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Study of Apomictic Seed Formation in Interspecific, *Gossypium barbadense* × *G. hirsutum*, Cotton Hybrids

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Abstract: The aim of the present study was to investigate the role of alien pollen and PGRs in obtaining apomictic seed in cotton. Emasculated pistils of cotton F_1 interspecific, *Gossypium barbadense* × *G. hirsutum*, hybrids were treated with alien pollen from *Hibiscus cannabinus* (kenaf) and/or plant growth regulators (gibberrellic acid+a-naphthylacetic acid) to investigate their reaction. Mixoploid progeny recovered after numerous alien pollinations were studied cytogenetically to establish their origin, documenting their reproductive basis. Root-tip chromosome counts among somatic cells ranged from 27 to 44. The aneuploid nature of the plants was also verified by flow cytometric analysis. The use of gibberrellic acid+ a-naphthylacetic acid (GA_3+NAA) after the alien pollination increased the percentage of apomictic plant induction by 55%. Apomictic frequencies were compared to those from emasculated and self-pollinated cotton pistils. These observations suggest that alien pollinations and plant growth regulators may have resulted in unusual reproductive events.

Key words: Mixoploidy, aneuploidy, heterosis, kenaf, PGR

INTRODUCTION

Heterosis or hybrid vigor in first generation (F_1) seeds, by crossing genetically distant breeding lines, is well known in breeding (Shull, 1952). Heterosis in cotton was reported in both intraspecific and interspecific crosses (Wu *et al*., 2004). In interspecific hybrids, particularly crosses between *G. barbadense* × *G. hirsutum*, economically valuable characters can occur in favorable combinations. Yet, interspecific crosses often lead to infertility, cytological abnormalities and distorted segregation (Saha *et al*., 2004). Therefore, the need to create a stable genotype that combines high yield and fiber quality requires new breeding techniques and a better understanding of the genetic basis of cotton hybrid reproduction.

Apomixis, an asexual reproductive process in flowering plants which results in seeds that are of the same genotype as that of the female parent (Koltunow, 1993) could be a good choice as a useful genetic tool for hybrid breeding. Apomixis is achieved through processes that occur in the ovule and lead to the avoidance of meiosis, fertilization-independent embryo development and, in some cases, autonomous development of the endosperm. The independence from meiosis and paternal genes during embryo formation imply that apomixis has the potential to fix the genotype of seeds. For this, it may prove to be a useful tool in agriculture if it can be used to fix and maintain the yield advantage provided by hybrid seeds between generations (Hanna and Bashaw, 1987; Koltunow *et al*., 1995). The process currently occurs in few agricultural crops and is dependent on the ability to introduce the trait and control its expression to produce genetically pure seed (Ramulu *et al*., 1999). Grossniklaus *et al*. (2001) suggested that if apomixis could be introduced into sexual crops, it would greatly simplify breeding schemes and allow the fixation of any genotype, including F_1 hybrids.

According to the origin of the embryos, apomixis has been classified into two types: adventitious embryony and gametophytic apomixis (Spillane *et al*., 2004). In adventitious embryony, embryos develop directly from unreduced somatic (sporophytic) cells in ovule tissues that are external to a sexual derived megagametophyte. So, adventitious embryony has been described sporophytic apomixis. In sporophytic apomixis, sexual embryo sac develops in the same ovule as the sporophytically derived, apomictic embryo and fertilization is required for

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apomictic seed development. The sexual embryo may abort but the sexual endosperm is required to nourish the asexual embryo (Koltunow, 1993). Therefore seeds in sporophytic apomixis contain endosperm with the normal 2M: 1P genomic ratio, which poses no imprinting-related problems. Most plants with adventitious embryo are commonly diploid (Asker and Jerling, 1992), suggesting that the adventitious embryo formation in sporophytic apomixis is not correlated with chromosome ploidy statue.

In gametophytic apomixis, the maternal progeny is the product of an apomictical development of unreduced megagametophytes that differentiate either from a megaspore mother cell which failed to enter meiosis (diplospory) or a somatic nucellar cell which begins gametogenesis in absence of sporogenesis (apospory). Endosperm development in gametophytic apomixes may follow pseudogamy or autonomous endosperm formation. Pseudogamy is the most common mode of the endosperm development and autonomous endosperm formation is rare (Asker and Jerling, 1992). Gametophytic apomicts, irrespective of the mechanism used, are almost invariably polyploids, yet sexual members of the same or closely related species are very commonly diploids (Asker and Jerling, 1992).

In sexual plants, distant pollination, treatment with Plant Growth Regulators (PGR) as well as pollination with irradiated or chemically treated pollen seems to favor apomictic seed formation (Asker and Jerling, 1992). In order to study apomixis in cotton, we analyzed the ability of alien pollen and PGRs to elicit seed development following their application to pistils of emasculated cotton flowers.

MATERIALS AND METHODS

Plant growth: Ten plants each from six hybrid crosses between two *G. barbadense* (Carnak and B403) and three *G. hirsutum* (Coker 310, Acala Sindos and 4S) commercial varieties were grown in the field with spacing of 1 m between rows and 30 cm within rows. Two rows of ten *H. cannabinus* (dowling kenaf) plants were also grown under the same conditions. The major flowering season of both cotton and kenaf plants in Greece is mid-July to mid-August. All accessions were grown in the experimental field on the farm of the Aristotle University of Thessaloniki and all experiments took place at the same location from 2003-2006.

Flower emasculation, controlled pollination and application of growth regulators: For each experiment, buds of each cotton hybrid were emasculated one day pre-anthesis. To avoid damage to the inflorescence meristem, fine forceps were used to remove sepals, petals and anthers, leaving an exposed pistil. Controlled self- or alien pollination was performed on anthesis stage pistils by dusting a freshly dehisced kenaf or cotton anther over the extended stigmatic papillae until pollen was seen adhering to the stigmatic surface. Alternatively, emasculated pistils were left un-pollinated as controls or treated with PGRs. Also, a set of self- and alien pollinated pistils were treated with PGR at 1, 3 and 5 Days after Pollination (DAP). Each pistil was uniformly coated from the tip of the stigmatic papillae to the pedicel in one-mL containing 10 mmol of GA_3+NAA, with 0.04% (v/v) Triton X-100 used as a surfactant. Each solution was buffered to pH 7.0. All emasculated and treated cotton pistils were protected from cross-pollination by paper bags, which were removed not earlier than five DAP. Boll development following pollination or/and PGR treatment was observed every 10 days and seed formation was measured at 56 DAP. Development data for each treatment was established by the examination of a minimum of three individual pistils per treatment from each separate plant (min n = 24). Experiments were carried out in July-August and repeated three times from 2003 to 2005.

Pistil receptivity to pollen: Emasculated cotton flowers (n = 24 pistils) were self- or alien pollinated and cotton styles with ovaries were harvested 1, 2 and 3 DAP to determine pistil receptivity to pollen. They were fixed in formalin: acetic acid: 70% ETOH; 1:1:8 and stored at 4°C. Fixed styles were rinsed with tap water, softened with a 8N NaOH solution for 8 h, rinsed again with tap water, stained with 0.1% aniline blue in 0.1 M potassium phosphate (Martin, 1959). They were then placed in a drop of glycerol on a glass slide, squashed with a cover slip and observed by fluorescence microscopy (Zeiss, Germany). In addition, ovules were dissected from ovaries and the possibility of fertilization was studied.

DNA flow cytometry and cytogenetics: To isolate and stain nuclei, approximately 2 cm^2 of young leaf tissue from the obtained seedlings were chopped with a razor blade in a plastic Petri dish containing 2 mL of lyses buffer (Partec HR-A solution). The suspension with released nuclei was passed through a 15 mL nylon mesh and with 2 mL of DAPI staining buffer (Partec HB-B solution). Finally, the sample tube was connected with the ploidy analyzer (Partec II, Germany) and the DNA-histogram was produced on the screen. All measurements were replicated twice per sample and an extra DNA sample from pea (*Pisum sativum*) was used as an internal reference standard (1C: 4.43 pg). Relative DNA content of individual plants was expressed using a DNA index (DI) calculated according to the formula:

$$DI=\frac{\text{Mean of relative DNA content of the Go/G1 nuclei of the sample}}{\text{Mean of relative DNA content of the Go/G1 nuclei of the standard}}$$

To determine the number of chromosomes in somatic cells, root tips from germinated seeds were pre-treated with 1 mM 1-bromonaphthalene for 3 h, fixed in 1:3 acetic acid: 70% ethanol and kept at 4°C for at least a week. Roots were hydrolyzed with 5 M HCl for 20 min, stained with 4% hematoxylin, squashed under a cover-slip and observed at 1000x with a light microscope (Zeiss, Germany). All the plants obtained from the alien pollinations were tested with or without PGR treatment and equal number of plants obtained from self-pollinations.

Statistical analyses: The data were analyzed on SAS JMP 8 using the general linear model and Tukey-Kramer HSD test for the separation of mean values.

RESULTS

Hybrid responses to pollination and chemical treatment: We investigated the existence of significant ($p<0.05$) differences between six interspecific cotton hybrids. Hybrids responded similarly without any of those to show divergence to percentage of seed formation for any treatment. The pollinations were repeated for three years and results showed repeatability given that here was no significant differences to percentage of seed formation between years.

Boll and seed formation: Ovaries from all hybrid combinations increased in fresh weight until 3 to 5 DPA and were 2- to 3-times bigger than their initial anthesis size. Other floral organs, excluding the developing ovaries, senesced soon after pollination and abscised during boll development. In self-pollinated cotton flowers, the ovaries grew exponentially, becoming green at 2 or 3 DAP. Unpollinated flowers also shed their floral organs, yet ovaries continued to grow slightly from their normal anthesis size at a considerably reduced rate of growth compared with pollinated bolls. The highest frequency of boll abscission of flowers pollinated with alien pollen and without PGR treatment occurred at 8 to 9DAP whereas the un-pollinated flowers without PGR treatment were abscised usually at 2 or 3 DAP. The percentage of mature bolls of the alien pollinations was significantly lower ($p<0.05$; mean percentage 4.83%) than that of self-pollinations which topped at 95.8% and significantly ($p<0.05$) higher than the control (un-pollinated flowers) (Table 1). More specifically, 600 flowers were pollinated with pollen from *H. cannabinus* without the presence of PGR giving 29 bolls that contained 2 to 10 seeds each, whereas 300 self-pollinated flowers resulted in 249 mature bolls with 3237 seeds (Table 1). The results obtained from PGR treatment of unpollinated, self-pollinated and alien pollinated cotton flowers are shown in Table 1. Treatment with GA_3 and NAA turned to be effective at preventing boll abscission, but it did not affect seed formation or the number of seeds per boll. It is characteristic that in pollinations with pollen from *H. cannabinus* abscission was detected later than 12 DAP instead of 8 or 9DAP without chemical treatment.

Response of cotton pistils to self and alien pollination: Pollen tube behavior in the styles was studied for self- and alien pollinated flowers. Fluorescence microscopy of germinated pollen grains between F_1 interspecific cotton hybrids and *H. cannabinus* showed abnormal germination with frequent branching of the tube and the presence of two or more tubes per grain. Pollen of *H. cannabinus* displayed low germination with a maximum mean of 35.4%, 2 DAP whereas pollen tubes did not penetrate the style (Table 2). In contrast, tube growth of self-fertilized pistils was normal. A large number of pollen grains germinated on the stigma and a bundle of pollen tubes grew vigorously in the style. More specifically, at one DAP several pollen tubes could be seen at the base of the style. Tubes penetrating the ovules were observed in all cases at 2 and 3 DAP. Percentage of ovules showing pollen tube entry ranged from 39.5% at one DAP to 81.6% at three DAP (Table 2).

Cytological study: It was possible to detect around 10 cells in metaphase per slide, of which at least five could be selected with well-scattered and contracted

Table 1: No. of flowers of interspecific cotton hybrids, *G. barbadense* x *G. hirsutum* emasculated and pollinated and/or treated with PGR, number and percentage of boll formation, seed content, no and percentage of seeds germinated, number of seedling produced and percentage of inductivity

Treatments	No. of treated flowers	No. and % of mature bolls	No. of seeds	No. and % of seeds germinated	No. of seedlings	Inductivity of seedlings (%)
Emasculation without pollination	300	0 (0.00)^{e}*	0	-	-	-
Emasulation and self-pollination	300	249 (83.00)^{b}	3237	1829 (56.52)	1734	53.57
Emasculation and alien pollination	600	29 (4.83)^{d}	169	75 (44.37)	43	25.44
Emasculation without pollination + PGR	150	4 (2.67)^{de}	0	-	-	-
Emasulation and self-pollination + PGR	150	142 (94.67)^{a}	2016	1085 (53.82)	978	48.51
Emasculation and alien pollination + PGR	300	32 (10.67)^{c}	272	137 (50.37)	102	37.50

*Mean separated with Tukey-Kramer test and levels connected with the same levels are not significantly different at $p<0.05$

Table 2: Percentage of pollen germination, tube growth and pollinated ovules per ovary in interspecific, *G. barbadense*×*G. hirsutum*, cotton pistils following self- or alien pollination

Treatments	DAP	Pollen germination (% ± SD)	Pollen tubes in the style (%)	Pollen tube growth*	Pollinated ovules per ovary (mean±SD)
Emasculation and self-pollination	1	75.0±0.8	85.3	N	39.5±2.6
	2	83.0±2.9	88.9	N	66.8±2.9
	3	81.3±1.7	87.6	N	81.6±4.2
Emasculation and alien pollination	1	28.8±2.1	0.0	N/A	0.0
	2	35.4±3.0	0.0	A	0.0
	3	29.1±2.7	0.0	A	0.0

*N: Normal; A: Abnormal

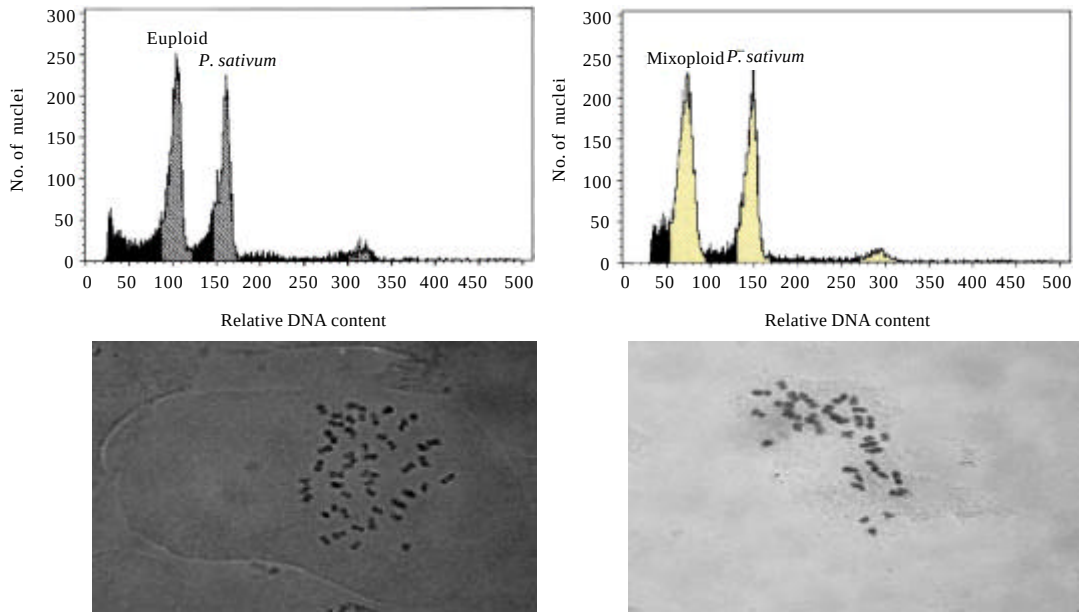

Fig. 1: On the left, histogram (top) of relative nuclear DNA content obtained after simultaneous analysis of nuclei isolated from an euploid plant obtained from self-pollination of interspecific, *G. barbadense x G. hirsutum*, cotton hybrids and *P. sativum* and mitotic metaphase plate (2n = 4x = 52). On the right, flow cytometry chart and metaphase plate from a hypoaneuploid plant (2n = 38) obtained from alien pollination of interspecific, *G. barbadense x G. hirsutum*, cotton hybrids

chromosomes. Among the 5253 seeds from self-pollinations and 441 seeds from alien pollinations a percentage of 55.47 and 32.88%, respectively developed normally (Table 1), but the rest were shriveled. All the cotton seedlings obtained from alien pollination with pollen from *H. cannabinus* examined cytologically and were classified as mixoploids (cells showing 27-44 chromosomes with mean number at 35) whereas seedlings harvested from self-pollinations were tetraploid (2n = 4x = 52) (Fig.1). The mixoploid plants were semi-fertile and less vigorous than the tetraploids (Fig. 2). Flow cytometric analysis of relative DNA content resulted in histograms with two dominant peaks representing the G1 nuclei of cotton and pea (Fig. 1). The G1 peaks were narrow with a coefficient of variation (CV) equal to 3.71%±0.10 (mean of 290 measurements±standard deviation × two times). The DNA index estimated for tetraploid plants (2n = 52) and mixoploid plants (2n = 27 -44) was equal to 0.683 and 0.556, respectively

Fig. 2: Mature boll and flower from an euploid (left) and hypoaneuploid (right) plant obtained from self-pollinations and alien pollinations of interspecific, *G. barbadense* × *G. hirsutum*, cotton hybrids, respectively

(mean of 145 measurements × two times for each group). The DNA content of tetraploid and mixoploid plants, expressed as DNA index, was highly correlated with chromosome number ($R^2 = 0.849$, significant at $p<0.001$).

DISCUSSION

Pollination in the ovary induces a coordinated sequence of cell division, expansion and differentiation events that result in fruit and seed structures. Neither the nature nor the succession of the signals that control pollination mechanisms is clear (Vivian-Smith and Kultunow, 1999). Alien pollinations of cotton interspecific, *G. hirsutum* ×*G. barbadense*, hybrids with pollen from *H. cannabinus* resulted to mixoploid plants, a result of intergeneric hybridization or apomictic ovule development. According to our study, hybridization via cotton × kenaf pollinations is not possible because none of the pollen tubes entered the cotton flower style. In addition, based on the lower chromosome numbers in the derived plants, the mean DNA values and the lack of characteristics or other indications that there were cotton×kenaf recombination events, intergeneric hybrid development has to be excluded. Thus, induction of apomictic development of normal egg cells remains the most likely alternative. Apomictic development of seeds after pollination of cotton flowers with pollen from *H. cannabinus* has been previously reported by Zhou *et al.* (1991), Shi-Qi *et al.* (1992) and Mavromatis *et al.* (2005). In *Arabidopsis* (Vivian-Smith and Koltunow, 1999), it has been observed that GA_3 induces parthenocarpy, results that may support this data according to which bolls that lack seeds were developed. In other species, like tomato (Bunger-Kibler and Bangerth, 1982) and crucifer rape (Srinivasan and Morgan, 1996), GA_3 treatment induces the expansion in mesocarp tissues. In addition, in garden strawberry biological induction was investigated with the use of alien pollen from two different species, *Potentilla anserine* and *Duschesnea indica* but no seedlings survived (Sukhareva *et al*., 2002).

It is known that apomixis is associated with polyploidy. Galitski *et al.* (1999) provided definitive evidence for a ploidy-dependent system of gene regulation in yeast. Isogenic yeast strains differing in ploidy from haploidy to tetraploidy showed that expression of several genes was induced or repressed when the number of chromosome sets was increased. The ploidy-dependent gene expression demonstrated by Galitski *et al.* (1999) in yeast may also have implications in the biology of higher plants.

We hypothesize that apomixis in tetraploid cotton plants is possible and alien pollen may induce the expression of apomixis-related genes. Although, the phenomenon is poorly understood, chromosome instability is believed to be one of the most common causes of apomixis (Hu *et al*., 1991).

Cytogenetic studies revealed that the chromosome number of the plants derived from alien pollinations ranged from 27-44 and flow cytometric analysis confirmed their aneuploid nature. Given that chromosome counting cannot be used to establish the ploidy of non-dividing cells in differentiated tissues, such as leaves (Roux *et al*., 2001), the flow cytometric assay incorporating an internal reference standard seems to be a more precise technique for detecting changes involving smaller numbers of chromosomes.

Introgression and synthesis should be considered in the making of a gametophytic apomict. Introgression has been tested in several species, generating a high degree of seed abortion (Spillane *et al*., 2004). Introgression of autonomous apomixis, which could circumvent the endosperm problem, is infeasible in cereals as this pathway is almost exclusively restricted to the *Asteraceae* (Spielman *et al*., 2003). Synthesis remains hypothetical, since genes involved are yet to be cloned and validated and probably it would be necessary to manipulate several factors for inducing the seed formation and endosperm development. The complexity of the genetic control of the apomixis trait is challenging the strategies for transferring apomixis to sexual crops by genetic engineering. Given that genetically controlled obligate apomixis in crop species is yet to be found, biological or chemical induction of apomictic seed formation should be further explored.

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