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Risk assessment of outcrossing of transgenic rapeseed to related species:
I. Interspecific hybrid production under optimal conditions with emphasis on pollination and fertilization

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Summary

The risk for a gene dispersal is reported for reciprocal crosses between a transgenic rapeseed variety resistant to the herbicide phosphinotricin and five related species. The first stages after pollination were cytologically observed and fertilized ovaries were established in *in vitro* culture for the production of interspecific hybrids. A similar classification was observed for the index of pollination compatibility and embryo yield. From the 243 embryos produced, 109 plantlets were obtained in a greenhouse. All the interspecific combinations tested were able to produce hybrid plants. A higher number of hybrids was obtained when rapeseed was used as the female parent. The hybrids had the expected triploid structure except for two amphidiploid, *B. napus* × *B. oleracea,* and one amphidiploid, *B. napus* × *S. arvensis,* plants with 56 chromosomes. The triploid hybrids were sterile or partially fertile but two of the amphidiploid plants, *B. napus* × *B. oleracea,* were fully fertile. The cytoplasm source did not seem to affect the fertility of the hybrids.

Introduction

Plant genetic engineering has provided new tools to the breeders for plant improvement (Goodman et al., 1987). For example, several genes have been cloned and transferred to plants in order to confer new resistances to herbicides, insects (Hilder et al., 1987), viruses (Powell et al., 1989) or to confer a male sterility (Mariani et al., 1990) or a change in seed quality (Guerche et al., 1990).

The first transgenic plants which might be proposed to commercialization, could be herbicide resistant ones. Several herbicide resistance genes have been introduced into plants. Their action is based either on the modification of the herbicide cellular target, on the detoxification of the herbicide or on the overproduction of the susceptible target protein (Comai & Stalker, 1986; De Block et al., 1987; Stalker et al., 1988; Schulz et al., 1990).

The production of genetically modified plants requires an assessment of gene dispersal risk (Hoffman, 1990). In fact, gene transfer by hybridization (horizontal transfer) could allow the introgression of alien genes into wild species. Rapeseed has been chosen as a model plant because of the presence of wild *Brassicaceae* species in the cultivated areas. Several studies from interspecific hybridization have revealed a relative genetic homology between rapeseed (*Brassica napus* L.) and different *Brassicaceae* (U, 1935; Mizushima, 1950; Harberd & McArthur, 1980; Prakash & Hinata, 1980).

In our study, transgenic rapeseed plants, resistant to the herbicide phosphinotricin, were crossed with five wild species. The transformation was per-

formed by Plant Genetic Systems (PGS, Belgium) (De Block et al., 1987). We will compare the results obtained by the pollination observations to the production of interspecific hybrids in optimal conditions.

Materials and methods

Plant material

A Canadian spring rapeseed variety, 'Westar', containing the bar gene conferring resistance to phosphinotricin (commercial name, Basta) was used. From progeny of two individual transformed plants, five and sixty plants were selected respectively and were crossed with the following three *Brassica* and two other *Brassicaceae* species:

- *B. oleracea* L. var. *acephala* (n = 9, CC), wild kale collected in Normandie and provided by G. Thomas (Le Rheu, INRA),
- *B. oleracea* L. var. *capitata* (n = 9, CC), a rapid cycling pure line provided by C. Doré (Versailles, INRA),
- *B. nigra* L. Koch (n = 8, BB), a German black mustard variety, 'Junius',
- *B. adpressa* L. (n = 7, AdAd),
- *Raphanus raphanistrum*L. (n = 9, RR),
- *Sinapis arvensis* L. (n = 9, SarSar).

The three last species were locally collected. They are common weeds occurring in rapeseed fields. Ten plants of *B. oleracea* var. *acephala* and around 30 plants of the other species were used for the interspecific crosses.

The plants were grown in a greenhouse under standard conditions. Reciprocal crosses and selfing of the different species were performed.

Methods

Observation of pollen germination and of pollen tube growth

For each cross, five flowers were pollinated. Pollen germination observations in the pistils were performed 24 and 48 hours after pollination according to the method of Martin (1959) with some modifications. The pistils were fixed in acetic acid: alcohol (3:1) for one hour, rinsed in water, dipped into a 6 N sodium hydroxide solution overnight for softening. After rinsing in water, and staining in 0.1 M K_3PO_4 and 0.1% anilin blue (w/v), they were observed for fluorescence microscopy at 350–400 nm UV light.

Twenty-four hours and forty-eight hours after pollinations, the germination percentage (G) was assessed for the 5 pistils. We considered that a pollen grain had germinated when the length of its tube was at least twice as long as its diameter.

To compare the different crosses, an index of pollination compatibility (I) estimating the growth of the pollen tubes into the pistil was established. The index proposed by Matsuzawa (1983) was modified and expressed by the x, y, z parameters. For an optimal estimation, the coefficients were defined by giving higher weights to the parameters involved in the final steps of pollination.

$$I = x + 2y + 3z$$

The x parameter was defined by the ratio number of germinated pollen grains/total number of pollen grains present on the stigma surface. A score of 0, 1, 2 or 3 was assigned to ratio values of 0, [0, 0.5], [0.5, 0.7] and [0.7, 1] respectively. The y parameter was defined by the number of pollen tubes present in the inferior part of the pistil. The scores were established according to the number of pollen tubes penetrating into the ovules. Scores 0, 1 and 2 corresponded to 0, 1 to 5 and more than 6 pollen tubes, respectively. The z parameter was defined by the number of pollen tubes penetrating into ovules. The same scores used for the y parameter was assigned. The index was calculated on the pistil showing the maximum value for the z parameter at 24 h and 48 h after pollination. We thus attempted to have the best estimation for fecundation probability.

Ovary culture

Before anthesis, buds were emasculated and polli-

nated. Four to six days after pollination, ovaries were excised and cultured *in vitro* on E12 medium according to the technique of Delourme et al. (1989). The cultures were incubated in a growth chamber at 22–23° C day/15° C night with a 16 h photoperiod. When seedlings emerged from the ovaries, they were placed at 4° C for 10 days and then transferred to Murashige & Skoog (1962) medium, containing 20 g/l of sucrose. The plantlets were transferred to pots at the 4 to 6 leaf stage, and then grown in the greenhouse. Simultaneously, in an attempt to produce seeds, pollinations of around 200 flowers were performed for each cross.

Chromosome counts

These were performed in root-tip dividing cells or in pollen mother cells using the technique described by Jahier et al. (1989).

Pollen fertility

The pollen fertility was determined by the percentage of pollen grains stained by aceto-carmine. Around 500 pollen grains were observed per plant.

Results

Pollen grain germination and pollen tube growth

For self pollination control, on the 5 pistils observed, numerous pollen grains adhered to *B. napus* (Fig. 1), *B. oleracea* and *B. nigra* papillae, whereas few self-pollen grains bound to *R. raphanistrum* and *S. arvensis* papillae (Table 1). Pollen germination was observed for all the species except for *S. arvensis*. Approximately 24% of germinated pollen penetrated into the *B. napus* ovary after 24 hours of selfing (Fig. 2). Some of the tubes had, at this time, penetrated into the ovules (porogamy) (Fig. 3). For *B. oleracea* var. *capitata* self-pollinations, a single pollen tube had penetrated the style 24 hours after pollination, whereas around 20 (more than 10% of the germinated pollen grains) tubes were observed into the ovary 48 hours after pollination. For *B. nigra* and *R. raphanistrum* selfing, a lower pollen germination rate was observed at 24 hours than at 48 hours. Therefore the comparison of germination percentages and of compatibility indexes, by the Chi square test ($P \leq 0.05$), was performed at 48 hours. Four and five classes were established for each criterion (Table 1).

Each of the interspecific crosses showed allopollen adhering to the stigma surface of the species used as female parent. No difference for pollen germination at 24 and 48 hours was observed, except for *B. napus* × *B. nigra*. The germination percentage in the latter cross increased from 7.3% at 24 hours to 19.0% at 48 hours after pollination. Therefore, only germination values at 48 hours were considered (Table 2).

When rapeseed was used as female, pollen germination percentages of related species were similar for *B. oleracea, R. raphanistrum,* whereas the germination percentages for *B. nigra* and *S. arvensis* pollen grains were lower. Approximately 51% of *B. oleracea* germinated pollen were observed in the rapeseed pistil. In spite of large numbers of *B. nigra* pollen tubes germinating on the rapeseed stigma, many of them failed to penetrate. Only a few pollen tubes could be followed in the ovary and into the ovules. Neither *R. raphanistrum* nor *S. arvensis* pollen tubes penetrated into *B. napus* styles. The germinating tubes were malformed and accumulated callose at the tips.

When rapeseed was used as male, the germination percentage of rapeseed pollen grains on *B. oleracea* stigma (75.5%) was significantly higher than from the percentages observed on *B. nigra* papillae (43.5%). However, the germination percentage for rapeseed pollen grains on *R. raphanistrum* and *S. arvensis* stigma (Fig. 4) were significantly lower, 11.8% and 10.8% respectively (Table 2). *B. napus* pollen tubes were observed 48 hours after pollination in *B. oleracea* ovary but without porogamy. In spite of the strong adherence of rapeseed pollen grains on the *B. nigra* stigmata, few pollen tubes grew into the pistil. The rapeseed pollen tubes failed to penetrate *R. raphanistrum* and *S. arvensis* stigma.

On the basis of pollen grain germination and of compatibility index, the classes defined for the interspecific crosses are reported in Table 2.

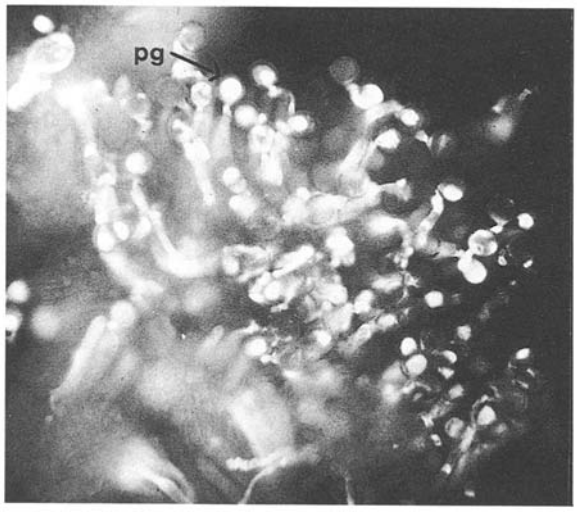

Fig. 1. Self pollen germination on *B. napus* papillae.
– pg: Germinated pollen grain.

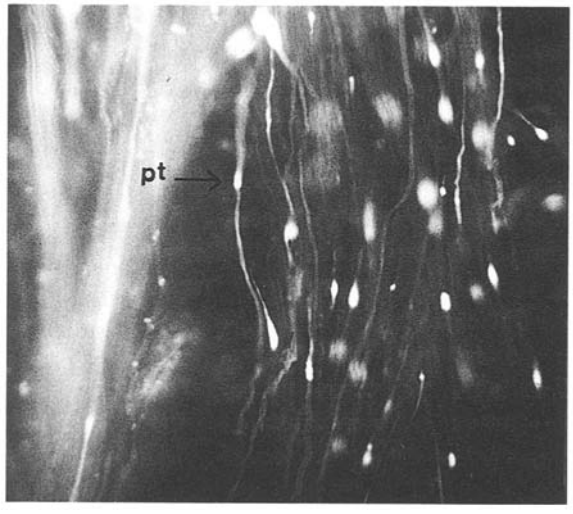

Fig. 2. B. napus pollen tube growth in *B. napus* pistil.
– pt: pollen tubes with callose plugs.

Embryo and hybrid production

From 243 embryos, 109 plants were produced by interspecific crosses after fertilized ovary culture (Table 3).

By using *B. napus* as female, the crosses with *B. oleracea* var. *acephala* gave the best results: 21.5 embryos per 100 fertilized ovaries. This value dropped significantly when this species was pollinated with *B. adpressa* and *B. oleracea* var. *capitata* (14.5% and 13.2%, respectively), and by pollinations with *B. nigra, S. arvensis* and *R. raphanistrum* (4.9%, 3.7% and 1.4%, respectively).

The classification of the related species based on the number of embryos obtained was different from the classification based on the number of hybrid plants (Table 3). Only two groups could be distinghuished: the first including only *B. oleracea* var. *acephala* (13.7 plants per 100 fertilized ovaries) and the second including all the other species with percentages of plantlets obtained ranging from 0.8% to 5.7%.

When using *B. napus* as male, the species used as female could be separated in two groups based on embryo yields: the first one including *R. raphanistrum* and *B. adpressa* with embryo yields ranging from 3.2% to 4.3%, the second one including *B. oleracea* var. *acephala, B. oleracea* var. *capitata, B. nigra* and *S. arvensis* with embryo yield ranging from 0.0% to 1.8%. No embryo was produced from *S. arvensis* × *B. napus* crosses. None of the four embryos from *B. nigra* × *B. napus* crosses grew

Table 1. Estimation of pollen germination and index of compatibility 48 hours after self pollination

	Total number of adhered PG	Germinated PG	Percentage of germination	Index of pollination compatibility (I)
B. napus	537	420	78.2 (a)[1]	12 (a)
B. oleracea var. *capitata*	250	194	77.6 (a)	10 (b)
B. nigra	134	20	14.9 (c)	6 (c)
S. arvensis	21	0	0.0 (d)	0 (d)
R. raphanistrum	58	18	31.0 (b)	1 (d)

[1] Each letter defines a class. Values with the same letters were not significantly different (Chi square test, $P \leq 0.05$)
PG = Pollen Grains

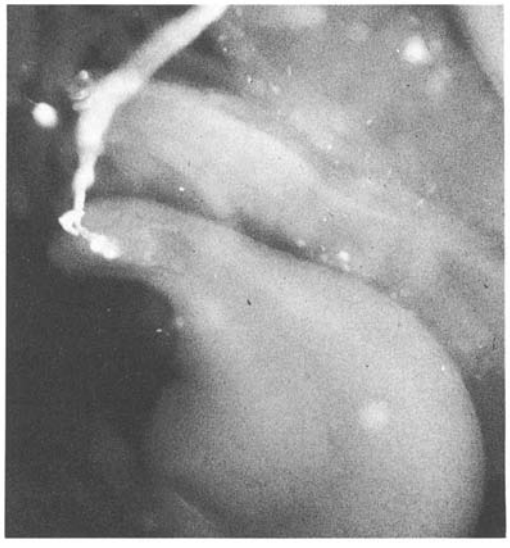

Fig. 3. B. napus pollen tube penetrating into a *B. napus* ovule.

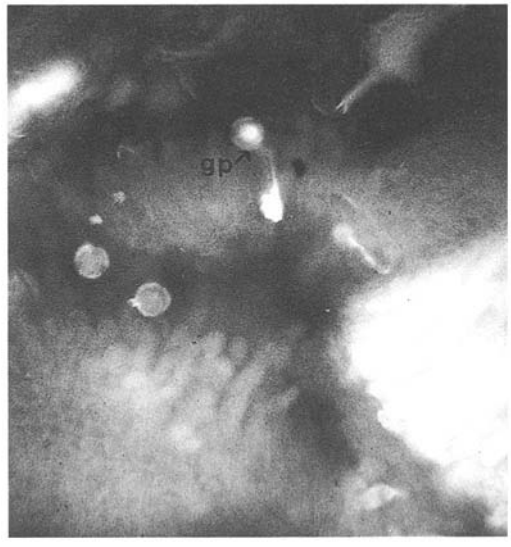

Fig. 4. B. napus pollen germination on *S. arvensis* papillae. – gp: Germinated pollen grain.

into plants. No difference was revealed between the interspecific hybrid plant yields.

Interspecic crosses did not produce any viable seed. Therefore, hybrids can only be obtained performing embryo rescue.

Cytogenetic studies

Chromosome counts in root meristem cells or pollen mother cells demonstrated that most of the hybrids had the expected triploid structure (ACX): one genome of rapeseed (AC) and one genome of the species used as the other parent (X) (Table 4).

However, when using rapeseed as the female parent, three amphidiploid plants with 56 chromosomes (AACCXX) were obtained: two by hybridization with the two *B. oleracea* accessions and one by hybridization with *S. arvensis* (Table 4).

Table 2. Estimation of pollen germination and index of compatibility 48 hours after cross pollination

Brassica napus	Related species	Total number of adhered PG	Germinated PG	Percentage of germination	Index of pollination compatibility (I)
F	*B. oleracea* var. *capitata*	121	39	32.2 (a)[1]	8 (a)
	B. nigra	358	68	19.0 (b)	6 (b)
	S. arvensis	91	18	19.8 (b)	1 (d)
	R. raphanistrum	193	66	34.2 (a)	5 (c)
M	*B. oleracea* var. *capitata*	180	136	75.5 (a)	7 (a)
	B. nigra	506	220	43.5 (b)	3 (b)
	S. arvensis	37	4	10.8 (c)	1 (c)
	R. raphanistrum	34	4	11.8 (c)	3 (b)

[1] Each letter defines a class. Values with the same letters were not significantly different (Chi square test, $P \leq 0.05$)
F = Female, M = Male, PG = Pollen Grains

Table 3. Interspecific hybrid production between transgenic rapeseed and related species

B. napus	Related species	Number of ovaries in culture O	Number of embryos obtained E	Number of plantlets obtained P	Percentage of embryos obtained E/O × 100	Percentage of plantlets obtained P/O × 100
F	*B. oleracea* var. *acephala*	205	44	28	21.5 (a)[1]	13.7 (a)[1]
	B. oleracea var. *capitata*	228	30	13	13.2 (b)	5.7 (b)
	B. adpressa	262	38	8	14.5 (b)	3.1 (b)
	B. nigra	325	16	11	4.9 (c)	3.4 (b)
	S. arvensis	808	30	18	3.7 (c)	2.2 (b)
	R. raphanistrum	368	5	3	1.4 (d)	0.8 (b)
M	*B. oleracea* var. *acephala*	445	8	3	1.8 (b)	0.7 (a)
	B. oleracea var. *capitata*	585	7	1	1.2 (b)	0.2 (a)
	B. adpressa	1117	36	15	3.2 (a)	1.3 (a)
	B. nigra	916	4	0	0.4 (b)	0.0 (a)
	S. arvensis	732	0	0	0.0 (b)	0.0 (a)
	R. raphanistrum	583	25	9	4.3 (a)	1.5 (a)

[1] Each letter defines a class. Values with the same letter were not significantly different (Chi square test, $P \geq 0.05$)
F = Female, M = Male

Pollen fertility

When using rapeseed as the female parent, the fertility of the triploid hybrids obtained with *B. oleracea* ranged from 4.5% to 59.2%. The fertility varied on the same plant: sterile and fertile florets were observed. On the other hand, the amphidiploid plants *B. napus* × *B. oleracea* had the same high fertility as the rapeseed parent (94%). The *B. napus* × *S. arvensis* triploid plants were either ster-

Table 4. Cytogenetic characterization of the interspecific crosses between transgenic *B. napus* and the related species

B. napus	Related species	Number of plantlets studied	Number of chromosomes	Genomic structure	Fertility percentage range
F	*B. oleracea*	27	28	ACC	7.6–59.2 (a)[1]
	var. *acephala*	1	56	AACCCC	94.1
	B. oleracea	9	28	ACC	4.5–40.0 (a)
	var. *capitata*	1	56	AACCCC	94.0
	B. adpressa	6	26	ACAd	0.0 (c)
	B. nigra	8	27	ACB	0.0–1.9 (c)
	S. arvensis	14	28	ACSar	0.0–39.8 (b)
		1	56	AACCSarSar	38.4
	R. raphanistrum	3	28	ACR	0.0 (c)
M	*B. oleracea* var. *acephala*	3	28	CAC	1.2–18.8 (a)
	B. oleracea var. *capitata*	1	28	CAC	16.6 (a)
	B. adpressa	11	26	AdAC	0.0 (c)
	B. nigra	0	–	–	–
	S. arvensis	0	–	–	–
	R. raphanistrum	8	28	RAC	0.0–7.1 (b)

[1] Each letter defines a class. Values with the same letter were not significantly different (Chi square test $P \leq 0.05$)
F = Female, M = Male

ile or partially fertile (never higher than 39.8%). The *B. napus* × *S. arvensis* amphidiploid plant had the same fertility as one of the triploid hybrids (34.8%). The hybrids *B. napus* × *B. nigra* were poorly fertile (from 0.0 to 1.9% of fertility). The hybrids obtained by crossing rapeseed with *B. adpressa, R. raphanistrum* were sterile.

For the reciprocal crosses, the fertility of *B. oleracea* × *B. napus* hybrids never exceeded 18.8%. The hybrids obtained with *B. adpressa* and *R. raphanistrum* were sterile except a single hybrid *R. raphanistrum* × *B. napus* (7.1%).

The classification of triploid hybrids, based on the percentage of pollen fertility, is given in Table 4.

Discussion

During a normal pollination process, the pollen grains adhere to the stigma papillae. After hydration, metabolic activity starts in the pollen grains, allowing germination if conditions are fulfilled. In the case of an incompatibility, the callose reactions in stigma surface and the formation of distorted pollen tubes in the style reveal that prezygotic barriers occur before fecundation (Dumas & Knox, 1983). From our results, the lack of pollen germination in the case of *S. arvensis* self pollination could be explained by an early autoincompatibility mechanism. Self pollination of *B. nigra, R. raphanistrum* and *S. arvensis* revealed partial self incompatibility. In interspecific crosses, the allo-pollen germinated, in all instances, on the stigma papillae of the pollinated species. However, very few pollen tubes grew into the style. The mechanisms of no-recognition were similar to a sporophytic auto-incompatible reaction. The hypothesis of an analogous S gene controlling sporophytic incompatibility has been proposed by Dumas & Knox (1983), Jingling & Houli (1987) and Elleman et al. (1989).

For the risk assessment of interspecific hybrid production, a similar classification was observed for compatibility index and embryo percentages when using rapeseed as female parent excepted for *S. arvensis*. In the reciprocal crosses, by studying the first stages of pollination, we were able to predict the low yield of hybrid plants. The compatible index seems to be a reliable measure to assess interspecific hybrid production. These results are in agreement with the work of several authors on other species (De Nettancourt et al. 1974; Gradziel & Robinson, 1991).

In fact, for the interspecific crosses, it seems that we successfully broke postzygotic barriers by *in vitro* embryo rescue, except for *S. arvensis* × *B. napus* crosses. Better results were obtained when rapeseed was used as female parent. Several studies have shown that the yield of hybrid embryos is always higher when the parent with the higher ploidy level is used as female (Mohapatra & Bajaj, 1987; Quazi, 1988). Nishiyama & Inomata (1966) and Johnston et al. (1980) proposed that the Endosperm Balance Number, determining the effective ploidy in the endosperm of each species, must be in a 2 maternal: 1 paternal ratio to get successfull hybridizations. The modifications of this ratio might explain the differences observed for the reciprocal crosses.

The efficiency of the interspecific hybridization was also reflected by the ratio of number of plants obtained / number of ovaries established in *in vitro* culture. The death of young embryos occur in all the crosses. Previously, *B. napus* × *B. adpressa* hybrids were reported (Harberd & McArthur, 1980). In the present study, we were able to generate these hybrids both ways. Reciprocal hybrids were also obtained for *B. napus* and *R. raphanistrum*. Until now, interspecific hybrids were reported for *B. napus* – *R. sativus* crosses by cytoplast-protoplast fusion (Sakai & Imamura, 1990) or by fertilized ovary culture (Eber, pers. com.). Varietal differences in hybrid yield were revealed in *B. oleracea*. More hybrids were produced by using var. *acephala* than var. *capitata* on *B. napus*. This might explain the lack of success for the *B. nigra* × *B. napus* and *S. arvensis* × *B. napus* crosses. However, these hybrids were obtained by Busso et al. (1987) and Mathias (1991). In agreement with our observations, Ripley & Arnison (1990) reported that production of *S. alba* × *B. napus* hybrids depends on the *B. napus* cultivar used.

Most of the hybrids had the expected triploid structure (ACX). The study of the meiotic beha-

viour of the hybrids is in progress: the higher the percentage of chromosome pairing will be, the higher the risk of gene insertion into the genome of the wild species could be. However, these hybrids were male sterile or poorly fertile representing low risk. The cytoplasm source did not seem to increase fertility which was similar whatever the origin of the mother-plant. However, three amphidiploid plants with 56 chromosomes were produced. Most likely, these plants arose from non reduced gametes in the parental plants. From several crosses, the same results were reported by Mizushima (1950), Heyn (1977) and Mathias (1991). Alternatively, chromosome doubling could be explained by endomitosis during the embryo culture. The probability of this event is very low, since embryos were well formed before subculture and it seems difficult that somaclonal variation occurred. Mizushima (1950) reported a *B. napus* × *S. arvensis* amphidiploid plant with 80% fertility. The amphidiploid we produced was as fertile as triploid hybrids. However, the two amphidiploid plants *B. napus* × *B. oleracea* had a fertility as good as rapeseed. The probability of getting progeny from these plants is high, thus the risk of gene dispersal is increased.

An important aspect to consider on gene dispersal is the assessment of the viability of interspecific hybrids. The female fertility will be assessed by the ability of the hybrids to produce a progeny after selfing or backcrossing with the wild species.

The study of the presence and the expression of the bar gene is in progress.

The work reported in this paper assesses the risk of interspecific hybridization with transgenic rapeseed maximizing conditions for hybrid embryo development. The next step of this study will be the research of correlations between the results obtained from *in vitro* culture and under natural conditions, by conducting field experiments. From preliminary data, less than 0.15 viable seeds per 100 flowers, between male sterile rapeseed and *B. adpressa,* were produced under field conditions (Renard, pers. com.). For the same combination, 3.1 plants per 100 ovaries were obtained by *in vitro* culture. Since more hybrids were produced when rapeseed was used as the female parent, the risk for gene dispersal is limited.

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