1190 Production of new cotton interspecific hybrids highly resistant to the reniform nematode

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To introgress resistance to *Rotylenchulus reniformis* from the East African wild diploid species *Gossypium longicalyx* Hutch & Lee into upland cotton, crosses involving *G. hirsutum* as the recipient species, *G. longicalyx* as the donor species and several D genome species (*G. thurberi*, *G. harknessii*, *G. davidsonii*, *G. armourianum*, *G. gossypoïdes*, *G. aridum*, *G. raimondii*) as bridge species were made. Only the combination [(*G. hirsutum* x *G. thurberi*)² x *G. longicalyx*] gave seeds (10 seeds from 34 crosses) that produced viable plants (9 plants). These hybrids were denoted as HTL. The use of SSR markers revealed the presence in the hybrid of *G. hirsutum*, *G. thurberi* and *G. longicalyx* specific alleles proving the success of the crosses. GISH analysis confirmed this result showing the presence of 13 *G. longicalyx* chromosomes among the 52 of the hybrid. Three HTL hybrid plants evaluated under controlled conditions following inoculation revealed a high level of resistance to *R. reniformis* equivalent to that of the diploid donor species. All HTL hybrid plants were self-sterile and photoperiodic. Backcrosses achieved using them as female parents gave seeds that produced viable plants. A total of 13 HTL BC1 plants were tested for their resistance to *R. reniformis*. Among them, nine were resistant (5 highly resistant and 4 resistant) and four were susceptible. The potential for development of commercial cotton with resistance to reniform nematodes is discussed.

Keywords: cotton; genetic resistance; *in situ* hybridization; interspecific hybridization; molecular markers; plant parasitic nematodes; *Rotylenchulus reniformis*. Introduction

Identified for the first time as a parasite of cotton (*Gossypium hirsutum* L.) in 1940, the reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) is becoming one of the most damaging nematode species in several cotton producing areas in the United States (Farias et al., 2002; Koenning et al., 2004; Westphal and Scott, 2005). Yield losses due to this nematode may be as high as 30 to 60% according to the levels of infestation and the environmental conditions (Yik and Birchfield, 1984; Farias et al., 2002). Moreover, plant root systems infected by the reniform nematode are more susceptible to secondary infections by fungi or bacteria (Robinson, 1999; Palmateer et al., 2004).

The only management practices used currently to reduce the infestation of *R. reniformis* in the fields are crop rotation and the application of nematicides (Koenning et al., 2004; Lafoe et al., 2005; Westphal and Scott, 2005). However, many growers prefer not to practice crop rotation. The use of nematicides is for various reasons not always efficient, can be dangerous for environment, and represents a considerable cost. Therefore, provided it were effective enough, genetic resistance would be the best management option to control *R. reniformis* because it should be more economical and less intrusive in the environment. Unfortunately, genetic resistance to the reniform nematode is not available in currently-available cotton cultivars (Lafoe et al., 2005; Westphal and Scott, 2005; Weaver et al., 2007).

The genus *Gossypium* L. (Malvaceae) contains 45 diploid species (2n=2x=26) distributed in 8 genome groups (A, B, C, D, E, F, G, K) and 5 allotetraploid species (2n=4x=52). The tetraploid species contain two distinct subgenomes related to the A and to the D diploid genomes (Wendel and Cronn, 2003). The tetraploid species *G. hirsutum* L. ($A_hA_hD_hD_h$), is very susceptible to the reniform nematode, and is the principal cultivated cotton throughout the world (95% of the world production of lint). Only a few wild cotton diploids can be used as sources of resistance to *R. reniformis*. Among them, *G. longicalyx* Hutch. and Lee (F genome) seems to be the most effective donor species (Yik and Birchfield, 1984). Two triple-species hybrids, HLA [(*G. hirsutum* x *G. longicalyx*)² x *G. armourianum*] and HHL [(*G. hirsutum* x *G. herbaceum*)² x *G. longicalyx*], developed by Dr. A. Bell of the U. S. Department of Agriculture in the 1980s, are being exploited in a breeding program (Robinson et al., 2005) aiming at introgressing the high resistance to the reniform nematode of *G. longicalyx* into upland cotton. In this paper, we report progress in developing fertile tetraploid cotton plants exhibiting the resistance trait to reniform nematodes from other trispecific hybrids involving *G. longicalyx*.

Materials and Methods

Plant materials and crosses

The pseudophyletic introgression method (Mergeai, 2006) was followed to try to produce trispecific hybrids involving *G. longicalyx* as the donor species of the resistance to *R. reniformis*. The goal of this approach is to create trispecific hybrids involving *G. hirsutum* and two diploid species. *G. hirsutum* is crossed directly with one of the diploid parents, creating a triploid hybrid. Chromosome doubling of the hybrid is intended to produce a fertile allohexaploid that is crossed to the other diploid species, resulting in the desired allotetraploid trispecific hybrid. The parents involved in the crosses are presented in Table 1. The hybrid plants were backcrossed to *G. hirsutum* to produce BC1 progenies. Morphological characteristics and pollen fertility were observed on the plants produced.

Plants were grown in the greenhouse and crosses were carried out in the following manner. Flowers were emasculated the afternoon before anthesis and the stigma was covered by a small plastic sachet; pollen was applied to stigmas between 08:00 and 11:00 hours the following morning. To avoid capsule shedding, a small piece of cotton wool, containing a drop of the growth regulator solution recommended by Altman (1988) was applied on the ovary just after pollination. The growth regulator solution was prepared with 100 mg β-naphtoxyacetic acid ($C_{12}H_{10}O_3$) + 50 mg gibberellic acid ($C_{19}H_{22}O_6$) in 1 1iter H_2O. For BC1 seed germination, *in vitro* culture was used because of the lack of germination vigor of the hybrid seed. Seeds were hulled, put on MS medium culture (Murashige and Skoog, 1962), and seedlings were transferred on sterile mixture of 3:2:1 (v:v:v) of compost, sand and peat, and then acclimated in a growth chamber programmed for 12-hour light (120–140.10^{-6} E/m².s), 28°C and 26°C respectively day and night temperatures, and 55%-60% air humidity.

DNA isolation and SSR markers analysis:

Young fresh leaf tissue was used for extraction of total genomic DNA. DNA was isolated by the protocol of Murray and Thompson (1980) modified by Vroh Bi et al. (1996). Eleven microsatellite markers BNL 1897, BNL 3989, BNL 4030, BNL 3992, BNL 1440, BNL 1604, BNL 2847, BNL 256, BNL 2495, BNL 2961 and BNL 3411 developed at Brookhaven National Laboratory and distributed respectively on the linkage groups c2 – c14, c3 – c17, c4 – c22, c5 – D08, c6 – c25, c7 – c16, c9 – c23, c10 – c20, c12 – c26, A02 – D03 and A03 – D02 of *G. hirsutum* (Nguyen et al., 2004) were used to verify that hybrids had been produced. Clone sequences used for primer definition are available at **http://ukcrop.net/perl/ace/search/cottonDB**.

Genomic *in situ* hybridization

To ensure the presence in the hybrid of the 13 chromosomes brought by *G. longicalyx*, the donor species of the resistance trait to *R. reniformis*, genomic *in situ* hybridizations were performed. Total genomic DNA was labelled by the nick translation method with digoxigenin-11-dUTP and biotin-14-dATP according respectively to the labeling protocol of Roche (version October 2004) and of Invitrogen life technologie (version 02/10/2003). Digoxigenin-11-dUTP was used to label total genomic DNA of G. *longicalyx* and biotin-14-dATP to label total genomic DNA of *G. hirsutum*. Mitotic metaphase chromosomes were prepared from the hybrid root tips and *in situ* hybridization was performed according to the protocol used by D'Hont et al. (1995). Chromosomes were observed and counted with an epifluorescence Nikon Eclipse E800 microscope with appropriate filters and equipped with a JVC KY-F 58E camera. Images were captured and processed with the software PhotoStudio 2000 and Paint Shop Pro 7.

Evaluation of the resistance to *R. reniformis* of the plant material produced

Resistance to *R. reniformis* of the hybrids, their parents and the BC_1 progenies were assessed in experiments in a growth chamber programmed for 12-hour light (120–140.10^{-6} E/m².s) and 12-hour dark with air day and night temperatures of 28°C and 26°C respectively. The *R. reniformis* nematodes used in this investigation was collected by the Agronomical Institute of Parana (IAPAR: Instituto Agronômico do Paraná) in Brazil and cultured in growth chambers and in the greenhouse on *G. hirsutum* (cv C2) plants at Gembloux Agricultural University. Inoculum preparation consisted of a suspension in water of *R. reniformis* eggs extracted from infested cotton roots obtained according to the centrifugal flotation technique of Caveness and Jensen (1955) and the *R. reniformis* inoculum preparation method of Walters and Barker (1993). Reniform nematode egg inoculations were performed on 30 day old plants planted in 5 liter pots filled with a 3:2:1 (v:v:v) sterile mixture of compost, sand and peat. The planting medium in each pot was infested with 6000 *R. reniformis* eggs by injecting the appropriate nematode eggs suspension 2 to 3 cm deep at four points located 2 cm apart from the stem. Sixty days after inoculation, the soil was removed by soaking the roots in water, and the entire root systems were gently harvested. Roots with nematode egg masses were blotted dry with absorbing paper and weighed. Eggs were extracted by macerating the roots in 0.25 % NaOCl solution with a blender for 30 seconds, removing root debris by filtration through nested 75 µm and 20 µm mesh sieves, and removing residual root debris by the centrifugal flotation technique using a kaolin powder and a MgSO4 solution of 1.18 specific gravity (Caveness and Jensen, 1955). Eggs were counted by using two 15 ml aliquots and the number of eggs per gram of root was determined for each plant. The host status was assessed according to the scale proposed by Yik and Birchfield (1984) where relative plant resistance is based on egg production per gram of root expressed as a percentage of egg production per gram on *G. hirsutum* control plants within the test. This scale contains the following classes: 0% = immune, 1-10% = highly resistant, 11-25% = resistant, 26-40% = moderately resistant, 41-100% = susceptible as check and above 100% = very susceptible.

Results

Trispecific hybrid production and morphological observations

Of the 184 crosses of the ten combinations only two produced seeds (Table 1). With the combination [(*G. hirsutum* x *G. longicalyx*)² ♀ x *G. armourianum* ♂], 36 crosses were made and only one seed was obtained. This seed produced a seedling which died after germination. With the combination [(*G. hirsutum* x *G. thurberi*)² ♀ x *G. longicalyx* ♂], 34 crosses were completed and 10 seeds were obtained. Nine of these seeds produced viable plants. These hybrid plants were denoted as HTL. They exhibited morphological traits that were intermediate to those of the parents without phenotypic segregation. All grew more than two meters high with erect mainstems (like *G. longicalyx* and *G. thurberi*), and had about 30 vegetative branches that were on average longer than 40 cm (like *G. longicalyx*). These branches carried generally tri-lobated leaves similar to the leaves of the hexaploid, but their size was bigger and closer to the ones of the *G. hirsutum* controls. Planted in May, the flowering of HTL plants only started in at the end of September when the day length in Gembloux was close to 12 hours. The *G. hirsutum* control plants sowed at the same time flowered about 50 days earlier than the hybrids. The flowers of HTL plants were a bit smaller than those of *G. hirsutum* but their general aspect was similar. The hybrid plants were all self-sterile. All the flowers aborted a few days after anthesis. The mean proportion of pollen grains stainable with acetocarmine was about 100% for *G. hirsutum* and only 9.0% for the HTL plants. The *G. hirsutum* control plants were 70 to 91 cm tall with 8 to 12 main stem nodes and the time from sowing to flowering was 64 to 84 days.

SSR markers analysis

Table 2 presents the results obtained with the SSR markers used to screen the HTL material and its parents. The 11 SSR markers used showed 64, 43, 39, 83 and 104 alleles respectively in *G. hirsutum*, *G. thurberi*, *G. longicalyx*, the (*G. hirsutum* x *G. thurberi*)² hexaploid and the [(*G. hirsutum* x *G. thurberi*)² x *G. longicalyx*] hybrid. Polymorphisms among the three parental species were detected, revealing specific alleles for each of them (39, 20 and 20 specific alleles for respectively *G. hirsutum*, *G. thurberi* and *G. longicalyx*). All alleles detected in *G. hirsutum* were present in the hexaploid and in the HTL hybrid. All the alleles revealed in *G. longicalyx* were also present in the HTL hybrid. For *G. thurberi*, of the 43 alleles revealed in the diploid parent, only 38 were present in the hexaploid and in the HTL hybrid. That is to say that 5 of the 20 specific alleles detected in this species were revealed neither in the hexaploid nor in the HTL hybrid. This observation was made with BNL 1897 (Figure 1), BNL 256 and BNL 2495. But for the other SSR markers used, the hexaploid exhibited specific alleles of both the parental species *G. hirsutum* and *G. thurberi* while the hybrid plants showed specific alleles of the three parental species *G hirsutum* , *G. thurberi* and *G. longicalyx*. These results confirm the success of the crosses and the hybrid character plants produced.

GISH analysis

Genomic *in situ* hybridization was performed on the hybrid chromosome preparation using *G. longicalyx* total DNA as a digoxigenin (DIG)-labelled probe and *G. hirsutum* total DNA as a biotin (BIO)-labelled probe. The DAPI filter shows 52 chromosomes (Figure 2a) resulting from the counterstaining of the chromosome preparation with DAPI. With the FITC filter, the hybrid chromosome sequences homologous to *G. longicalyx* total DNA fluoresced green resulting from the detection of the *G. longicalyx*-labelled DNA with FITC. With the Texas Red filter, the hybrid chromosomes sequences homologous to *G. hirsutum* total DNA fluoresced red resulting from the detection of the *G. hirsutum*-labelled DNA with Texas Red. When FITC detection and Texas Red detection were superimposed (Figure 2b), three populations of chromosomes are differentiated. Thirteen chromosomes appeared green and should be those from *G. longicalyx*. Thirteen chromosomes appeared yellow-orange and should be those from *G. hirsutum* A_h subgenome. Twenty six small chromosomes appeared red and can be those from *G. thurberi* and the D_h subgenome of *G. hirsutum* (the D genome is comprised of small chromosomes - Phillips and Strickland, 1966). Because of the homology between *G. hirsutum* D_h subgenome and *G. thurberi* D genome it has not been possible to differentiate the 13 chromosomes from these two genomes. These results prove the presence in the hybrid of the 13 chromosomes brought by the donor species *G. longicalyx.*

Resistance to *R. reniformis* of the HTL hybrid

Among the parental genotypes tested, *G. hirsutum* had the greatest number of eggs per g root (506 eggs.g^{-1} root) and *G. thurberi* had 310 eggs.g^{-1} root (Table 3). *G. longicalyx* and the HTL hybrid had >2.5 % of the eggs per g root of the susceptible *G. hirsutum*.

Production of HTL BC1 plants and assessment of their resistance to *R. reniformis*

Having confirmed that the HTL plants were true hybrids and that the hybrids were resistant to *R. reniformis*, all plants were backcrossed to *G. hirsutum*. Of 183 backcrosses attempted, 22 seeds were obtained (with generally one seed per boll). All these seeds germinated on MS culture medium, but when the seedlings were transferred to the growth chamber, some of them were unable to grow and remained stunted or died. Of 22 germinated seeds only 13 gave viable plants. Four HTL BC1 plants were susceptible to the reniform nematode, four BC1 plants were resistant, and five BC1 plants highly resistant (Table 4).

Discussion

Of the 10 *Gossyium* species combinations attempted in 184 crosses in order to create a trispecies cotton hybrid of *G. hirsutum* and *G. longicalyx* by means of a diploid D genome bridge species, only two combinations gave seeds. The tetraploid cotton *G. hirsutum* contains two distinct subgenomes (A_h and D_h) with 26 chromosomes for each (Wendel and Cronn, 2003). Since the F genome of *G. longicalyx* is more closely related to the A_h subgenome than to the D_h subgenome, and the D_h subgenome retains appreciable chromosome homology with species of the D genome group (Brown and Menzel, 1952; Endrizzi, 1957; Philips and Strickland, 1966), the strategy was to substitute an A_h subgenome chromosome set for an F genome chromosome set from the donor *G. longicalyx*, to substitute a D_h chromosome set for a D genome chromosome set from the bridge species. Such a strategy would create a hybrid with the same number (52) and type of chromosomes as upland cotton. Such a hybrid might offer the possibility of successfully backcrossing it on *G. hirsutum* cultivars to produce viable genetic stocks carrying the nematode resistance trait. Theoretically, hybrid seeds could have been obtained from all the combinations tried here. Incompatibility barriers in cotton germplasm are often less important than in other genera and viable seeds can be obtained for almost all the possible crosses without using *in vitro* culture techniques on immature embryos, provided a sufficiently large number of pollinations are carried out and an effective hormone mixture is applied at pollination (Mergeai, 2004). Notable exceptions concern the species *G. gossypoides*, *G. davidsonii*, *G. klotzchianum*, and the "*sangineum*" race of *G. arboreum* because of the presence of complementary lethal genes that condition embryo or seedling death in hybrids with standard tetraploids (Stewart, 1995). The unsuccessful combinations observed here could be linked to stress conditions experienced by the plants cultivated in pots under greenhouse conditions. Probably with a larger number of crosses, some of the unsuccessful combinations could have produced seeds. The success of the [(*G. hirsutum* x *G. thurberi*)² x *G. longicalyx*] combination leads us to suppose that it more easily produces seeds compared to the other species tested.

SSR markers analysis on the HTL plants showed the presence of specific alleles of the three parental species indicating the success of the crosses. However, some specific SSR alleles of *G. thurberi* were not observed in the hexaploid and in the HTL hybrid plants. As the GISH observations made on the HTL materials confirmed their tetraploid nature, the decrease in the number of *G. thurberi* specific SSR markers observed should thus not be related to a problem of chromosome transmission caused by irregular meiosis during the development of the hybrids structure as was observed by Faure et al. (2002) in the crosses between cultivated sunflower and perennial *Helianthus* species. At least three different explanations can be proposed to account for the observed phenomena: (i) Heterozygosity in *G. thurberi* can explain the variability observed in the amplification of the specific SSR alleles of this species in the interspecific hybrids; (ii) The lack of amplification of a SSR allele in a genotype can be the result of divergence in the sequences flanking the microsatellite, creating a null-allele (Lavi et al., 1994); (iii) The lack of amplification of a SSR allele in a genotype can result from the production of an undetectable amount of PCR product (Smulders et al., 1997) which means that the DNA sequences are present but cannot be amplified. Farelly et al. (1995) showed that a reduction of the relative proportion of target to non target DNA in the template concentration can cause a reduction of the amplification potential. In our hexaploid and HTL trispecies hybrid, as it was noted for RAPD markers by Heun and Helentjaris (1993) for intraspecific maize hybrids, the association of diploid and tetraploid cotton chromosomes in large genomic structures may induce competition for amplification amongst SSR fragments, and *G. thurberi* SSR products seem to be less competitive.

SSR data were globally consistent with morphological observations made. Indeed the hybrids exhibited various parental traits related to growth habit, leaf shape and flower morphology. The 52 chromosomes revealed through GISH analysis, confirms this hybrid status. Theoretically, this hybrid must contain a 13 chromosomes set from each constituent parent (F genome, A_h subgenome, D_h subgenome and D genome). With fluorescent hybridization, the 13 chromosomes from the donor species *G. longicalyx* were detected, confirming that the hybrid created carries a complete F-genome set of chromosomes inherited from the resistant parent.

Results of the evaluation of the resistance to *R. reniformis* of the HTL hybrid and its parents showed that *G. hirsutum* and G *thurberi* are susceptible whereas *G. longicalyx* and the three HTL hybrid plants tested were highly resistant. These data confirm the high resistance of *G. longicalyx* to the reniform nematode evidenced by Yik and Birchfield (1984) and Robinson et al. (2006). They also reveal the expression of this resistance trait in the HTL hybrid. The resistance of all the HTL hybrid plants tested implies that resistance to this nematode could be a dominant trait. In addition to this useful trait, the hybrid showed undesirable characters such as an elongated and slender growth habit, a very long growth cycle, linked to photoperiodism and self-sterility. To get rid of these traits from the wild donor and bridge species, a program of backcrosses with the recipient species *G. hirsutum* will be necessary. The first backcrosses carried out with *G. hirsutum* cultivars to HTL hybrids gave a total of 22 HTL BC1 seeds from 180 pollination attempts. Despite the use of *in vitro* culture, only 13 viable BC1 plants were obtained. This result is not surprising for such material. Within cotton tri-species hybrids, BC1 seed typically have low viability. In addition to low germination, seedlings may stop growing and remain stunted or die after a short period of development (Kammacher, 1966). With the BC1 seeds obtained from the backcrosses of *G. hirsutum* to the [(*G. hirsutum* x *G. arboreum*)² x *G. raimondii*] trispecies hybrid, Kammacher (1966) noted a low level of viability (40%). These low levels of viable BC1 materials could be due to chromosomic disequilibrium or to chromosome recombinations that would have been able to generate lethal gene associations called "synthetic lethality" (Kammacher, 1967).

Nine of the 13 HTL BC1 viable plants investigated in this study were resistant to *R. reniformis* (5 plants were highly resistant and 4 plants were resistant) and four were susceptible. It is to be noted that 5 of the 9 resistant BC1 plants had relative low root weight that might be affecting apparent levels of resistance. Insufficient BC1 plants were available for any conclusions regarding the inheritance of the resistance to *R reniformis*. However, the results obtained so far are consistent with the hypothesis made by Bell and Robinson (2004) for whom the resistance trait to reniform nematode in cotton could be determined by a single dominant gene. For the moment, it can be stated that the characteristics of the [(*G. hirsutum* x *G. thurberi*)² x *G. longicalyx*] triple hybrid plants make them good candidates to obtain the introgression of the desired resistance to reniform nematodes in upland cotton.

Table 1. Crosses achieved to create a tri-species hybrid including *G. hirsutum* as recipient species, *G. longicalyx* as donor species and a diploid D genome species as bridge.

Crosses	No. of	No. of	No. of	No.

	crosses	abortion	bolls harvested	of seeds
[(*G. hirsutum* x *G. harknessii*)²♀ x *G. longicalyx* ♂]	20	0	17	0
[(*G. hirsutum* x *G. thurberi*)² ♀ x *G. longicalyx* ♂]	34	1	31	10
[(*G. hirsutum* x *G. aridum*)² ♀ x *G. longicalyx* ♂]	1	0	1	0
[(*G. hirsutum* x *G. raimondii*)² ♀ x *G. longicalyx* ♂]	14	4	10	0
[(*G. hirsutum* x *G. longicalyx*)² ♀ x *G. harknessii* ♂]	2	0	2	0
[(*G. hirsutum* x *G. longicalyx*)² ♀ x *G. davidsonii* ♂]	45	6	24	0
[(*G. hirsutum* x *G. longicalyx*)² ♀ x *G. armourianum* ♂]	36	2	24	1
[(*G. hirsutum* x *G. longicalyx*)² ♀x *G. thurberi* ♂]	24	2	22	0
[(*G. hirsutum* x *G. longicalyx*)² ♀ x *G. gossypoïdes* ♂]	2	0	2	0
[(*G. hirsutum* x *G. longicalyx*)² ♀ x *G. raimondii* ♂]	6	0	6	0
Total	**184**	**15**	**136**	**11**

Table 2. Frequency distribution of the alleles revealed with the 11 SSR markers used to screen the [(*G. hirsutum* x *G. thurberi*)² x *G. longicalyx*] tri-species hybrid (HTL), the (*G. hirsutum* x *G. thurberi*)² hexaploid (HT) and the parental species *G. hirsutum* (H), *G. thurberi* (T), *G. longicalyx* (L).

Linkage group	SSR	Total alleles					Common alleles								Specific alleles		
		H	T	HT	L	HTL	H&T	H&HT	H&L	H&HTL	T&HT	T&L	T&HTL	L&HTL	H	T	L
C2 – C14	BNL 1897	6	3	7	2	8	1	6	1	6	1	0	1	2	4	2	1
C3 – C17	BNL 3989	10	8	12	8	14	5	10	6	10	8	6	8	8	4	2	1
C4 – C22	BNL 4030	6	4	9	3	12	1	6	0	6	4	0	4	3	5	3	3

C5 – D08	BNL 3992	6	4	8	4	11	2	6	2	6	4	1	4	4	4	2	3
C6 – C25	BNL 1440	4	2	5	2	6	1	4	1	4	2	0	2	2	2	1	1
C7 – C16	BNL 1604	6	4	6	4	10	4	6	0	6	4	0	4	4	2	0	4
C9 – C23	BNL 2847	10	6	13	6	16	3	10	2	10	6	1	6	6	5	2	3
C10 – C20	BNL 256	4	2	5	2	6	0	4	0	4	1	0	1	2	4	2	1
C12 – C26	BNL 2495	4	4	5	3	6	1	4	1	4	2	2	2	3	3	2	1
A02 – D03	BNL 2961	4	3	7	3	8	0	4	1	4	3	1	3	3	3	2	1
A03 – D02	BNL 3411	4	3	6	2	7	1	4	1	4	3	1	3	2	3	2	1
Total		64	43	83	39	104	19	64	15	64	38	12	38	39	39	20	20

Table 3. Results of the evaluation of the resistance to *R. reniformis* of the HTL hybrid and its parents.

Genotypes	Total number of eggs per plant	Root weight per plant (g)	Eggs No. per gram roots	% f eggs per gram roots compared to *G. hirsutum* control	Host status[z]
G. hirsutum (cv C2) (2 plants)	53 894	106.54	506	100	S
G. thurberi	7 407	23.91	310	61.27	S
G. longicalyx (2 plants)	253	21.20	12	2.38	HR
HTL (3 plants)	314	79.69	4	0.80	HR

[z] 0%= immune (I), 1-10% = highly resistant (HR), 11-25% = resistant (R), 26-40% = moderately resistant (MR), 41-100% susceptible (S).

Table 4. Results of the assessment of the resistance to *R. reniformis* of HTL BC1 plants

Genotypes	Total number of eggs per plant	Root weight per plant (g)	Eggs No. per gram roots	% of eggs per gram roots compared to *G. hirsutum* control	Host status[z]
G. hirsutum (cv C2): 2 plants	147 167	175	841	100 %	S
HTL BC1-1	123 334	315	392	46.56 %	S
HTL BC1-2	29 500	182	162	19.27 %	R
HTL BC1-3	142 667	217	658	78.17 %	S
HTL BC1-4	5 700	289	20	2.35 %	HR
HTL BC1-5	30 000	317	95	11.25 %	R
HTL BC1-6	146 667	224	655	77.86 %	S
HTL BC1-7	128 667	239	539	64.01 %	S
HTL BC1-8	4 334	63	69	8.18 %	HR
HTL BC1-9	14 167	74	192	22.76 %	R
HTL BC1-10	44 167	205	216	25.62 %	R
HTL BC1-11	500	44	12	1.35 %	HR
HTL BC1-12	3 000	35	86	10.19 %	HR
HTL BC1-13	84	16	6	0.62 %	HR

[z] 0%= immune (I), 1-10% = highly resistant (HR), 11-25% = resistant (R), 26-40% = moderately resistant (MR), 41-100% susceptible (S).

List of captions for figures

Figure 1 : SSR electrophoresis profile of the primer BNL 1897 showing the triple species character of the HTL hybrid *G. hirsutum x G. thurberi x G. longicalyx* and the absence of some specific alleles of *G. thurberi* in the (*G. hirsutum* x *G. thurberi*)² hexaploid and in the HTL hybrid: 1) *G. hirsutum*; 2) *G. thurberi*; 3) *G. longicalyx*; 4) the (*G. hirsutum* x *G. thurberi*)² hexaploid; 5) nine plants of the [(*G. hirsutum* x *G. thurberi*)² x *G. longicalyx*] HTL hybrid; 6) specific allele of *G. thurberi* present in the hexaploid and in the HTL hybrid; 7) specific allele of *G. hirustum* present in the hexaploid and in the HTL hybrid; 8) specific allele of *G. longicalyx* present in the HTL hybrid; 9) specific allele of *G. thurberi* absent in the hexaploid and in the HTL hybrid.

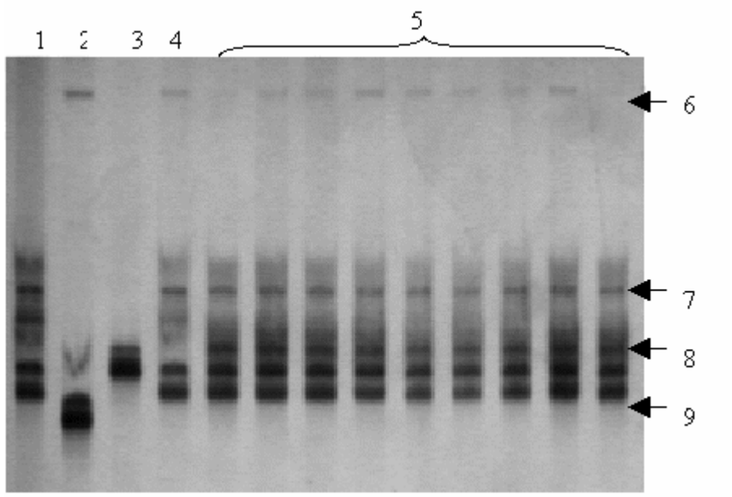
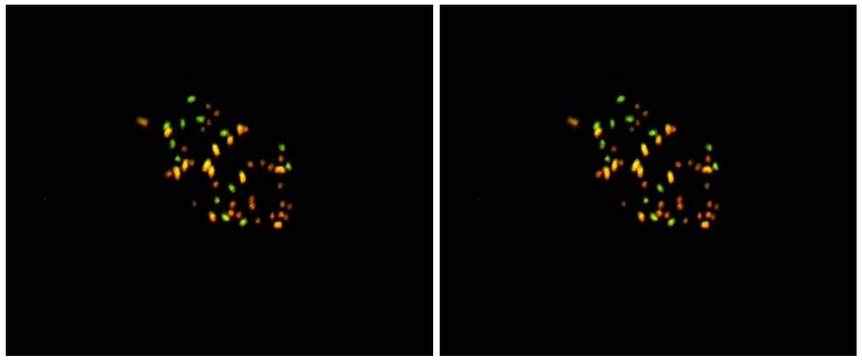


Figure 2 : Genomic *in situ* hybridization on mitotic metaphase chromosomes of the [(*G. hirsutum x G. thurberi*)² *x G. longicalyx*)] tri-specis hybrid (HTL): a) 52 chromosomes of the HTL hybrid revealed by counterstaining with DAPI; b) 13 green chromosomes from *G. longicalyx*, 13 yellow-orange chromosomes from the A_h subgenome of *G. hirsutum* and 26 red chromosomes from *G. thurberi* and the D_h subgenome of *G. hirsutum* revealed in the HTL hybrid after the superimposition of FITC detection and Texas Red detection.



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