the segregating chromosomes. Furthermore, Skovsted (1934), working on the production of hybrids between diploid Asiatic and tetraploid New World cotton, obtained a hybrid with 52 chromosomes and hypothesized the production of 2n gametes in the diploid parent. Likewise, in cytogenetic studies of cotton cultivars grown in Iran, the occurrence of large pollen grains was assumed to be potential unreduced gametes (Sheidai, 2008; Noormohammadi *et al*. 2012), although other methods are considered to be necessary to confirm the correlation between size and ploidy in pollen (Dewitte *et al*. 2012). For the same type of sterile hybrids (triploids 2n = 39, one tetraploid 2n = 52) obtained by crossing A-genome Asiatic cottons with tetraploid New World cottons, Amin (1940) reported that backcrossing to New World cottons gave occasional success in boll setting on the F1s. Such bolls contained very few seeds, and the plants developed from them had varying degrees of fertility. In this context, by crossing one of our triploid hybrid (H1) as female with *G. hirsutum* (25 flowers emasculated and hand pollinated with *G. hirsutum* pollen) we obtained a single large seed. Its germination gave rise to a plant with 2n=65 chromosomes and a putative $A_1A_tA_tD_t$ structure (data not shown). The structure may be the result of unreduced gametes

from the triploid hybrid. This ability of *Gossypium* to produce such gametes from diploid parents and from F1 hybrids might point to an alternative explanation for the formation of the allotetraploid cultivated species, *G. hirsutum*. In fact, it has been hypothesized the formation of unreduced gametes offers the possibility to generate a triploid bridge which can be at the origin of polyploid species (reviewed in Mason and Pires 2015). Interspecific hybridization has often been carried out to provide new sources of germplasm for incorporation into breeding programs (Chee et al. 2016). However, it is not possible to obtain fertile hybrids from crosses between cultivated tetraploid *G. hirsutum* and wild diploids species without treating the floral bud at the time of fertilization with growth substances in order to prevent boll abscission (Meyer, 1974; Altman, 1988). Brubaker *et al*. (1999) have shown that, in the best case, the natural crossing of diploid wild Australian species with tetraploid cotton cultivars only resulted in the production of sterile hybrids. However, fertility can be restored by doubling the number of chromosomes using colchicine to produce hexaploid plants (2n=78 chromosomes). This approach has been widely used in interspecific hybridization to improve cotton plants, but such a doubling has never been observed occurring spontaneously in nature (Mergeai, 2004, 2006). 2n gametes have already been exploited to create new cultivars at higher ploidy levels, but more interestingly, they have also been used in various species to create a bridge for the transfer of desirable genes from wild diploid species into the cultivated polyploid gene pool in various species (reviewed in Dewitte *et al*. 2012). This approach is particularly relevant for cultivated cotton, as the wild diploid species of *Gossypium* are rich in rare desirable attributes that are not available in the germplasm of cultivated species (Stewart, 1995; Mergeai, 2006). This methodology has been used to transfer genetic diversity from diploids through 2n gametes to polyploid crop varieties, as demonstrated for example in potato and alfalfa (Peloquin *et al*. 1999; Ramanna and Jacobsen, 2003). The production of autopolyploids to overcome the difficulty of transferring desirable traits from wild or

cultivated diploids to *G. hirsutum* was investigated by Mehetre *et al*. (2003). The use of unreduced gametes would facilitate the direct production of such AAAD hybrids either by direct crosses (H3 hybrid) or by crossing an AAD hybrid with *G. hirsutum*. Even though the production of unreduced gametes has rarely been described in cotton, various methods have been tested in other species to increase their occurrence (Brownfield and Kohler, 2011, Dewitte *et al*. 2012, Younis *et al*. 2014). New strategies (Ravi *et al*. 2008; Chan, 2010; Ravi and Chan, 2010) developed on the model plant *Arabidopsis thaliana* (reviewed in Crismani *et al*. 2013) are considered by the authors to be possibly applicable to crops.

The possibility of obtaining an interspecific hybrid between tetraploid cultivated and diploid wild cotton through fertilization with an unreduced gamete raises the question of its evolution in natural populations. We have shown that this hybrid produced no seeds and its pollen has very low viability. In conclusion, the likelihood of natural transfer of chromosomal material from cultivated cotton to the diploid *G. herbaceum* is very low; even though one hybrid with a particular genomic structure arose via an unreduced gamete of the diploid parent, it did not produce seeds after selfing. However, the assessment of the hybrids' ability to produce a backcross progeny would require further experimentation, such as cultivating hybrids in the presence of *G. herbaceum*. Furthermore, the hybrid materials obtained in this study, with different genome dosages (triploids AAD and unreduced gamete-derived hybrid AAAD), could provide useful tools for the study of the expression of duplicated genes and the effects of allele dosage from A and D cotton genomes.

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1