

# Interspecific hybridization between *Brassica napus* and *Brassica rapa* ssp. *chinensis* genotypes through embryo rescue and their evaluation for crossability

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## Abstract

The genus *Brassica* contains several important crop species that are used for a variety of purposes. *Brassica napus* and *Brassica rapa* are the most important ones for their use as oilseed crops. In *Brassica*, interspecific hybridization is a potential and useful method for transferring valuable traits between species of commercial interest. For breeding Brassicas resistant to some diseases, i.e. clubroot, attempts of transferring resistant genes (CR) through interspecific hybridization have also been reported. The main step in the introgression process is the production of interspecific hybrids between the two species. However, the cross-incompatibility occurring in wide hybridization might hamper the possibility of obtaining hybrid progenies. For that reason, the crossability study is essential and may give an insight into the cross-compatibility relationship among the species, the direction of success of crossing, and the crossability barriers of some combinations, if any. In the present study, interspecific reciprocal crosses between *B. napus* and *B. rapa* ssp. *chinensis* genotypes were carried out in order to determine their crossability and to produce their F1 hybrids. Crossability was analyzed based on the pollen germination index (PGI) and the development of hybrid embryos in *in vitro* cultures. It was observed that the unilateral interspecific incompatibility occurred in crosses between *B. napus* × *B. rapa* ssp. *chinensis*. When *B. napus* cultivars were used as a maternal parent, pollen grains of *B. rapa* germinated well on the stigmas, while only a small number of pollen tubes could elongate near the ovules, resulting in a low development of hybrid embryos. Using the *B. rapa* ssp. *chinensis* as the pistillate parent in the crosses, it was found that the crossability was relatively higher than that in the case of the reciprocal cross. This resulted in a greater number of embryos obtained. Generally, it was concluded that the selection of parental components for hybridization is an important step for obtaining breeding success.

**Key words:** interspecific hybridization, crossability, embryo rescue, Brassicas, clubroot resistance

## Introduction

Many *Brassica* species and allies are wild and weedy with useful genes which could be incorporated into breeding programs, including research into the cytoplasmic and nuclear male sterility; the resistance to diseases; insect or nematode pests; and the tolerance of cold, salt, and drought conditions (Chen et al., 2011). Thus, interspecific hybridization is still a useful approach for the introgression of these favorable agronomic traits from wild species into cultivated crops (Mei et al., 2010; Niemann et al., 2012, 2014). It is interesting, mainly because of the growing yield losses in oilseed rape caused by various pathogens among which one of the most dangerous is *Plasmodiophora brassicae* (Pageau et al., 2006). For breeding Brassicas resistant to clubroot, attempts of

transferring CR through interspecific hybridization have been reported (Gowers, 1982; Diederichsen et al., 2009; Niemann et al., 2015). Among the two progenitor species of *Brassica napus*, clubroot resistance is more frequently found in turnips (*Brassica rapa*; A genome;  $n = 10$ ) (Hirai, 2006). This is why the crosses between *B. napus* and the three genotypes of *B. rapa* ssp. *chinensis* with potentially increased resistance to clubroot were made. However, interspecific hybridization between allotetraploid and diploid *Brassica* species is difficult; as a result, cross-incompatibility often hampers the production of those hybrid progenies. While most attempts on interspecific hybridization were based on conventional techniques (hand pollination), the frequency of hybrids was low. In addition, hybrids were obtained at relatively higher

frequency when *B. rapa* was used as a female parent, indicating a strong incompatibility in the reverse cross (Ammitzboll et al., 2005). Therefore, the main goal of our study was to evaluate the crossability between *B. napus* and *B. rapa* ssp. *chinensis* genotypes and to obtain F1 hybrids between those genotypes using an embryo rescue technique.

## Materials and methods

### Plant materials and crosses

Five *B. napus* seed cultivars, i.e. Jet Neuf, Lisek, Skrzyszowicki, Californium, and Zhongshuang 9, were selected from the resources of the Genetics and Plant Breeding Department, Poznań University of Life Sciences, while seeds of *B. rapa* accessions (A: Chinese Cabbage PI430485 98CI; B: Pak Choi 08 007569; C: Chinese Cabbage 08 006169) came from the Warwick HRI Genetic Resources Unit. The reciprocal crosses were carried out in the glasshouse of Genetics and Plant Breeding Department during spring 2013. Each genotype was represented by four to six plants. The pollen of pollinators was placed on stigmas immediately after emasculation, which was done at the closed bud stage. In each cross-combination, around 11 to 90 flower buds were pollinated.

Pollinated pistils were divided into two parts. Half of them were allocated for the microscopic examinations. The rest of pistils were left on the plants to silique formation.

### The evaluation of crossability based on the PGI

Observations of pollen grain germination and pollen tube (PT) growth were conducted after cross-pollination (CP) of the chosen *Brassica* genotypes. For this purpose, pistils of three *B. napus* cultivars i.e. Jet Neuf, Californium, Skrzyszowicki, and one *B. rapa* ssp. *chinensis* genotype (08 006169) were collected 48 h after pollination. They were next fixed and stained with aniline blue (Antkowiak and Wojciechowski, 2006). By applying a fluorescent microscopy technique, the assessment of pollen tube growth was made. Six pistils were analyzed in each combination. The intensity of pollen grain germination and pollen tube penetration was expressed by the six degree scale (Niemann et al., 2014), where 0 was the absence of PT, 1-4 was the intermediate number of PT, and 5 was the largest number of PT. The crossability

(CC: cross-compatibility; CI: cross-incompatibility) was evaluated on the basis of the PGI according to Kaneko et al. (2009); and also, pistils were classified into five specimens: 1) the number of pistils with pollen grains, 2) the number of pistils in which pollen grains do not germinate, 3) the number of pistils in which pollen grains germinate on the stigmas, 4) the number of pistils in which pollen tubes enter the style tissue, and 5) the number of pistils in which pollen tubes penetrate close to or enter the ovules.

The value of PGI was obtained from the formula of  $PGI = (1b+2c+3d+4e)/(a+b+c+d+e)$ .

In the case of PGI equal or higher than 2, it was concluded that there existed compatibility.

### The effectiveness of crosses

All hybridizations were performed with the application of an *in vitro* culture of isolated embryos according to the method described by Wojciechowski (1998).

The immature embryos were isolated from young siliques at different developmental stages, i.e. heart, early torpedo, torpedo, late torpedo, or nearly matured embryos, 14-19 days after pollination. For the embryo culture, basal White (W, 1963), Murashige and Skoog (MS, 1962), Murashige and Skoog modified by Keller and Armstrong (MS<sub>k</sub>, 1977), and Nitsh and Nitsh (H<sub>3</sub>, 1969) media were applied (Table 1).

The effectiveness of an interspecific hybridization of *B. napus* with chosen *B. rapa* genotypes was expressed in three different ways: by the number of embryos obtained to the mean number of well-developed ovules, by the fertility (silique/pollinated flowers (%)) – Figure 1), and by the number of plants regenerated in the soil.

## Results

### Evaluation of crossability based on the PGI

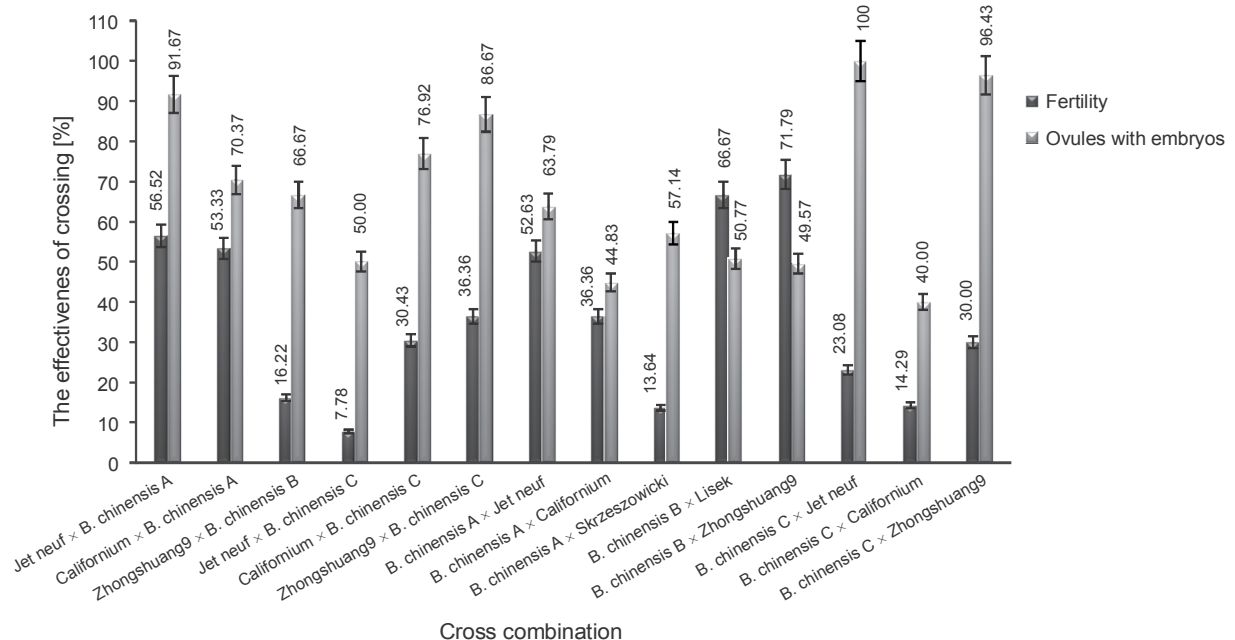
Generally, a different intensity of pollen tube growth and the differences at PGI value were observed in most cross-combinations, depending on which form was used as a maternal parent. It was observed that after cross-pollination of *B. napus* cultivars with *B. rapa* ssp. *chinensis* pollen grains, the intensity of pollen tube growth was low, and the pollen tubes were observed mainly in the style or in the ovary. Occasionally, a low number of ovules were penetrated by pollen tubes (PGI value less than 2) – Figure 2E. The crossing between three *B. na-*

**Table 1.** Pattern of *Brassica* embryos cultures in *in vitro* conditions

No.	Media	Embryo/plant developmental stage	<i>In vitro</i> culture duration	Conditions of <i>in vitro</i> cultures
1.	White	H*, ET	7 days	Growth room**
2.	Murashige & Skoog	T, LT, NM	3-4 weeks	
3.	Murashige & Skoog in Keller modification	explants developing callus and first leaves	5-11 weeks	
4.	Nitsch & Nitsch	seedlings	2 weeks	
5.	Soil	well rooted seedlings	7 days until matured plants	greenhouse

\* H – heart, ET – early torpedo stage, T – torpedo stage, LT – late torpedo, NM – nearly matured embryo,

\*\* conditions: temperature 26°C; photoperiod: 16 h light, 8 h dark



**Fig. 1.** Effectiveness of reciprocal crosses between *B. napus* cultivars with *B. rapa* ssp. *chinensis* genotypes expressed by the fertility and ovules with embryos (%). Error bars indicate within subject standard error

*napus* cultivars and the tested genotype of *B. rapa* ssp. *chinensis* showed that these two species do not suit each other concerning their compatibility, and in these cases, there are some prezygotic incompatibility barriers. Quite a different situation was observed in crosses in which *B. rapa* ssp. *chinensis* plants were used as a maternal and *B. napus* cultivars as a pollen donor. In those cases, the intensity of pollen tube growth was higher, and PGI ranged from 2.11 (*B. rapa* ssp. *chinensis* × *B. napus* cv. Californium) to 2.69 (*B. rapa* ssp. *chinensis* × *B. napus* cv. Jet Neuf) with a mean value of 2.32 (Table 2, Fig. 2A-D).

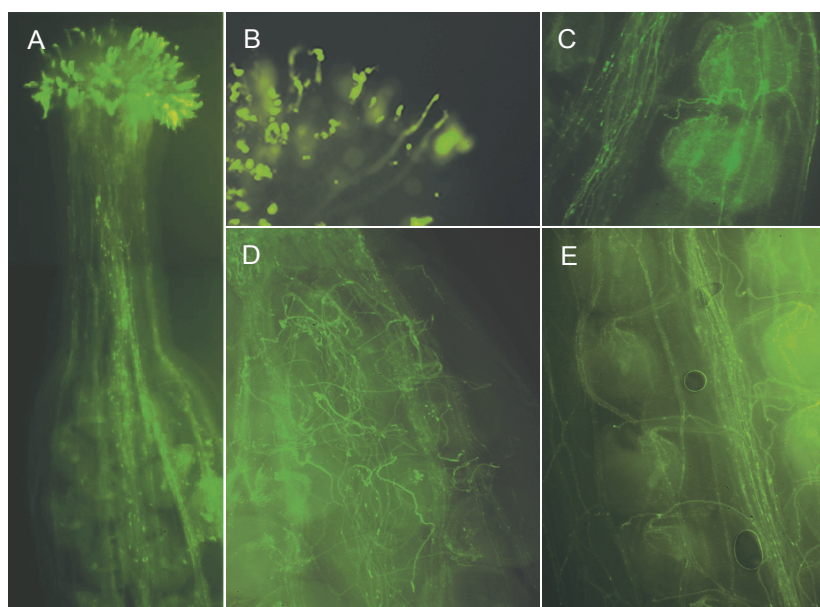
#### The effectiveness of crosses

The highest fertility was observed in crosses between *B. rapa* ssp. *chinensis* B and *B. napus* cv. Lisek and Zhongshuang 9. It was 66.67% and 71.79%, respectively (Fig. 1). Whereas in one of the reverse crossing, i.e. *B. napus* cv. Lisek × *B. rapa* ssp. *chinensis* B, there were no siliques obtained at all. Similarly, no siliques were received in *B. napus* cv. Lisek × *B. rapa* ssp. *chinensis* C and *B. napus* cv. Skrzyszowicki × *B. rapa* ssp. *chinensis* A. Moreover, in all cross-combinations, quite a high percentage of ovules with embryos were obtained

**Table 2.** Pollen germination index (PGI) in reciprocal crosses of chosen *Brassica* genotypes

Combination of pollination		PGI 48 h after pollination
<i>B. napus</i> cv. Jet Neuf	<i>B. rapa</i> ssp. <i>chinensis</i> C	1.94
<i>B. napus</i> cv. Californium		1.81
<i>B. napus</i> cv. Skrzyszowicki		1.88
Mean		1.88 ± 0.06*
<i>B. rapa</i> ssp. <i>chinensis</i> C	<i>B. napus</i> cv. Jet Neuf	2.69
	<i>B. napus</i> cv. Californium	2.11
	<i>B. napus</i> cv. Skrzyszowicki	2.17
Mean		2.32 ± 0.32

\* Mean ± SD



**Fig. 2.** Pollen germination and pollen tube elongation in pistils of analyzed *Brassica* genotypes: A) The pistil of *B. rapa* ssp. *chinensis* A after pollination with *B. napus* cv. Californium; B) Pollen grains germinating on the stigma, *B. napus* cv. Zhongshuang 9 × *B. rapa* ssp. *chinensis* A; C-D) pollen tubes in the ovary, *B. rapa* ssp. *chinensis* A × *B. napus* Jet Neuf; E) Pollen tube penetrating the ovule, *B. napus* cv. Lisek × *B. rapa* ssp. *chinensis* A

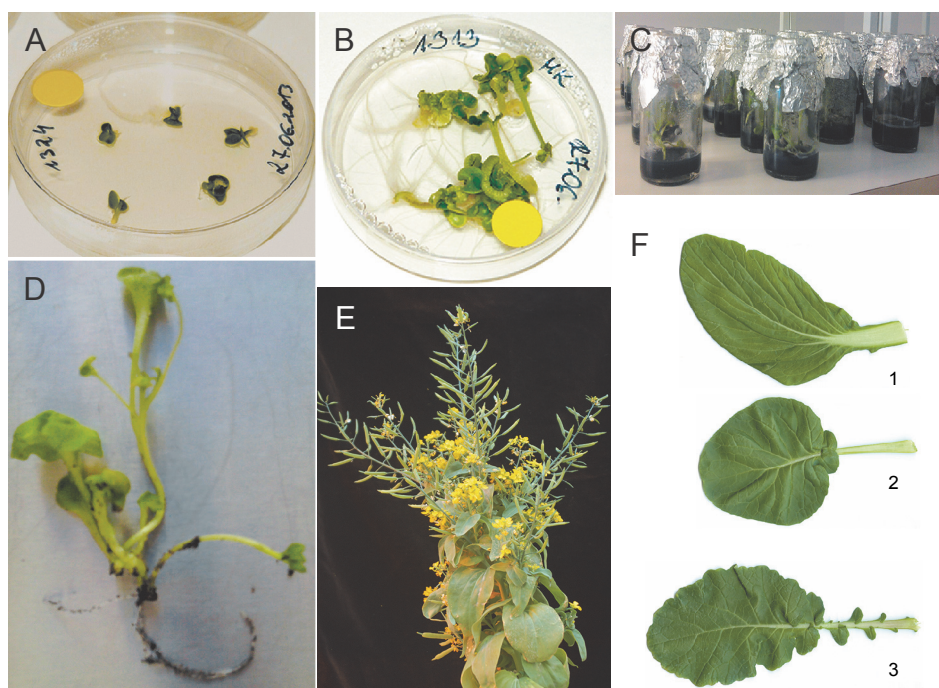
ranging from 40% in *B. rapa* ssp. *chinensis* C × *B. napus* cv. Californium to 96.43% in *B. rapa* ssp. *chinensis* C × *B. napus* cv. Zhongshuang 9. In the case of the last-mentioned cross-combination, plants were obtained from 51.85% of the isolated embryos.

In this experiment, from 236 pollinated flowers, up to 452 embryos (Fig. 3A) were received after crosses between all *B. rapa* ssp. *chinensis* × *B. napus* genotypes. In the case of reverse hybridization, a lower number of embryos were formed out only 80, despite the pollination of a greater number of flowers (273). However, the

efficiency of embryo rescue presented as the ratio of the regenerated plants to the isolated embryos was higher in these variants of crosses, and it was 73.7% (*B. napus* × *B. rapa* ssp. *chinensis*), while only 35% of plants were regenerated from the *B. rapa* ssp. *chinensis* × *B. napus* crosses. Generally, in all tested cross-combinations, 509 flowers were pollinated and 220 plants were regenerated through embryo cultures, wherein most plants were obtained from the crosses between *B. rapa* ssp. *chinensis* × *B. napus* genotypes – 161 (Fig. 3B-F, Table 3). In those cross-combinations, the highest number of em-

**Table 3.** Effectiveness of interspecific crosses between *B. napus* cultivars and *B. rapa* ssp. *chinensis* genotypes expressed by the number of regenerated plants in the soil

Cross-combination		No. of pollinated flowers	No. of siliques	No. of isolated embryos			No. of plant rooted in H <sub>3</sub>	No. of plant in soil		
♀	♂			Total	In different developmental stages					
					H	ET			LT	
<i>B. napus</i>	Jet Neuf	<i>B. rapa</i> ssp. <i>chinensis</i> A	23	13	11	0	0	11	7	7
	Californium		15	8	19	0	8	11	12	12
	Skrzeszowicki		13	0	0	0	0	0	0	0
	Lisek	<i>B. rapa</i> ssp. <i>chinensis</i> B	11	0	0	0	0	0	0	0
	Zhongshuang9		37	6	2	0	0	2	0	0
	Jet Neuf	<i>B. rapa</i> ssp. <i>chinensis</i> C	90	7	2	0	0	2	2	2
	Lisek		28	0	0	0	0	0	0	0
	Californium		23	7	20	0	7	13	19	18
	Zhongshuang9		33	12	26	0	18	8	26	20
Total		273	53	80	0	33	47	66	59	
<i>B. rapa</i> ssp. <i>chinensis</i> A	<i>B. napus</i>	Jet Neuf	19	10	74	24	50	0	22	22
		Californium	22	8	39	20	19	0	10	8
		Skrzeszowicki	66	9	68	12	50	6	60	57
Lisek		15	10	66	29	35	2	0	0	
Zhongshuang9		39	28	173	64	80	29	61	59	
Jet Neuf		13	3	1	0	1	0	1	1	
Californium		42	6	4	1	3	0	0	0	
Zhongshuang9		20	6	27	0	0	27	14	14	
Total		236	80	452	150	238	64	168	161	
General		509	133	532	150	271	111	234	220	



**Fig. 3.** Regeneration of plants in *in vitro* embryo cultures after crossing *B. rapa* ssp. *chinensis* A × *B. napus* cv. Jet Neuf: A) Fully matured embryos on MS medium; B) Regenerated seedlings on MS<sub>k</sub> medium; C) Regenerated plant on rooting H<sub>3</sub> medium; D) Regenerated plant just before being transferred to soil; E) Regenerated plant in the soil; F) Comparative leaf morphology of F1 hybrid plants: 1 – *B. rapa*, 2 – F1 hybrids, 3 – *B. napus*

bryos was isolated at the heart and early torpedo stages, while after *B. napus* × *B. rapa* crosses, no embryos have been obtained at the heart developmental stage.

### Discussion

To date, successful attempts of hybridization between *Brassica rapa* and *Brassica napus* have been published in numerous scientific papers (Fitz et al., 2007; Choudhary et al., 2012). Crossability between *B. napus* and *B. rapa* has also been examined, specifically in terms of the genes introgression from *B. napus* to *B. rapa*, which is mainly considered a weed in America and Europe (Tsuda et al., 2014). However, to the best of our knowledge, this is one of the first studies focusing directly on the potential of hybrid production and crossability between diploid *B. rapa* ssp. *chinensis* genotypes and allotetraploid *B. napus* in terms of the portability of the resistance genes. It is known that in *B. rapa*, a high level of genetic diversity was observed, and moreover, species of *B. rapa* vary in their level of cross-compatibility (Olsson, 1960).

Thus, it is difficult to compare our results with those presented in literature, particularly because *B. rapa* is

a species consisting of various widely cultivated subspecies including *pekinensis*, *chinensis*, the turnip and the turnip rape (*B. rapa* subsp. *oleifera*). Within the species, different genotypes may differ in their propensity to cross with other species depending on their crossability or self-incompatibility. For this reason, experimental hybridization studies for plant breeding often incorporate diverse genotypes. This means that even if one genotype crosses with a wild relative, others within the same species may not. Although, according to Downey et al. (1980), *B. rapa* varieties are generally self-incompatible (Indian brown sarson and toria types as well as North American and European *B. rapa* cultivars), some of them are highly self-compatible (Indian yellow sarson form of *B. rapa*).

Despite the fact that *B. rapa* is one of the most comprehensively studied crops, as with *B. napus*, relatively few studies report success of hybrid production quantitatively (Fitz et al., 2007).

It is commonly known that canola (*B. napus*, AACC genome) and *B. rapa* (AA genome) have a common set of chromosomes, which facilitates interspecific gene flow between these two species. Spontaneous hybridization

in the field is possible, and natural *B. napus* × *B. rapa* hybrids have been reported in several countries, including Canada (Yoshimura et al., 2006), Denmark (Hansen et al., 2001), and the United Kingdom (Allainguillaume et al., 2006). In field trials, under natural conditions, the success of hybridization varies widely, depending on the experimental design and the direction of crosses (Pallett et al., 2006). However, subsequent introgression of alleles from *B. napus* to *B. rapa* occurs infrequently (Leflon et al., 2006). In the laboratory experiments, *B. rapa* pollen has a significantly lower degree of fitness on *B. napus* than conspecific pollen, and hybrid zygote survival is markedly reduced in comparison to conspecific zygotes (Hauser et al., 1997). In addition, *B. rapa* × *B. napus* hybrids have been found to have reduced fertility and lower seed set compared to either parental species (Jorgensen and Andersen, 1994).

The results of our investigation showed that the biggest number of embryos was received when *B. rapa* ssp. *chinensis* was treated as a female parent. These results are consistent with those obtained from the literature data (Ammitzbol et al., 2005) because they demonstrate that hybridization rates are significantly higher if *B. rapa* is a female parent, rather than vice versa, due to the self-incompatibility of *B. rapa*.

Our results confirmed that there is unilateral crossability between *B. rapa* ssp. *chinensis* and *B. napus* genotypes. When *B. rapa* ssp. *chinensis* was a female parent and *B. napus* was a male parent in all cases, cross-compatibility was observed with a mean PGI value =  $2.32 \pm 0.26$ . In reciprocal crosses, generally there was incompatibility (PGI =  $1.87 \pm 0.05^*$ ). According to Long et al. (1992), *pekinensis* group was more compatible with *Brassica raphanus* than *rapifera* group in *B. rapa*. In addition, kales were even more compatible than other varieties of *B. oleracea*, which suggests that the selection of certain species, subspecies, varieties or cultivars as pollen parents is important and may improve the cross-compatibility between them. Although, we obtained hybrid plants in both directions of crosses *B. rapa* ssp. *chinensis* × *B. napus* and *B. napus* × *B. rapa* ssp. *chinensis* (161 and 59, respectively). A greater number of regenerated plants were obtained when *B. rapa* ssp. *chinensis* was a female parent. Those results are coinciding with our observation of PGI value. This is particularly interesting in view of our previous studies on crossability in different *Brassica* species (Niemann

et al., 2014). In those cases, crosses between self-compatible (SC) cultivar Californium (*B. napus*) and self-incompatible (SI) *B. rapa* ssp. *pekinensis* were successful only when SC cultivar was used as a maternal form. Reciprocal crosses (SI × SC) failed. Fitz et al. (2007) made a similar observation related to the crossability barriers. Crosses between *B. napus* and *B. rapa* (a progenitor of *B. napus*) were very successful with a median rate of 2.29 hybrids/pollination when *B. napus* was the female parent in the cross but lower (0.44 hybrids/pollination) when *B. napus* was the male parent in the cross. In both directions, the rates of production ranged widely across trials (Fitz et al., 2007). The same authors proved that crosses between *B. napus* and *B. rapa* were more successful when the amphidiploid species, i.e. *B. napus*, was used as a female parent. Reciprocal crosses were generally unsuccessful. Moreover, Choudhary et al. (2012) demonstrated that amongst crosses involving three varieties/forms of *B. rapa* with *B. napus*, yellow sarson produced a maximum number of hybrids (18.0%) followed by brown sarson (11.3%) and toria (6.9%). Thus, the success rate of cross-fertility of three varieties of *B. rapa* with *B. napus* was in the order yellow sarson > brown sarson > toria. These observations are similar to our results indicating that the success of interspecific crosses depends not only on the species but also on the form/varieties of the species involved. That is why we observed differences in the number of *B. napus* × *B. rapa* ssp. *chinensis* hybrids obtained through embryo rescue. The latest was probably related to interspecific crossability between genotypes.

## Conclusions

- 1) Observations of the pollen tube growth and embryo set indicate unilateral interspecific compatibility in the crosses where *B. rapa* ssp. *chinensis* was used as a maternal form. However, the number of obtained embryos depended on the genotype.
- 2) The highest number of hybrids was obtained from crosses between *B. rapa* ssp. *chinensis* × *B. napus*.
- 3) The degree of hybridization between *B. rapa* and *B. napus* varied depending on subspecies of *B. rapa*, which were used in the crosses and the direction of crossing.

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