PROTOPLAST CULTURE AND FUSION BETWEEN *BRASSICA CARINATA* AND *BRASSICA NAPUS*

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Abstract

In this study, attempts were made to select in vitro responsible genotypes and to fuse the isolated protoplasts of Brassica carinata and Brassica napus breeding lines (BC DH Dodolla, BC DH -1, BC DH-6 and BN OP-1, BN-SL-03/04), obtained from our previous experiments. Combination of three different PEG concentrations (20%, 25% or 30%) and two different treatment durations (15 and 20 min.) were tested. Our experiments identified several genotypes (Brassica carinata DH BC-6 and DH BC-1, Brassica napus DH OP-01) with satisfactory regeneration ability of calli from protoplast cultures. Proper combinations of concentration and treatment time of PEG determined protoplast fusion frequency between genotypes used. Although the 30% PEG solution was evaluated to be the best concentration, large amount of multifusants, unwanted in practical applications, was detected especially in Petri dishes with longer PEG treatment. In general, 25% PEG combined with 20 minutes treatment duration produced satisfactory fusion frequency and good rate of viability was obtained as well.

Key words: *Brassica carinata*, *Brassica napus*, protoplast culture, protoplast fusion

INTRODUCTION

The genus *Brassica* includes a wide range of crop species with great economic value worldwide. Therefore, they attracted not only breeders using conventional methods but also those concerned with biotechnological methods. In recent years, major efforts in *Brassica* research have focused on utilization of tissue culture technology for crop improvement. Microspore culture and protoplast culture techniques are most frequently used for the manipulation of foreign gene to broaden genetic diversity. The techniques of protoplast culture and fusion are becoming increasingly useful accessories for breeding programs in various crop plants. The most widespread use of protoplasts is for somatic hybridization experiments either to overcome barriers in sexual crosses or to modify cytoplasmic traits by altering organelle populations (Kumar and Cocking, 1987). Somatic hybridization has been used in *Brassica* improvements programs for the transfer of chloroplast-encoded triazine resistance into cytoplasms carring mitochondria-encoded male sterility (Pelletier et al., 1983) and for the transfer of male sterile cytoplasm from spring to winter rapeseed (Barsby et al., 1987).
Numbers of studies have observed that the *in vitro* tissue culture and the regeneration of plants can lead to alterations in traits conditioned by the cytoplasm (Gengenbach et al., 1981; Kemble et al., 1984). As outlined above, *Brassica* intergeneric, interspecific and intraspecific somatic hybrids and cybrids have been obtained and used for the transfer of agronomically important nuclear and cytoplasmic traits.
Plant regeneration has been increasingly realized via organogenesis and somatic embryogenesis by means of various explants. Protoplast culture improvements have been focused on various factors including genotypes, media additives and effects of enzyme treatment on viability of protoplasts at different time durations.
The aim of our study was to identify *Brassica carinata* and *Brassica napus* genotypes or inbred lines that offer protoplasts with satisfactory regenerative capacity, to optimize current methods for establishing protoplast culture in selected genotypes of *Brassica carinata* and *Brassica napus*, to develop an efficient and reliable protoplast culture protocol for fusion experiments in selected *Brassica carinata* and *Brassica napus* genotypes.

MATERIALS AND METHODS

Plant material

High productive doubled haploid (DH) lines of winter oilseed rape (*Brassica napus* L.) as a source of mesophyll protoplasts BN-OP-01 and BN-SL-03/04 and genotypes of Ethiopian mustard (*Brassica carinata* A. Braun) – DH line BC Dodolla and breeding materials BC-1 and BC-6 – as a source of hypocotyl protoplasts were used for experiments. To obtain sterile plant material for establishing protoplast cultures, seeds of above mentioned genotypes were surface sterilized in 70% ethanol for one minute, followed by immersion in 30% commercial bleach Savo (on the basis of NaClO) for twenty minutes and finally by washing procedure

with sterile distilled water three times. MS medium without growth regulators was used for germination of seedlings. Seeds of genotypes intended for hypocotyl protoplasts were incubated in a thermostat at 25°C in the dark; those for mesophyll protoplasts were kept in a cultivation room and cultivated under controlled environmental conditions (25°C and 16/8h day/night photoperiod and light intensity 260 µmol/m²/s).

Protoplast isolation and fusion

Seven-day old hypocotyls and leaves from one-month-old *in vitro* plants were cut into 1mm segments and treated separately in different Petri dishes with the enzyme solution, containing 0.25% macerozyme R10 (Serva) and 1% cellulase Onozuka R 10 (Serva) in W5 salt solution (Potrykus and Shillito, 1986, Mukhopadhyay et al., 1994). Cut tissue was incubated for 18 hours in a thermostat at 25°C without shaking. Incubated mixtures were filtered separately through a 50 µm nylon mesh and transferred to 10 ml centrifuge tubes. Suspensions were centrifuged at 100 g for 5 minutes. After careful supernatant removal, 4 ml of 20% sucrose solution was mixed with the protoplast suspension and then gently covered with 2 ml W5 salt solution without disturbing the protoplast suspension. Suspensions were again centrifuged at 100g for 5 minutes. Floating protoplasts, forming a ring between two layers of W5 and sucrose solutions, were collected by sterile Pasteur pipette and diluted in the W5 solution (Menczel et al., 1981) and centrifuged at 100 g for 5 minutes.

Protoplasts, intended for fusion experiments were after supernatant removal finally resuspended in 0.1–0.5 ml of M+C medium, suspensions of mesophyll and hypocotyl protoplasts were then mixed in the ratio of 1 : 1 and the density was adjusted to $1–2 \times 10^5$ protoplasts per 1 ml. 3 × 50 µl (3 drops) of the protoplast suspension was added to the 35 mm Petri dish and sedimented for 20 minutes. Polyethylene glycol solution (PEG) was gently added to each drop of sedimented protoplasts and incubated for 15 or 20 minutes. Three different concentrations of PEG 6000 (Molecular weight) were used separately (20%, 25% or 30%) for each set of experiments. The PEG solution was carefully removed and 200 µl of STOP solution was added to each drop. After 20 minutes of incubation the STOP solution was replaced by 0.5 ml of liquid cultivation medium B.

Protoplast cultivation

Protoplasts after isolation, purification and, eventually after fusion experiments were cultured in the liquid medium B (Pelletier et al., 1983), containing 0.25 mg/l 2.4-D, 1 mg/l NAA, 1 mg/l BAP, 2% glucose, pH 5.8 at the density of 2.0×10^4/ml. Petri dishes with protoplast suspension were sealed with Parafilm and incubated in the dark in a thermostat at 25°C. Low osmotic medium C (Pelletier et al., 1983) without 2.4-D was added in amount 0.5 ml per each Petri dish containing medium B. Medium C was added twice every third day. After three weeks of cultivation in the dark, cultures were transferred to a cultivation room and further maintained under controlled environmental conditions (25°C and 16/8h day/night photoperiod and light intensity 260 µmol/m²/s). When microcalli reached the size of 0.5–1.0 mm in diameter, they were transferred to solid induction medium E (Pelletier et al., 1983). The microcalli (calli with typical prolonged cells) formation frequency was calculated by the number of microcalli formed to the total number of protoplasts cultivated.

Plant regeneration

After the growth period of approximately 15 days on the solid E medium calli reached the size of 5–10 mm in diameter. Well-developed calli were transferred to the regeneration medium F (Pelletier et al., 1983) to form shoots. Well-formed shoots were subsequently transferred to MS regeneration medium (Murashige and Skoog, 1962) without growth regulators. After a culture period of 1–2 months on hormone free medium most shoots regenerated roots and were transferred to soil and gradually adapted to a greenhouse environment.

Analysis of data

To prepare the sets of measured values for the analysis of variance (i.e. to follow normal distribution of errors), the percentage data was converted via square-root transformation (Bartlett 1936). Analytic software STATISTICA (StatSoft, Inc., Tulsa, OK, USA) was used for both data analysis and the preparation of graphs.

RESULTS

Regeneration of microcalli

Formation of the cell wall and, subsequently, cell divisions (Figure 6) after 4–7 days of the culture and the regeneration of microcallus structures after four weeks of cultivation (Figure 7) was observed in all genotypes tested and in all biological replications (Table 1). According to the statistical analysis of variance, the effect of a genotype on the regeneration of microcalli was significant, while the influence of individual biological replications and the interaction between genotypes and replication were not relevant (Table 3). The highest frequency of microcalli was obtained in the genotype BC-6 (31.4×10^{-2}%). Significantly less efficient were genotypes BC-1 (22.3×10^{-2}%) and BC Dodolla (18.9×10^{-2}%), followed by BN-OP-01 (13.7×10^{-2}%) and BN-SL-03/04 with 13.4×10^{-2}% of regenerated microcalli per cultivated protoplasts

(Figure 1). Homogeneous groups and contrast values derived from multiple comparisons between means of microcalli regeneration are shown in Table 1.

Regeneration of calli

Induction of callus structures with prolonged cells was detected in all genotypes tested. However, in some biological replications no conversion from microcalli to calli was observed in case of the genotype BN-SL-03/04 (Table 1). These differences were confirmed by means of statistical calculations, where the effects of biological replications (and the genotype, respectively) were evaluated as significant (Table 4). In general, the efficiency of conversion from microcalli to calli was significantly the highest in the genotype BC-6 (23.6%); less efficient were genotypes BC-1 (13.6%) and BN-OP-01 (10.0%). Poor regeneration was observed in case of genotypes BC Dodolla and BN-SL-03/04, where the conversion rates from microcalli to callus structures was only 2.7 and 0.8% (Figure 2). Since there were some differences between biological replications, analyses for individual replications were made as well (Figure 3). Homogeneous groups and contrast values derived from multiple comparisons between means of the callus regeneration are shown in Table 1.

Plant regeneration

In total, sixteen plants with properly developed stems and leaves were regenerated from calli possessing meristematic structures (Figure 8) of genotypes BC-6, BC-1 and BN-OP-1 (Table 1, Figure 9 and 10). Plants with well-developed leaves, regenerated on the solid F medium were transferred to the medium MS without growth regulators. After one week of cultivation on the solid MS medium, regeneration of the root system entered. Plants with properly developed roots were then subsequently multiplied from nodal segments with axilary meristems (Figure 11 and 12). Calli of other genotypes tested were not responsible to regenerate shoots or even meristematic structures and shoot primordia in the callus tissue and turned brown after several subcultivations.

Protoplast fusion

Statistical calculations of the data on the efficiency of the PEG treatment demonstrate its significant impact on the fusion frequency between genotypes used (Table 5). Remarkable differences were detected between individual biological replicates as well (Table 5). In general, the best combinations of 'PEG concentration × time duration' were 25% for 20 minutes and both time duration (15 and 20 minutes) of 30% PEG with fusion frequencies 7.9, 7.8 and 9.1%, respectively. Other combinations were significantly less efficient (Figure 4). More detailed results, i.e. for individual biological replications are shown in Figure 5, homogeneous groups and contrast values based on multiple comparisons between means of the fusion frequency are demonstrated in Table 2. Calli, obtained from fusions between *B. carinata* BC-6 and *B. napus* BN-OP-01 turned yellow and stopped their development (Figure 13).

DISCUSSION

Although the formation of cell divisions and microcallus structures were detected in all genotypes used in our experiments and the progress of the development was similar to the discovery of other researchers (Glimelius, 1984; Hu et al., 1999; Chen et al., 2004, Deep Kaur et al., 2006) there were remarkable differences between particular genotypes not only in the productivity (i.e. in the number of microcalluses per cultivated protoplasts) but also in the morphological and physiological characteristics of obtained microcalli. While the genotype BC-6 proved to be the most productive (31.4×10^{-2}% of regenerated microcalli per cultivated protoplasts), genotypes BN-OP-01 and BN-SL-03/04 demonstrated rather low level of regeneration ability. In addition, above mentioned, low productive genotypes often formed microcalli that gradually turned brown at early stages of development. Such structures may exude various substances, which can negatively affect the development of other, normally dividing microcalli (Chen et al., 2004). Strong impact of the genotype on the initialisation of cell divisions and further development of dedifferentiated tissue in the protoplast culture was presented by various authors like Hu et al., 1999 in *B. napus* and *B. juncea*; Chen et al., 2004 in *B. oleracea*; Narasimhulu et al., 1992 in *B. carinata.*

Successful conversion of microcalli to calli was observed in all genotypes used; however, outstanding differences in regeneration ability were detected not only between individual genotypes but also between some biological replicates within particular genotypes. The relevance of both factors was later confirmed via statistical analyses of measured data. Moreover, in some biological and technical replicates no regeneration of calli, characterised by formation of prolonged cells, was detected, although such genotypes produced satisfactory amount of calluses in other replications. For example, viable calli were regenerated only from one biological replication (B) of the genotype BN-SL-03/04. The differences in the efficiency of the callus formation between genotypes have been reported by many authors, for instance Hu et al. (1999); Deep Kaur et al. (2006). Significant differences between some biological (i.e. successive) replications of the same genotype can be explained by the heterogeneity of the biological material used for establishing of protoplast cultures and was observed also in the experiments of Chen et al. (2004).

Plants with properly developed leaves and stems were derived from bright green calluses with dark green meristematic structures inside callus tissue. No shoots were regenerated from white and yellow calluses without meristematic zones. In total, only sixteen calluses of genotypes BC-1, BC-6 and BN-OP-01 regenerated shoots; several previously mentioned calli produced more than one shoot. All regenerated plants were characterized by certain level of hyperhydricity; such type of plants was described also by Jourdan and Earle (1989) in the protoplast culture of four *Brassica* species, Hu et al., 1999 in *Brassica napus and B. juncea*. This undesirable physiological state was eliminated by repeated subcultivation on MS medium without growth regulators.

It is evident, that only the proper combination of PEG 6000 concentrations and time durations can provide satisfactory fusion frequency. From results can be concluded that all the 30 % PEG solutions provided good results (about 8% of fusants) together with 25% PEG solution with the period of 20 minutes. The efficiency of these combinations is comparable with the results, obtained by Gurel et al. (2002). Even higher frequency of fusants was observed by Beranek et al. (2006) on protoplast fusions between *B. carinata* and *B. rapa*, where the 30% PEG 6000 MW treatment for 10 minutes achieved about 25% of fusants. Both 20% PEG concentrations and shorter period of 25% concentration showed rather low efficiency. However, both combinations with 30% PEG solution increased the number of multifusants, unfavourable for practical applications. Analogous to the production of calli, significant differences between biological replicates in fusion frequency were detected as well. These circumstances might be explained by the heterogeneity of fused material during experiments even if all measurable environmental conditions were maintained. Protoplasts of fused cultures regenerated cell walls and first divisions were observed after 4th day of the culture. Small calli, approx. 1-2 mm in diameter turned brown-yellow and did not develop desired callus structures. Similar results were defined by Beranek et al. (2007) in *Brassica carinata* and *Brassica rapa* genotypes.

CONCLUSION

Regeneration of microcalli and calli was achieved in all examined genotypes; strong effect of the genotype on the regeneration of both types of dedifferentiated tissue was confirmed by means of statistical analysis. *Brassica carinata* BC-6 and BC-1 were proved the best genotypes in terms of the formation of microcalli and calli from isolated protoplasts. On the contrary, not satisfactory level of regeneration ability was reported in genotypes *B. carinata* BC Dodolla and *B. napus* BN-SL-03/04. However, significant differences were detected also between biological replicates within some genotypes tested; this phenomenon might be caused by certain level of the heterogeneity of biological material used for successive replications.

Whole plants with well-developed roots were derived from three genotypes; only calli with dark green meristematic structures were able to regenerate shoot primordia and subsequently shoots, however, hyperhydrated breakable stems and leaves were observed in all achieved regenerants. Consequently, repeated subcultivations on the solid MS medium were necessary to obtain vigorous plants.

Proper combinations of PEG concentrations and treatment time determined the fusion frequency between genotypes used. Although the 30% PEG solution was evaluated to be the best concentration, large amount of multifusants, unwanted in practical applications, was detected especially in Petri dishes with longer PEG treatment.

Our experiments identified several genotypes with satisfactory regeneration ability of calli from protoplast cultures. However, poor regeneration of whole plants and their physiological state (i.e. hyperhydricity) extended the regeneration of vigorous plants and thus complicated transferring such plants to non-sterile conditions.

Although callus-like aggregates, achieved in fused cultures, did not formed calli with the ability to undergo further regeneration, optimal concentrations and time durations of the fusion agent polyethylene glycol were evaluated for *B. carinata* BC-1 and *B. napus* BN-OP-01. These observations could help in further research not only on fusion experiments with closely related species of genus *Brassica.*

Tab. 1: Microcalli, calli and plant regeneration in the protoplast culture of selected genotypes of genus *Brassica*

Genotype	Replication (+)	Petri Dish No. (++)	(A) No. of microcalli (*)	(A) No. of calli (*)	(A) Regeneration of microcalli (**) [x 10 -2 %]	(A) Regeneration of calli (***) [%]	(A) No. of plants per Petri Dish (*)	(B) No. of microcalli (*)	(B) No. of calli (*)	(B) Regeneration of microcalli (**) [x 10 -2 %]	(B) Regeneration of calli (***)	(B) No. of plants per Petri Dish (*) [%]	(C) No. of microcalli (*)	(C) No. of calli (*)	(C) Regeneration of microcalli (**) [x 10 -2 %]	(C) Regeneration of calli (***) [%]	(C) No. of plants per Petri Dish (*)
BC Dodolla	1		238.5	0.0	18.3	0.0	0	184.6	15.4	14.2	8.3	0	269.3	7.7	20.7	2.9	0
	2		207.7	0.0	16.0	0.0	0	253.9	7.7	19.5	3.0	0	176.9	0.0	13.6	0.0	0
	3		261.6	7.7	20.1	2.9	0	230.8	7.7	17.8	3.3	0	392.3	15.4	30.2	3.9	0
Mean			235.9	2.6	def 18.1	EF 1.0		223.1	10.3	def 17.2	CD 4.9		279.5	7.7	cde 21.5	DE 2.3	
BC-6	1		407.7	107.7	31.4	26.4	2	469.3	138.5	36.1	29.5	1	538.5	115.4	41.4	21.4	1
	2		323.1	69.2	24.9	21.4	0	338.5	84.6	26.0	25.0	0	438.5	69.2	33.7	15.8	0
	3		338.5	92.3	26.0	27.3	0	430.8	100.0	33.1	23.2	2	384.7	84.6	29.6	22.0	0
Mean			356.4	89.8	abc 27.4	A 25.0		412.9	107.7	ab 31.8	A 25.9		453.9	89.8	a 34.9	AB 19.7	
BC-1	1		307.7	46.2	23.7	15.0	2	284.6	23.1	21.9	8.1	0	269.3	30.8	20.7	11.4	1
	2		284.6	30.8	21.9	10.8	2	338.5	61.5	26.0	18.2	0	415.4	69.2	32.0	16.7	0
	3		215.4	23.1	16.6	10.7	0	238.5	30.8	18.3	12.9	0	253.9	46.2	19.5	18.2	2
Mean			269.3	33.3	cde 20.7	B 12.2		287.2	38.5	cde 22.1	B 13.1		312.8	48.7	bcd 24.1	AB 15.4	
BN-OP-01	1		253.9	23.1	19.5	9.1	0	146.2	23.1	11.2	15.8	0	192.3	15.4	14.8	8.0	1
	2		323.1	38.5	24.9	11.9	2	153.9	23.1	11.8	15.0	0	92.3	0.0	7.1	0.0	0
	3		200.0	23.1	15.4	11.5	0	76.9	7.7	5.9	10.0	0	169.2	15.4	13.0	9.1	0
Mean			259.0	28.2	cde 19.9	BC 10.8		125.7	18.0	g 9.7	D 13.6		151.3	10.3	fg 11.6	D 5.7	
BN-SL-03/04	1		161.6	0.0	12.4	0.0	0	200.0	7.7	15.4	3.8	0	115.4	0.0	8.9	0.0	0
	2		92.3	0.0	7.1	0.0	0	176.9	0.0	13.6	0.0	0	200.0	0.0	15.4	0.0	0
	3		246.2	0.0	18.9	0.0	0	238.5	7.7	18.3	3.2	0	138.5	0.0	10.7	0.0	0
Mean			166.7	0.0	fg 12.8	F 0.0		205.1	5.1	efg 15.8	DE 2.4		151.3	0.0	fg 11.6	F 0.0	

(*), per 35 mm Petri Dish; (**), per No. of cultivated protoplasts; (***), per No. of obtained microcalli, (+), Biological replicates; (++), Technical replicates
Letters in rows and columns indicate homogeneous groups derived from multiple comparisons between means of microcalli (a – f) and calli regeneration (A – F) (LSD; $P = 0.05$)

Tab. 2: Fusion frequency between *B. carinata* BC-6 and *B. napus* BN-OP-01 genotypes obtained from different treatments with PEG solution

Replication (+)		(A)			(B)			(C)		
Concentration of PEG solution	Spot No. (+)	No. of protoplasts (*)	No. of fusants (*)	Fusion frequency [%]	No. of protoplasts (*)	No. of fusants (*)	Fusion frequency [%]	No. of protoplasts (*)	No. of fusants (*)	Fusion frequency [%]
	1	31.2	1.2	3.8	36.6	0.2	0.5	69.8	1.6	2.3
PEG 20%	2	56.8	1.0	1.8	87.2	1.0	1.1	75.0	4.0	5.3
15 minutes	3	47.6	0.2	0.4	49.8	0.4	0.8	65.4	2.8	4.3
	Mean	45.2	0.8	fg 2.0	57.9	0.5	g 0.8	70.1	2.8	def 4.0
	1	77.0	1.0	1.3	56.6	1.4	2.5	83.2	2.0	2.4
PEG 20%	2	65.8	0.8	1.2	39.0	0.4	1.0	49.0	1.4	2.9
20 minutes	3	59.2	1.0	1.7	77.6	0.0	0.0	63.0	4.0	6.3
	Mean	67.3	0.9	fg 1.4	57.7	0.6	g 1.2	65.1	2.5	def 3.9
	1	80.8	2.2	2.7	62.6	0.0	0.0	88.2	2.8	3.2
PEG 25%	2	44.2	1.0	2.3	75.2	1.6	2.1	76.6	4.6	6.0
15 minutes	3	95.4	3.6	3.8	93.2	2.2	2.4	84.0	5.0	6.0
	Mean	73.5	2.3	efg 2.9	77.0	1.3	g 1.5	82.9	4.1	cde
	1	74.4	4.0	5.4	69.0	2.0	2.9	105.2	6.8	6.5
PEG 25%	2	100.6	5.6	5.6	51.4	3.8	7.4	71.8	9.0	12.5
20 minutes	3	62.2	8.2	13.2	85.0	8.0	9.4	91.8	7.6	8.3
	Mean	79.1	5.9	abcd	68.5	4.6	bcde	89.6	7.8	abc
	1	37.6	3.6	9.6	45.8	1.6	3.5	67.2	7.2	10.7
PEG 30%	2	87.2	5.2	6.0	38.6	3.6	9.3	59.8	4.8	8.0
15 minutes	3	99.0	5.0	5.1	69.2	5.2	7.5	81.0	8.2	10.1
	Mean	74.6	4.6	bcd	51.2	3.5	bcde	69.3	6.7	ab 9.6
	1	71.8	6.6	9.2	69.0	5.2	7.5	81.2	14.0	17.2
PEG 30%	2	60.6	4.2	6.9	64.4	4.0	6.2	95.0	11.4	12.0
20 minutes	3	89.0	7.4	8.3	96.6	3.8	3.9	74.8	7.8	10.4
	Mean	73.8	6.1	abc	76.7	4.3	bcde	83.7	11.1	a 13.2

(*) per 1 mm^2 of fused spots

(+) Biological replicates; (++) Technical replicates

Letters a–g in rows and columns indicate homogeneous groups derived from multiple comparisons between means of fusion frequency (LSD; $P = 0.05$)

Tab. 3: Test of significance (*P*) of individual sources of variability and their interactions for the regeneration of microcalli

Effect	SS	DoF	MS	F	P
Genotype	0.241811	4	0.060453	20.684	0.000000*
Replication	0.001300	2	0.000650	0.222	0.801899
Genotype x Replication	0.046789	8	0.005849	2.001	0.080878
Error	0.087680	30	0.002923		

Tab. 4: Test of significance (*P*) of individual sources of variability and their interactions for the regeneration of calli

Effect	SS	DoF	MS	F	P
Genotype	113.8755	4	28.4689	53.2550	0.000000*
Replication	5.9839	2	2.9919	5.5969	0.008597*
Genotype x Replication	6.9016	8	0.8627	1.6138	0.162608
Error	16.0373	30	0.5346		

Tab. 5: Test of significance (*P*) of individual sources of variability and their interactions for fusion frequency between *B. carinata* BC-6 and *B. napus* BN-OP-01

Effect	SS	DoF	MS	F	P
Replication	7.9239	2	3.9620	14.6779	0.000022*
PEG solution	26.0091	5	5.2018	19.2711	0.000000*
Replication x PEG solution	0.9852	10	0.0985	0.3650	0.953861
Error	9.7174	36	0.2699		

Two-way ANOVA; significant effects (*P*-value ≤ α-level = 0.05) are marked with an asterisk (*)
SS – Sum of Squares, DoF – Degrees of Freedom, MS – Mean Square, F – F-Statistic, *P* – Significance (*P*-value)

Figure 1: The impact of individual genotypes on the regeneration of microcalli; pooled data for three successive biological and three technical replicates

Bars represent individual 95% confidence intervals
Letters a-c designate homogeneous groups (LSD; *P* = 0.05)

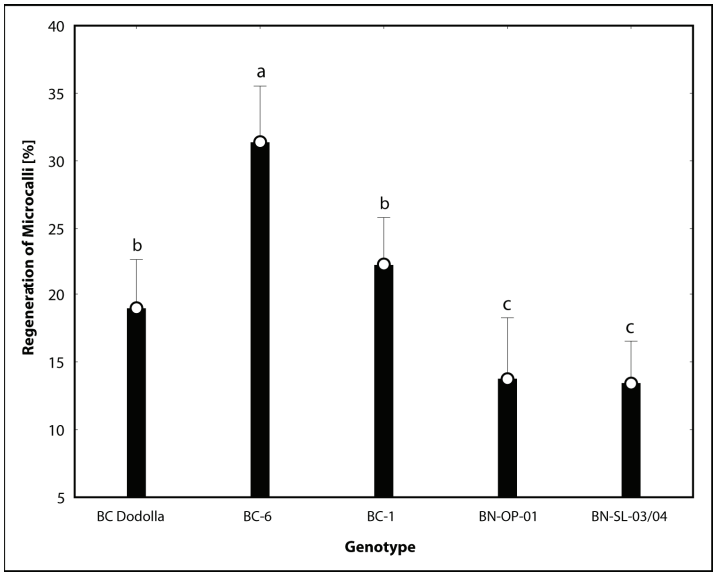


Figure 2: The impact of individual genotypes on the regeneration of calli; pooled data for three successive biological and three technical replicates

Bars represent individual 95% confidence intervals
Letters a–d designate homogeneous groups (LSD; *P* = 0.05)

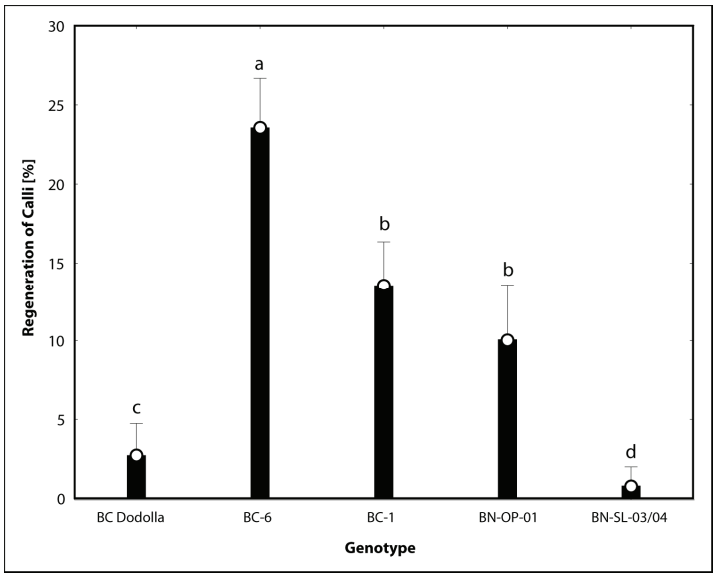


Figure 3: The impact of particular genotypes on the regeneration of calli within individual biological replicates (%); pooled data for three technical replicates

Bars represent individual 95% confidence intervals
Letters a–c, A–C, and α–γ designate homogeneous groups (LSD; $P = 0.05$)

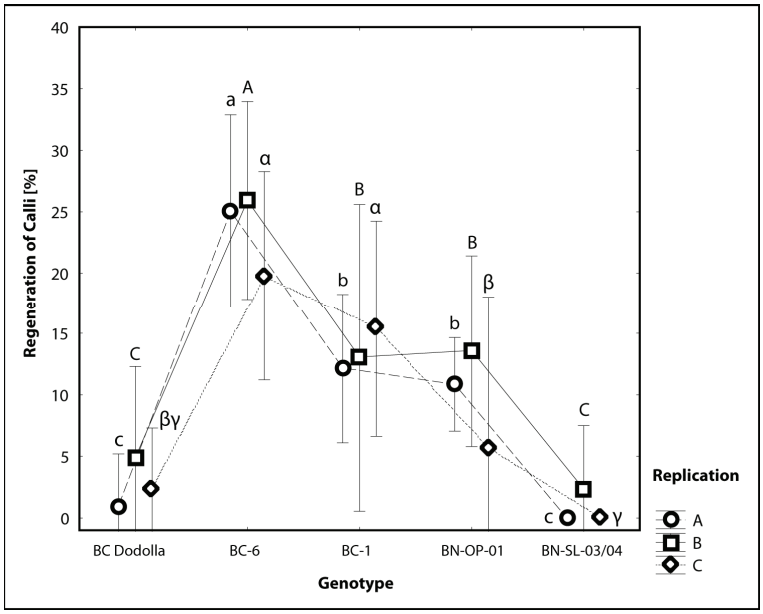


Figure 4: The impact of different PEG treatments on the fusion frequency between *B. carinata* BC-6 and *B. napus* BN-OP-01 genotypes (%); pooled data for three successive biological and three technical replicates

Bars represent individual 95% confidence intervals
Letters a and b designate homogeneous groups (LSD; $P = 0.05$)

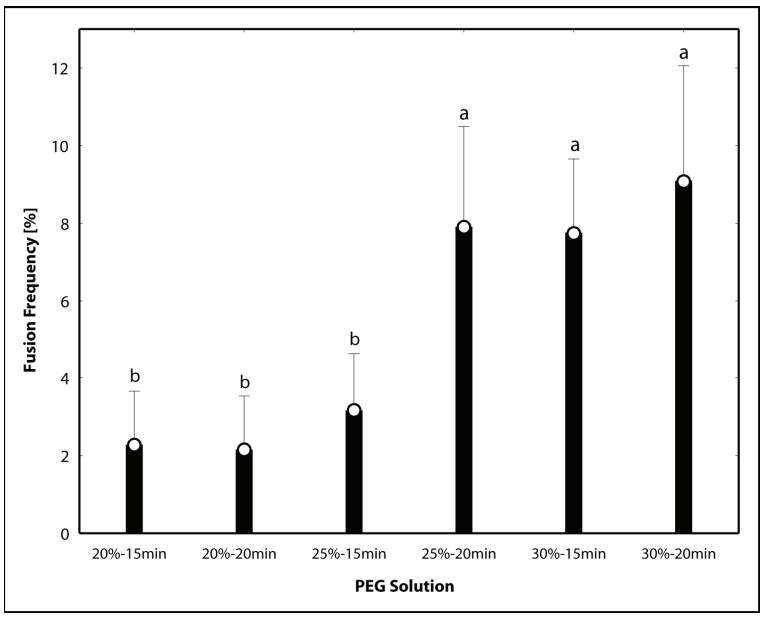


Figure 5: The impact of different PEG treatments on the fusion frequency (%) between *B. carinata* BC-6 and *B. napus* BN-OP-01 genotypes, within individual biological replicates; pooled data for three technical replicates

Bars represent individual 95% confidence intervals
Letters a–b, A–B, and α–γ designate homogeneous groups (LSD; $P = 0.05$)

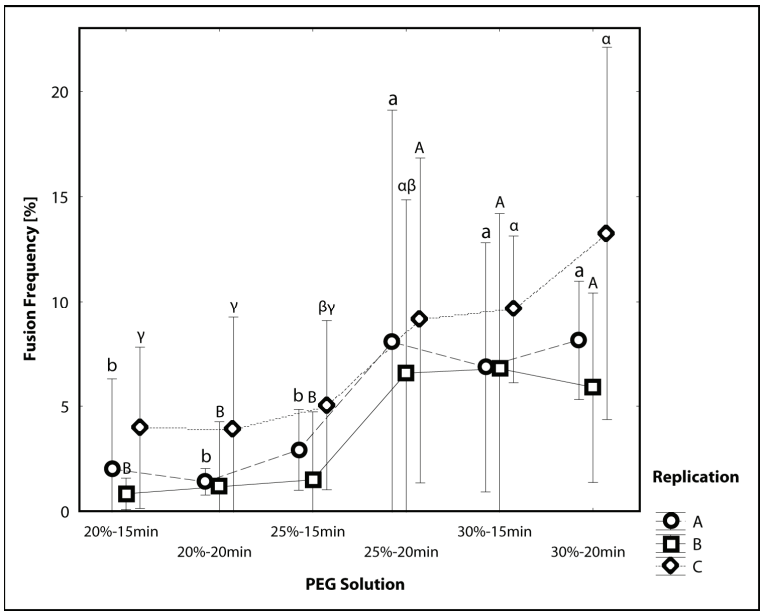


Figure 6: Cell divisions in the protoplast culture of the genotype BC-6 (Liquid C medium)

Bar = 50 micrometers

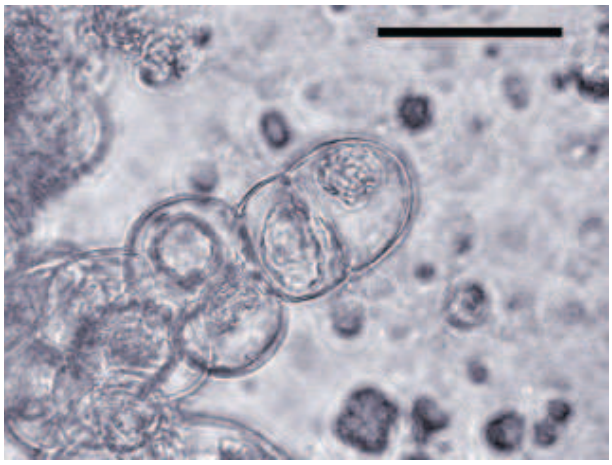


Figure 7: Formation of the microcallus in the protoplast culture of the genotype BC-6 (Liquid C medium)

Bar = 50 micrometers

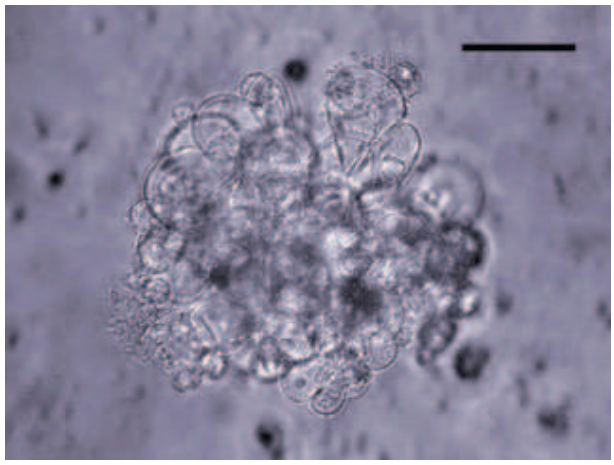


Figure 8: Protoplast-derived with meristem-like structures of the genotype BC-6 (Solid F medium)

Bar = 5 millimetres

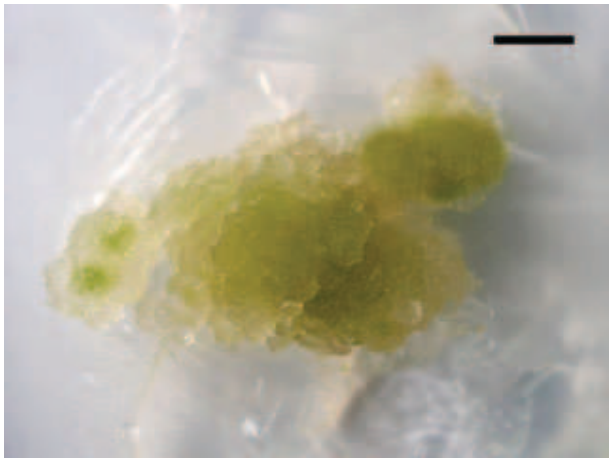


Figure 9: Plant regeneration from protoplast-derived callus tissue of the genotype BN-OP-01 (Solid F medium)

Petridish diameter = 90 millimetres

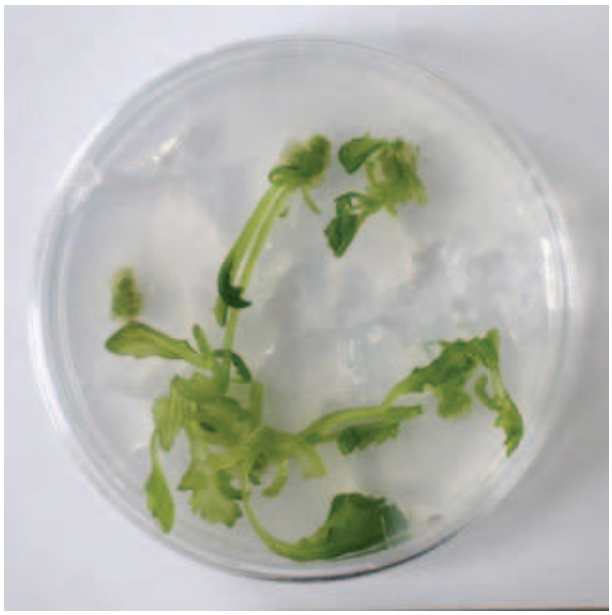


Figure 10: Plant regeneration from protoplast-derived callus tissue of the genotype BC-1 (Solid F medium)

Petridish diameter = 90 millimetres

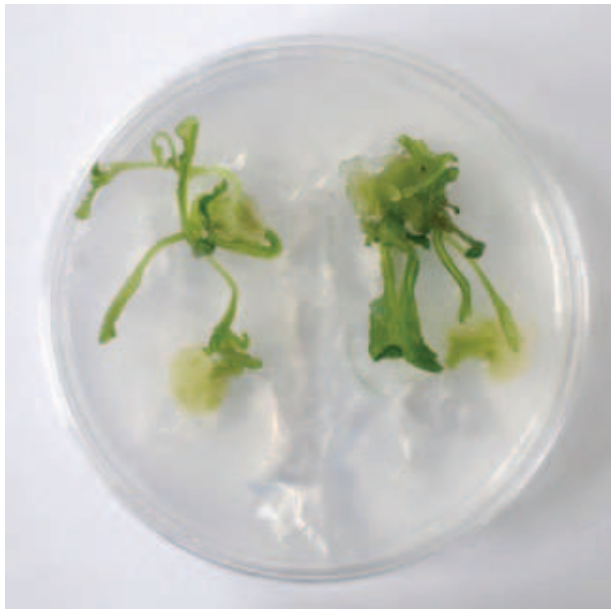


Figure 11: Whole plants of the genotype BN-OP-01 on the solid rooting medium MS

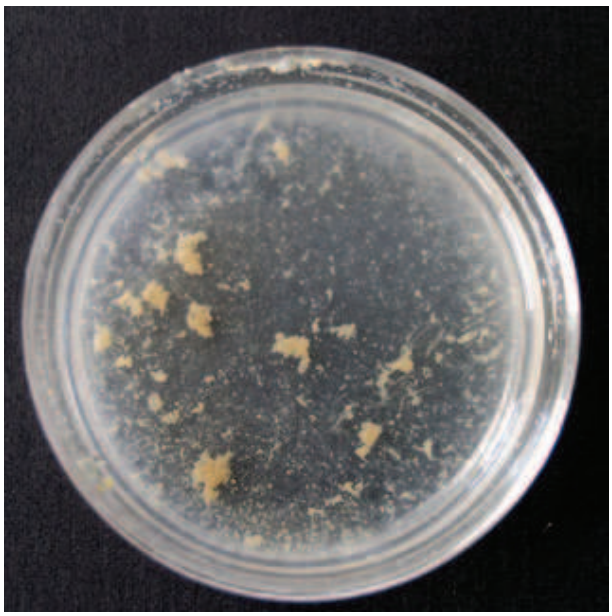
Flask height = 100 millimetres

Figure 12: Whole plants of *Brassica carinata* genotypes BC-1 and BC-6 on the solid rooting medium MS

Flask height = 100 millimetres

Figure 13: Small calli, regenerated from fused culture of *B. carinata* BC-6 and *B. napus* BN-OP-01 genotypes (Liquid C medium)

Petridish diameter = 35 millimeters



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