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Assessment of possible hybridization between Bt cotton (*Gossypium hirsutum* L.) and other Malvaceae species (*Abelmoschus* spp. and *Hibiscus* spp.) cultivated in Burkina Faso

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The cultivation of transgenic cotton in Burkina Faso should consider the possible hybridizations with cultivated cotton relatives like okra (*Abelmoschus* spp.) and roselle (*Hibiscus* spp.), when these crops are often sympatric and co-flowering. The possibility for Bt cotton and these crops to outcross was investigated in Farako-Bâ research centre, in the south sudanian zone. The study was carried out in 2007 using both open-pollinated and artificial pollination experimental designs. Results show that the studied species are effectively reproductively isolated because of low overlapping of their optimum flowering periods. In the case of the artificial crossings (direct and reciprocal) between the studied species and the Bt cotton, the results show that fruits were produced at different rates depending on the species being crossed and the crossing direction. Immunological tests done on progeny seeds resulting from the crosses with transgenic cotton as the male parent confirmed that there was a transfer of transgenes from Bt cotton to the conventional cotton (99.2%). The absence of the transgenes in the seeds of okra and roselle means that these seeds are not the result of intergeneric hybridization but could be attributed to self-pollination or parthenogenetic development of un-fertilized ovules.

Key words: Transgenic cotton, relatives' species, intergenera hybridizations, immunological test, Burkina Faso.

INTRODUCTION

Burkina Faso flora is rich in cotton relatives, having about 20 species of Malvacea family (Spcnge, 1999). Cotton plays an important role in the country economy and related species as okra and roselle are more and more valued. For cotton pest management, Burkina Faso opted to introduce Bt cotton which is resistant to bollworms. First studies of transgenic flow in Burkina, as elsewhere, showed that flow is inevitable between transgenic and conventional cotton, especially in the case of immediate proximity of the two types (Karieva and al., 1994; Allen

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Table 1. Some characteristics of species used in the study.

Specie	Variety name	1st flower (das) blooming	Pollination mode	Sowing to maturity cycle (das)	Yield potential
Conventional cotton	FK37 (*G. hirsutum*)	65	Partial self-pollinated	130	2,6 t/ha (seed cotton)
Transgenic cotton	FK37 BG2 (*G. hirsutum*)	60	Partial self-pollinated	130	3,5 t/ha (seed cotton)
Okra (*A. esculentus*)	Kéléya	30 to 60	Self-pollinated	100	500g à 1t/ha (seeds) 10-40t/ha (fresh okra)
	BF-locale1	30 to 60	Self pollinated	-	-
	BF-locale2	60	Self-pollinated	-	-
Roselle *(H. sabdarifa)*	R72-1 (red)	Short days	dominant self-pollinated	150	60 à 1000 kg/ha (dried flowers)
	R147-1 (green)	Short days	dominant self-pollinated	180	50 à 800 kg/ha (dried flowers)

and al., 2005; Bourgou, 2006). Isolation precautions taken during the cropping-season can minimize and possibly avoid the intraspecific flow of transgenes.

However, little precise knowledge exists on possible genetic interactions between cotton and its related species while they co-habit the cropping systems (Perlak et al., 2001). Moreover, these species carry chromosomal variations, floral biology similarities and known allopoly-ploidization that could justify possible hybridizations. Authors such as Vysotskii, (1960) and Mehetre et al. (1980) reported the production, although difficult, of "true" hybrids between cotton and some hibiscus species but no hybrid of this type is recognized in nature. More recently, more authors (Zou et al., 1991; Stewart, 1992; Mavromatis et al., 2005; Vlachostergios et al., 2007) have suggested a parthenogenetic origin for progeny of crosses between cotton and its related species.

Because prior reports provide disconcordant answers, the question of intergeneric hybridization in the Malvacea family remains unanswered. For the commercial release of Bt cotton in Burkina Faso, the risk of Bt cotton out-crosses with okra and roselle is germane. This study focused on an opportunity to use immunological tests, to detect transgenes where they have been introgressed. The objectives were to: i) monitor the response of plants to artificial pollination (direct and reciprocal) between Bt cotton, okra and roselle and ii) screen for presence of the Bt transgene using an immunological test in the seeds of okra, roselle and conventional cotton either derived from open or artificial pollination.

MATERIALS AND METHODS

Study site

The study was carried out in Farako-Bâ during the cropping season of 2007. Farako-Bâ is located in the western cotton zone of Burkina Faso, between the isohyets 800 and 1200 mm at 405 m of altitude, 4°20'O of longitude and 11°06'N of latitude. The vegetation is woody land with abundance of pollinator insects. Soils are ferasols with low clay and organic matter content, with a deficiency of nitrogen and phosphorus (Bado, 2002). Rainfall during the 2007 season was 1113.6 mm for 80 rainy days. Between March and November, the averages of minimum and maximum temperatures were respectively 21.6 and 33.51°C.

Plant material

The study's plant material consisted of three different cultivated species (cotton, okra and roselle) of three genera of the Malvacea family: FK37 and FK95 BG2 (*Gossypium* genus); Kéléya and two local okra species (*Abelmoschus* genus) and two roselle varieties R72-1 and R147-1 (*Hibiscus* genus).

FK95 BG2 is a transgenic variety produced by a cross followed by backcrosses between the conventional variety FK37 and a transgenic variety DP50 BGII. Table 1 presents some characteristic of the varieties used in this study.

Experimental designs

Open-pollinated in field design

For the open-pollinated or free-pollination design (Figure 1), plants were planted in a circular pattern that permits maximal gene flow between species (Scheffler et al., 1993). It consists of a central plot of transgenic cotton FK95 BG2 (113.04 m^2) surrounded by 3 plots (22.3 m^2 each), altering conventional cotton, okra or roselle. Seeds were sown manually July 12, 2007 in rows spacing 80 cm apart. Plant to plant spacing was 40 cm. For okra, some re-sowings were done July 19 and 25, 2007. After emergence, a thinning to 2 plants/hill was done. Mineral fertilization and pest control were carried out according to the local standard.

Artificial pollination design in pots

This experimental design constituted of 49 pots of transgenic cotton placed at the center of the design and surrounded by 20 pots of conventional cotton, 20 pots of okra and 20 pots of roselle. Okra, roselle and conventional cotton pots were alternated by group of five pots and by species (Figure 2). Seeds were sown in 15 L plastic pots containing a mixture of soil (2/3) and farm yard manure

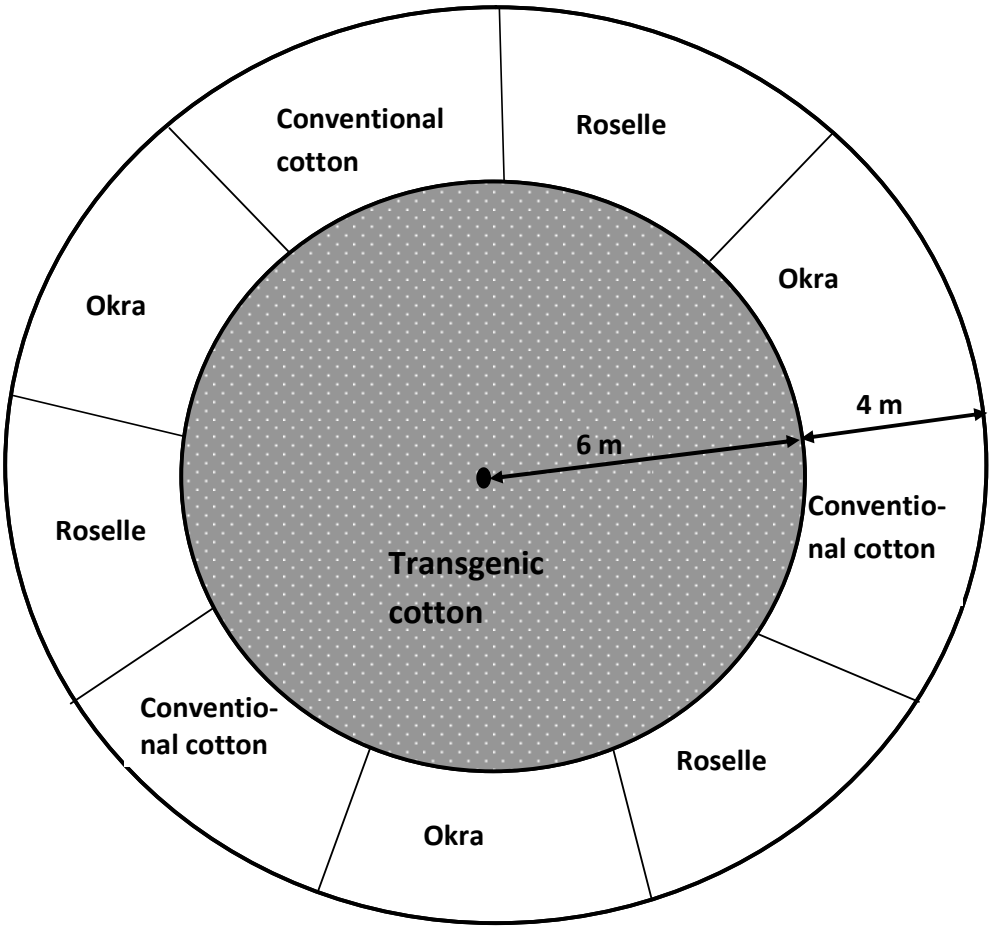

Figure 1. Illustration of the open-pollinated experimental design.

(1/3). Sowing date was June 27, 2007 and the thinning was 2 plants/hill two weeks later. Plants were provided water two times a day to avoid effect of water deficit stress on crosses. Mineral fertilization and pest control were assured according to the local standard. Okra variety Kéléya was exploited in the crossings to compensate for the weak floral production of local okra.

Manual crossings

Plants grown in pots were used for manual crossings. Each variety was used in crosses as male parent first then as female parent. Emasculation and manual pollination were done as described by Laçon (1993), Hamon (1988) and Akhond et al. (2000). The following crosses were made: transgenic cotton (♂) x conventional cotton (♀); transgenic cotton (♂) x okra (♀); transgenic cotton (♂) x roselle (♀); okra (♂) x roselle (♀); okra (♂) x transgenic cotton (♀); roselle (♂) x transgenic cotton (♀); roselle (♂) x okra (♀); conventional cotton (♂) x transgenic cotton (♀) (♂ = male; ♀ = female). For each species, 50 self-fertilized flowers and 50 emasculated flowers left un-pollinated were used as controls.

Emasculation of the flowers

Emasculation consisted of manually and completely removing petals and sepals to facilitate access to stamens. Then, staminal columns bearing immature anthers were removed and the style covered with rigid transparent plastic. Emasculation was done in the afternoon one day prior to anthesis or earlier in the morning of the day of anthesis, before the flowers opened and before the pollen and stigma of the pistil are ready for pollination.

Manual pollination

The controlled hand pollination method conducted in the morning resulted in the deposition of the fresh chosen pollen grains on the stigmas of the emasculated flowers. Pollen grains were obtained from stamens from whole flowers or staminal columns of the chosen male parent. After hand pollination, a protective bag was immediately placed over the manipulated flower which was identified with a label.

Parameters recorded on plants

In field as well as in pots, the parameters we observed were: First flower blooming date corresponding to the number of day after sowing (das) when 50% of observed plants had atleast one opened flower; flowering volume that corresponds to the number of flowers produced per species; rate of fruit set, that is the ratio between number of mature fruits divided by the number of flowers used in manual crossings.

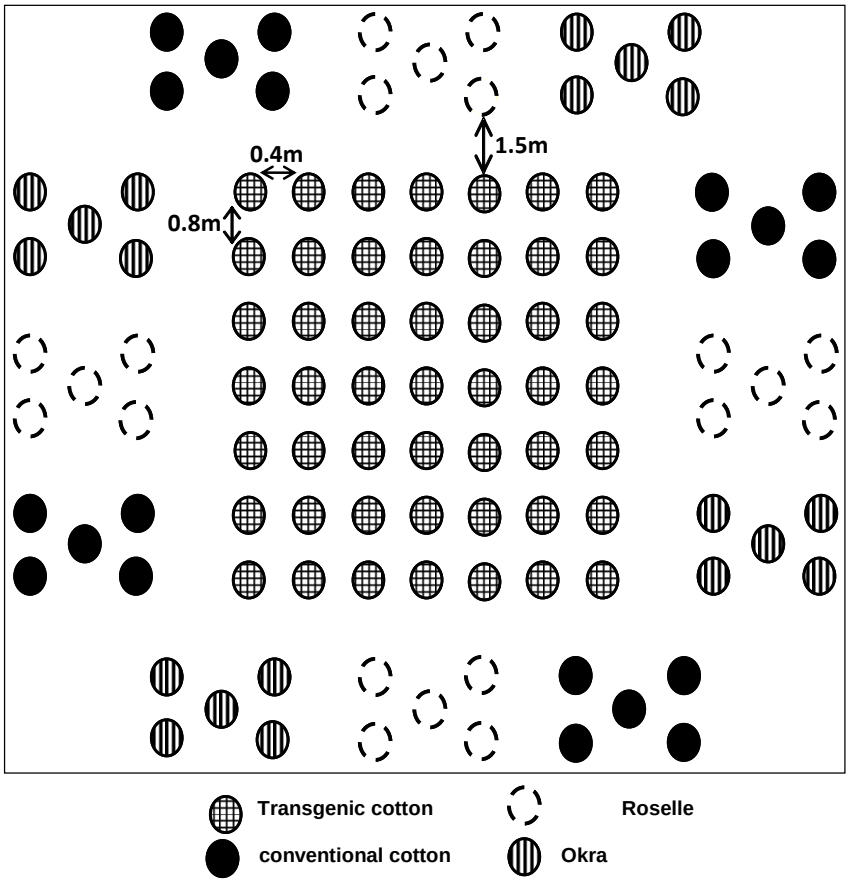

Figure 2. Illustration of the manual crossings experimental design.

Laboratory analysis

Analysis principle and immunological tests to reveal the transgenes

The tests principle is based on the ability to specifically detect (by immunological tests) proteins produced by transgenes in seed if these seeds are "true" hybrids where a transgenic plant contributed as the male parent. The presence of target proteins points out the presence of transgenes.

An immuno-enzymatic detection kit (Seed B2R Test Strips & Combs) set up in 2004 by Strategic Diagnosis Inc. (111 Pencader Drive Newarks, OF 19702, techservice@sdix.com) was used. The technique takes advantage of a test strip on which two specific antibodies of Cry proteins fixed on a paper support are used to specifically and instantaneously detect Cry1Ac and/or Cry2A proteins if present in extracts from tested seeds. Tests were done individually for presence or absence of these proteins on 500 seeds per species (conventional cotton, okra and roselle) from open-pollination. From the hand crossings with the transgenic cotton as male parent, 1000 progeny seeds of each conventional cotton, okra and roselle were tested.

Processing the transgenic detection test

Before testing unknowns, a control test was conducted with transgenic cottonseed to make sure the trait sample extraction buffer and lateral flow strips were functional. Each seed to be tested was cracked into small pieces using pliers. 1 to 2 g of the cracked cottonseed was put into a 1.5 ml Eppendorf tube and ground. To this, 1 ml of the trait seed sample buffer (extraction buffer) provided with the kit was added. Then, we let the tube with the cottonseed sample extract stand for 4 min while shaking intermittently to extract the proteins. 5 min after we stopped shaking, one seed B2R lateral flow strip test provided with the kit was inserted into each Eppendorf tube containing prepared sample extract and the sample migrated up the strip by capillarity action. Each test strip was left in the tube for about 10 min to develop and show lines to be used for interpreting the test.

Interpreting the results of the test

For a good test, at least one line (the control line) should be present. Results are positive for transgenic presence when 2 or 3 lines are observed on the strip (including the control) at the end of capillary migration phase (Figure 3): upper line (control line) indicates that the strip functioned properly. This line appears even in absence of the target proteins; median line indicates a positive result for Cry1Ac protein; lower line indicates a positive result for Cry2Ab protein.

Data recorded after laboratory analysis was the proportion of transgenes present in seeds tested as calculated according to the following formula (Van Deynze et al., 2005):

Transgene outflow (%) = Number of seeds testing positive/ Total number of seeds tested x 100.

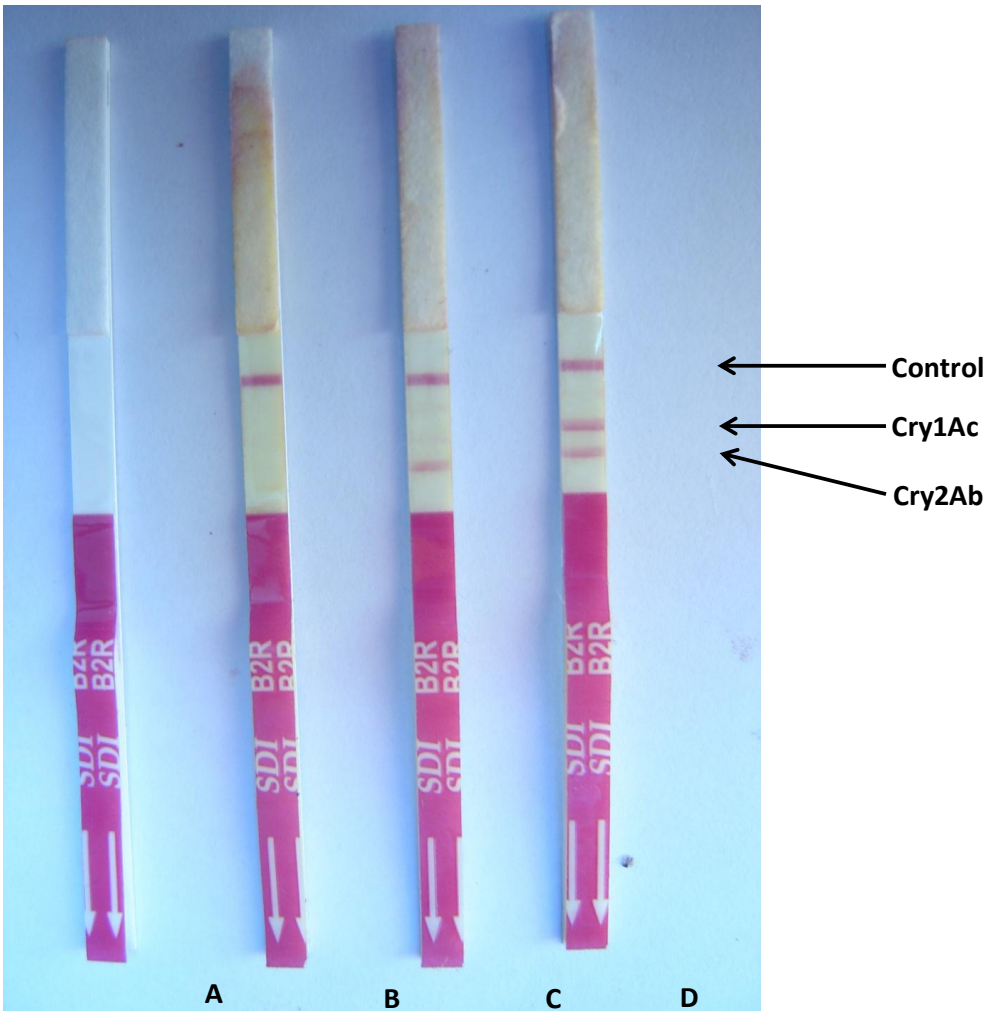

Figure 3. Illustration of test strip results. A = unused strip; B = negative test; C = positive test for Cry2Ab; D = positive test for Cry1Ac and Cry2Ab.

Data analysis

Analysis of variance was carried out using XLSTAT 6.1.9 Software. The averages were compared by the NEWMAN and KEULS test at 5% significance level.

RESULTS

First flower blooming and flowering volume

Variance analysis of 1st bloom and flowering volume are presented in Table 2. In the field, okra, conventional and transgenic cottons 1st flowers opened respectively 77.00, 85.00 and 87.00 days after sowing (das). In pots, the 1st flower blooms appeared at 81.00, 83.00 and 86.00 das for the same species in the same order. Roselle started flowering later that is 108.00 das in the field and 125.00 das in pots. However, variance analysis showed that roselle produced on average significantly more flowers per cycle than cotton or okra in field tests as well as in pots.

Rate of fruit set

Results show that some fruits were produced from flowers used in the manual crosses (Table 3). Following evening emasculations, crossings between conventional cotton and transgenic cotton resulted in significantly, the highest fruit set rate (50.8%, P = 0.0001). The crossings okra x roselle did not produced fruit (0%) with evening emasculations but in general, intergenera crossings (transgenic cotton x okra, transgenic cotton x roselle, okra x roselle) were equivalent. With morning emasculation, there was no significant difference regarding seeds presence following intergenera crosses (p = 0,086). But, it is essential to note that crossing between the roselle (♂) and transgenic cotton female (♀) gave the lowest rate of set fruits (2%) followed by the cross okra (♂) x roselle (♀) (7%).

Table 2. 1st flower blooming and flowering volume on field and pots.

Test	Specie or variety	First flower blooming (das)	Flowering volume (number of flowers)
	conventional cotton	83.00[b]	156.00[b]
	Okra	81.00[b]	70.33[c]
Test in pots	Roselle	125.00[a]	776.00[a]
	mean	96.30 ± 21.8	334.1 ± 334.4
	probability	0.0001[HS]	0.0001[HS]
	conventional cotton	85.00[b]	641.67[b]
	Okra	77.00[b]	64.67[c]
Test at field	Roselle	108.00[a]	2235.00[a]
	mean	90.00 ± 14.8	980.00 ± 985.0
	probability	0.002[HS]	0.0001[HS]

das, Date after sowing ; HS= highly significant. Numbers followed by the same letter in each column are not significantly different at 5% mean separation test.

Table 3. Relation between fruit set, number of crossings and the crossing period.

Intra or inter-gender Crossing	Mean number of crosses		Mean of fruit set rate (%)	
	Evening emasculation	Morning emasculation	Evening emasculation	Morning emasculation
Transgenic cotton x conventional cotton	93.50[ab]	171.50[a]	50.80[a]	55.95[a]
Transgenic cotton x okra	90.00[ab]	209.50[a]	4.35[b]	15.55[a]
Transgenic cotton x roselle	125.00[a]	260.00[a]	0.40[b]	14.25[a]
okra x roselle	44.00[b]	163.00[a]	0.00[b]	19.85[a]
Mean	88.12 ± 33.6	201.00 ± 50.8	13.89 ± 22.9	26.40 ± 20.8
probability	0.042[S]	0.202[NS]	0.0001[HS]	0.086[NS]

NS = Non significantive; S = significant; HS= highly significant. Numbers followed by the same letter in each column are not significantly different at 5% means separation test.

Table 4. Number of tests and rate of positive tests for Cry proteins in free pollination.

Experimental design	Sampled specie (variety)	Number of seeds tested	Number of seeds tested positive	Rate of positive tests %
	conventional cotton	1000	163	16.3
Free pollination	Okra	1000	0	0
	Roselle	1000	0	0
	conventional cotton	500	496	99.2
Artificial crossing	Okra	500	0	0
	Roselle	500	0	0

Transgenic revelation by immunological tests

Field (open-pollination) tests results did not reveal presence of Cry1Ac and Cry2A in any seeds form okra or roselle. Inversely, the transfer of these proteins to conventional cotton resulted in 16.3% of seeds harvested from plants that were 0.8 to 2.4 m from the source of transgenes; that is the transgenic cotton (Table 4). We also noted that tests done on seeds resulting from manual crosses between transgenic cotton as male parent and okra or roselle as female parents were negative for Cry1Ac and Cry2A. However, 496 out of 500 seeds, that is 99.2% of the seeds resulting from conventional cotton (♀) x transgenic cotton (♂) resulted in positive tests for the target Cry proteins (Table 4).

DISCUSSION

Results on flowering parameters show that compared to cotton, okra flowered earlier and has an especially brief period of flowering. Roselle, with photoperiodic responses, starts its flowering in short days suddenly and abun-

dantly. These flowering patterns resulted in a little overlap of the optimum flowering periods of the studied species. Thus, the species are in practical terms reproductively isolated which effectively decreases inter-crossing possibilities in natural or even in experimental settings (Renno et al., 1997).

Proportion of fruit set varied according to the type of manipulation and especially according the species being crossed and the direction of crossing. The low fruits set rates could be explained by the traumatic effect of manipulations (emasculation and hand pollination) on the flowers in addition to the natural "shedding" known on Malvacea in response to stress. If possible, it would be of interest to make the crosses without emasculation (Hamon and Koechlin, 1991). Crosses between transgenic and conventional cottons were made readily which is not surprising given that they are the same genus and the same species. But, the present study show that intergenera hybridizations in the Malvacea family are very rare at best, as has been reported by other authors.

Thus, the Vysotskii (1960) report of the production of "real hybrid fruits" by crossing cotton with a hibiscus is not convincing, especially since Mehetre et al. (1980) got only one cotton boll by crossing 2000 cotton flowers with pollen grain from *Hibiscus pandureaformis* and Stewart (1992) did not get a fruit by crossing cotton as male parent and *H. acetosella* or *H. syriacus* as female parents. These difficulties could be explained by poor or even a total absence of *Hibiscus* spp pollen germination on the stigma of cotton flowers (Mehetre et al., 1980).

Using pollen of okra on the stigma of cotton, Vlachostergios et al., (2007) produced cotton seed. Brown (1947) mentioned by Hau and Hofs (2005), and Stewart (1992) produced, at variable rates, fruits of cotton and okra by direct and reciprocal crosses. In both cases, it was revealed that the putative hybrids are not actual hybrids but seeds produced by other mechanisms (Stewart, 1992; Mavromatis and Roupakias, 1995).

Immuno-enzymatic tests indicate that the seeds from conventional cotton are introgressed with transgenes at 16.3% (open-pollination) and 99.2% (hand crossings). It is evidence that crosses occur freely between transgenic and conventional cottons when they are sympatric and co-flowering (Karieva et al., 1994; Van Deynze et al., 2005; Bourgou, 2006). To the contrary, transgenic presence has not been revealed either in okra or in roselle seeds harvested after free pollination or from manual attempts at fertilization. That is to say, seeds obtained in these cases are not the result of hybridization between transgenic cotton and these two species. According to Harlan and De Weit (1971), Hau and Hofs (2005), the criteria of sexual compatibility do not favor the exchanges of gene beyond genera.

Two hypotheses exist for the origin of the seeds resulting from the manual crossings when transgenic cotton is male and okra or roselle is female. The first hypothesis is self-pollination that starts before the anthesis of the flower or which is caused by contamination during emasculation operations (Chandra and Bhatagar, 1975). Hamon and Koechlin (1991) report that after 8 pm., less than 38% hybrids can be obtained by manual self-pollination on non emasculated okra flowers, meaning a high frequency of selfing is occurring. The second hypothesis explains the origin of these seeds as the results of parthenogenetic development of unfertilized ovules of okra or roselle given that the flowers have been emasculated. Zhou et al. (1991) and Mavromatis et al. (2005) produced some parthenogenetic cotton seeds by using *Hibiscus cannabinus* pollen whereas Stewart (1992) and Vlachostergios et al. (2007) got cotton seeds after attempts to fertilize ovules with the pollen of okra. Lastly, in the family of Poacea, Oury et al., (1993) produced parthenogenetic haploids of wheat (*Triticum* genus) using corn (*Zea* genus) pollen.

Conclusion

During this study, we first investigated flowering parameters and on plant fruit set potential after direct and reciprocal cross fertilizations between cotton, okra and roselle. Secondly, we screened for transgenic transfer from transgenic cotton to okra or roselle. In both the first and second cases, our aim was to appreciate the "real" possibilities of transgenic cotton to transfer genes to, and thus to "pollute" okra or roselle.

We found no risk of transgenic transfer into the relative species okra and roselle, even if volunteer pollinations due to sympatric and co-flowering coincidence because: Firstly, in natural conditions, transgenic cotton did not transfer transgenes to its relative species okra or roselle, most likely as a result of flowering patterns that create reproductive isolation; secondly, deliberate hybridizations did not result in transgenic transfer; even in cases where seeds were found, they did not include the target transgenes. In our tests transgene flow from transgenic cotton occurred only within the species.

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