CYTOGENETIC STUDIES OF *BRASSICA NAPUS-SINAPIS ALBA* HYBRIDS FROM OVARY CULTURE AND PROTOPLAST FUSION. ATTEMPTS TO INTRODUCE *ALTERNARIA* RESISTANCE INTO RAPESEED

A.M. CHEVRE (1), F. EBER (1), H. BRUN (1), J. PLESSIS (1), C. PRIMARD (2), M. RENARD (1)

(1) INRA - BP 29 - F.35650 LE RHEU
(2) INRA - ROUTE DE ST CYR - F.78026 VERSAILLES CEDEX

INTRODUCTION

Interspecific crosses are valuable to introduce missing characters into cultivated species and to enlarge genetic variability. In order to overcome crossing barriers, protoplast fusion or embryo rescue are used.

White mustard (*Sinapis alba* L., SalSal, $2n = 24$) bears tolerance to drought and to black spot (*Alternaria brassicae* Berk. Sacc.) (Brun *et al.* 1987) which occasionally causes severe yield losses in rapeseed. So it is an interesting species for rapeseed (*Brassica napus* L., AACC, $2n = 38$) improvement. Sexual hybridizations have been accomplished between rapeseed as female (Heyn 1977) or as male (Ripley and Arnison 1990) and white mustard. Somatic hybrids have been also obtained by protoplast fusion (Primard *et al.* 1988). The characterization of these somatic hybrids revealed differences between them for mitochondrial and chloroplast DNA and for their meiotic behavior. In the present paper, reciprocal sexual hybrids (*Sinapis alba* x *B. napus*) and their backcrossed progeny to rapeseed were produced and compared to somatic hybrids from the same cultivars and their progeny.

MATERIAL AND METHODS

Plant material : As for somatic hybridization, we used the Spring oilseed line "Brutor" and the white mustard variety cv "Carine". Ten plants of the latter, previously selected for tolerance to *Alternaria*, were used as parents. Nine somatic hybrids (B, C, F, G, H, P, R, U, V) produced by Primard *et al.* 1988 were studied. All the hybrids were backcrossed to "Brutor" as recurrent parent. The procedure for obtaining the different progeny is shown in Figure 1.

Ovary culture : For obtaining sexual hybrids, five to six days after pollination, ovaries were excised and subjected to *in vitro* culture as previously described by Delourme *et al.* 1989.

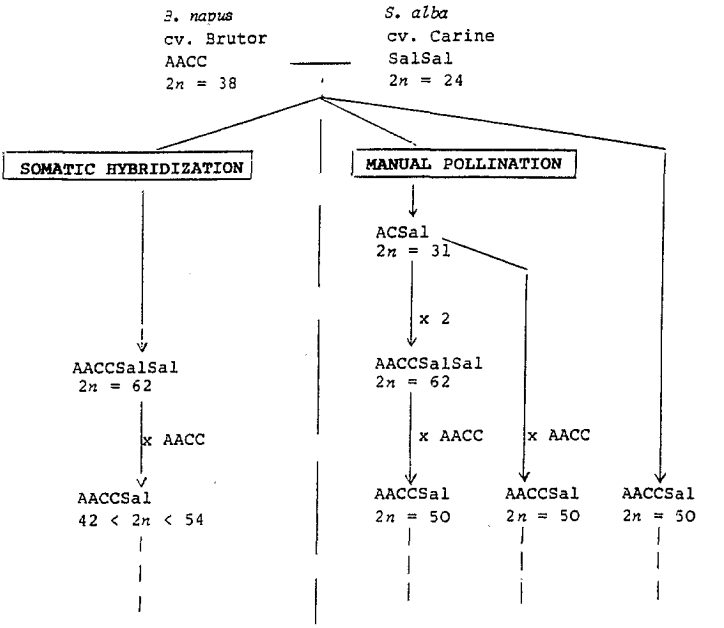
Meiotic behavior : Floral buds were fixed in Carnoy solution (ethanol, chloroform, acetic acid 6 : 3 : 1) for 24 hours and stored in 50 % ethanol solution at 4°C. After squashing an anther in a drop of aceto camine solution, pollen mother cells (PMCs) at Metaphase I stage of meiosis were observed.

Male fertility : It was estimated by pollen stainability in aceto-carmine. Three flowers and at least 800 pollen grains were observed per plant.

Colchicine treatment : Cuttings of the hybrids were dipped in a 0.3 % colchicine solution for 2 hours. After rinsing with running water, they were transplanted in pots.

Alternaria resistance test : We used the method of Brun *et al.* (1987) with some modifications. After pregermination, plants were incubated in a growth chamber for the first ten days and after at 10°C. Droplets of 5×10^4 spores/ml solution were placed the 10^{th} day on cotyledons under 100 % humidity for 36 to 40 hours. Plants were scored 48 and 92h later.

Fig. 1 :Procedure for obtaining the different hybrids between *B.napus* and *S.alba*



RESULTS

Production of hybrids and successive backcrosses.

Hybrids were obtained by ovary culture from the interspecific reciprocal crosses (Table 1). Although no significant differences were observed for the total number of plants produced, the genomic structure seemed to be influenced by the female parent used. Half of the hybrids produced on white mustard cytoplasm were hybrids of ACSal genomic constitution whereas only one similar hybrid was obtained on rapeseed cytoplasm. Higher frequency of hybrid plants of AACCSal genomic structure was observed when rapeseed was used as female parent. Plants with $2n = 4$ chromosomes (AC SalSal) were only obtained from crosses where white mustard was the female parent.

Table 1. Production of interspecific hybrids using ovary culture

Brutor ♂						Brutor ♀					
Number of ovaries	Embryos		Plants			Number of ovaries	Embryos		Plants		
	Nbr.	%	Nbr.	Genomic structure	%		Nbr.	%	Nbr.	Genomic structure	%
412	21	5.1	5	ACS_a	1.21	586	16	2.73	1	ACS_a	0.17
			1	$AACCS_a$	0.24				10	$AACCS_a$	1.71
			3	ACS_aS_a	0.73						
T.412	21	5.1	9	-	2.18	586	16	2.73	11	-	1.88

Table 2. Meiotic behavior of F1 hybrids, amphidiploids and somatic hybrids

		2n	Number of PMC	chromosome pairing per PMC				% of pollen fertility
				Univalents	Bivalents	Trivalents	Quadrivalents	
F_1 hybrids	a	31	72	13.65 (7.20)*	8.22 (4.11)	0.04 (0.1)	0.19 (0.1)	11.5 to 35.15
	b	31	58	13.36 (9.21)	7.21 (3.10)	-	0.67 (0.2)	26.75
Amphidiploids	a	62	26	2.92 (0 .8)	28.92 (27.31)	-	0.31 (0.1)	87.32
	b	62	31	2.84 (0.8)	28.87 (27.31)	-	0.35 (0.1)	-
Somatic Hybrids	F	62	22	1.73 (0.6)	30.05 (28.31)	-	0.04 (0.1)	-
	G	62	17	2.47 (2.6)	29.76 (28.30)	-	-	41.9
	R	62	18	4.67 (2.8)	28.56 (27.30)	-	0.06 (0.1)	68.7
	U	62	14	0.57 (0.5)	28.5 (25.31)	0.14 (0.1)	1 (0.3)	32.1
	V	62	14	3.43 (2.8)	28.86 (25-30)	-	0.21 (0.1)	37.9

a : Hybrids on white mustard cytopalsm
b : Hybrids on rapeseed cytoplasm
* : Range

By ovary culture, 0.98 % and 0.68 % of BC1 plants of expecte constitution AACCSal(2n = 50) were produced after backcrossing to rapeseec the ACSal hybrids on white mustard and rapeseed cytoplasms respectively.

The chromosomes of only one F1 hybrid plant from each cytoplasmi origin was sucessfully doubled after colchicine treatment.The resultin amphidiploids (AACCSalSal, 2n = 62) and the somatic hybrids gave seed after backcrossing to rapeseed without the need of embryon rescue.

Cytogenetic studies.

The plants of the same ploidy level were compared : ACSal hybrids amphidiploids and BC1 plants (figure 1).

The meiotic behaviors of the F1 hybrid with 31 chromosomes were no significantly different from each other (table 2). Their pollen fertilit was low ranging from 11.5 to 35.15%.

No cytoplasmic effect on meiotic behavior (table 2) was observed i the amphidiploids (AACCSalSal). The pollen fertility of the amphidiploid o white mustard cytoplasm was better than that F1 hybrids. At the same ploid level, the study of 6 somatic hybrids showed that only 2 of them (V and G were comparable to sexual amphidiploids for average of univalents (test t $p > 0.05$). The other four were different. The pollen fertility of th somatic hybrids was highly variable.

For the sexual hybrids, plants of AACCSal genomic constitution fro three different origins were studied : hybrids from direct crosses Carine Brutor, from F1 hybrids x Brutor, from amphidiploids x Brutor (fig.1). Al of them had 50 chromosomes and a meiotic behaviour close to the expecte one (12 univalents of white mustard + 19 bivalents of rapeseed). Few cell (0 to 8.62 %) with less than 12 univalents were observed (table 3). Thei pollen fertility ranged from 36 to 90 %.

Table 3. Study of plants with genomic constitution AACCSal, 2n=50, from different origins : percentage of pollen mother cells (PMCs) wit less than 12 univalents

	Natural hybrids						Somatic hybrids							
	I		II		III									
	a	b	a	b	a	b	C	F	G	H	P	R	U	V
Number of plants	1	8	7	2	3	2	2	2	5	2	1	2	1	5
Number of PMCs	29	227	190	36	73	58	53	52	82	48	19	31	15	96
% of cell with less than 12 univ.	3.4	7.9	0	0	0	8.6	15.1	23.1	4.9	29.2	47.4	22.6	40	5.21

I : Hybrids obtained from crosses Carine X Brutor
II : Hybrids obtained from crosses F1 hybrids X Brutor
III : Hybrids obtained from crosses amphidiploids X Brutor
a : Hybrids on white mustard cytoplasm
b : Hybrids on rapeseed cytoplasm

Three somatic hybrids (F,C,P) after backcrosses to rapeseed produce only plants with 50 chromosomes but they showed a high frequency of cell with less than 12 univalents (table 3). As at the amphidiploid level, n significant differences were observed between the BC1 plants from V and somatic hybrids and hybrids from sexual crosses (table 3). However the also produced plants with a chromosome number ranging from 48 to 54. Fro

other three somatic hybrids (H,R,U), the meiotic behavior of plants with 50 chromosomes (table 3) and the production of plants with various chromosome number (48 to 54) revealed important differences with the sexual hybrids. The five plants produced from the B somatic hybrid had 42 to 51 chromosomes and none of them showed 50 chromosomes. The pollen fertility was estimated to 52 to 89 %.

The chromosome number of BC2 progeny ranged from 38 to 47 and no significant difference was observed between plants from somatic or sexual hybrids.

Until now, all the plants from sexual hybrids which showed *Alternaria* resistance carried additionnal chromosomes. In the BC2 and BC3 progeny of H and B somatic hybrids respectively, resistant plants with 38 chromosomes (19 bivalents) were selected. After 4 or 3 selfings, 12 % and 49 % of plants were tolerant from H and B somatic hybrids respectively.

DISCUSSION

Usually in *Brassicaceae*, interspecific crosses are most successful when plant with the highest chromosome number is used as female (Quazi 1988, Kerlan *et al.* 1991). From crosses with *Sinapis alba*, Heyn (1987), Mohapatra and Bajaj (1987) reported hybrid production by using *B. napus* and *B. juncea* as female, respectively. On the contrary, Ripley and Arnison (1990) obtained hybrids between *S. alba* and *B. napus* using white mustard as female. In the present study, reciprocal hybrids were produced. However it seems that the plant of *S. alba* used as female had a great influence on the genomic structure of the hybrid produced. These results are in agreement with those reported by Ripley and Arnison (1990) which showed an influence of the varieties used. The relatively high frequency of unreduced gametes observed in the *Brassiceae* (Heyn 1977) could explain the genomic structure AACCSal and ACSalSal. In the same way, BC1 hybrids obtained from crosses between F1 hybrids and rapeseed probable resulted from unreduced gametes of F1 hybrids. As the F1 hybrids had a poor fertility, the frequency of unreduced gametes is probably very low. This observation could explain why the number of plants produced for 100 ovaries was lower than for F1 hybrid production. The same result was reported by Delourme *et al.* 1989 from crosses between *Diplotaxis erucoides* and *B. napus*.

In the F1 hybrids, more univalents and bivalents but less multivalents were observed compared to the average meiotic behavior reported by Ripley and Arnison 1990. The high allosyndetic pairing in rapeseed haploids (AC) (Renard and Dosba 1980) and the presence of 21 univalents in an hybrid between *B. oleracea* (CC) and *S. alba* (Mizushima 1980) suggested that most of the pairing observed involved rapeseed chromosomes. However some non homologous recombination could occur because of the high frequency of bivalents and the presence of multivalents.

This hypothesis was also supported by the presence of some multivalents in 33 % of cells of the amphidiploids obtained from F1 hybrid colchicine doubling. In these plants only 7 % of cells showed 31 bivalents. The high ploidy level ($2n = 6x = 62$ chromosomes) could explain the meiotic irregularities observed.

All the somatic hybrids were symetric whereas many asymmetric hybrids were reported after interspecific protoplast fusion (Sjodin and Glimelius 1989, Landgren and Glimelius 1990). The meiotic behavior of somatic hybrids and of their BC1 progeny compared to those of plants obtained from sexual crosses suggested that protoplast fusion or plant regeneration may induce chromosome rearrangements. Similar results were reported in *Solanum* species (Ehlenfeldt and Helgeson 1987).

Cytoplasmic effect does not seem to explain the differences observed. The meiotic behaviors of the F1 hybrids, their amphidiploids and the BC1 plants were similar on the two cytoplasms and somatic hybrids and their BC1 progeny with the same cytoplasmic DNA (Primard *et al.* 1988) showed different meiotic behavior. These results are in agreement with those

reported from natural interspecific hybrids (Busso *et al.* 1987) and from somatic hybrids (Landgren and Glimelius 1990).

The occurrence of intergenomic pairing has been confirmed by the selection of *Alternaria* tolerant plants with 38 chromosomes in the progeny of two somatic hybrids. The study of the *Alternaria* tolerance genetic determinism is in progress in these plants.

At the same time, addition lines from natural hybrids will be selected in an attempt to find markers linked to the resistance gene(s).

We have shown in this study that whatever the mean used to produce sexual AACCSal plants after manual pollination, the probability to induce recombination between white mustard and rapeseed chromosomes is very low. On the contrary, it seems that by protoplast fusion it is possible to induce chromosome rearrangments.

REFERENCES

BRUN, H., PLESSIS, J., RENARD, M. 1987. Resistance of some crucifers to *Alternaria brassicae* (Berk)Sacc. 7th International Rapeseed Congress. Poznan 11-14 May 1987 :1222-1227

BUSSO, C., ATTIA, T., ROBBELEN, G. 1987. Trigenomic combinations for the anlysis of meiotic control in the cultivated *Brassica* species. Genome 29 : 331-333

DELOURME, R., EBER, F., CHEVRE, A.M. 1989 Intergeneric hybridization of *Diplotaxis erucoides* with *Brassica napus*. I Cytogenetic analysis of F1 and BC1 progeny. Euphytica 41 : 123-128

EHLÆNFELDT, M.K., HELGESON, J.P. 1987 Fertility of somatic hybrids from protoplast fusions of *Solanum brevidens* and *S. tuberosum*. Theor. Appl. Genet. 73 :395-402

HEYN, F.W. 1977 Analysis of unreduced gametes in the *Brassiceae* by crosses between species and ploidy levels. Z. Planzenzüchtg. 78 : 13-30

KERLAN, M.C., CHEVRE, A.M., EBER, F., BOTTERMAN, J., DEGREEF, W. 1991 Risk assessment of gene transfer from transgenic rapeseed to wild species in optimal conditions. 8th International Rapeseed Congress Saskatoon

LANDGREN, M., GLIMELIUS, K. 1990 Analysis of chloroplast and mitochondrial segregation in three different combinations of somatic hybrids produced wihin *Brassiceae*. Theor. Appl. Genet. 80 : 776-784

MIZUSHIMA, U. 1980 Genome analysis in *Brassica* and allied genera. In : *Brassica* crops and wild allies. TSUNODA, S., HINATA, K., GOMEZ-CAMPO, C. (eds) Japan Scientific Societies Press, Tokyo. pp 89-106

MOHAPATRA, D., BAJAJ, Y.P. 1987 Interspecific hybridization in *Brassica juncea X Brassica hirta* using embryo rescue. Euphytica 36 : 321-326

PRIMARD C., VEDEL, F., MATHIEU, C., PELLETIER, G., CHEVRE, A.M. 1988 Interspecific somatic hybridization between *Brassica napus* and *Brassica hirta* (*Sinapis alba* L.) Theor.Appl. Genet. 75 : 546-552

QUAZI, M. H. 1988 Interspecific hybrids between *Brassica napus* L. and *Brassica oleracea* developed by embryo culture. Theor. Appl. Genet. 75 : 309-318

RENARD, M., DOSBA, F. 1980 Etude de l'haploïdie chez le colza. Ann. Amélior. Plantes 30 : 191-209

RIPLEY, VAN L., ARNISON, P.G. 1990 Hybridization of *Sinapis alba* L. and *Brassica napus* L. via embryo rescue. Plant breeding 104 : 26-33

SJODIN, C., GLIMELIUS, K. 1989 *Brassica naponigra*, a somatic hybrid resistant to *Phoma lingam*. Theor. Appl. Genet. 77: 651- 656