A Simple Procedure for Mesophyll Protoplast Culture and Plant Regeneration in *Brassica oleracea* L. and *Brassica napus* L.

Narpal Deep Kaur[1], Miroslava Vyvadilová[2], Miroslav Klíma[2] and Miroslav Bechyně[1]

[1]*Institute of Tropics and Subtropics, Czech University of Agriculture in Prague, Prague, Czech Republic;* [2]*Research Institute of Crop Production, Prague-Ruzyně, Czech Republic*

Abstract: An improved protocol for *Brassica* protoplast culture and plant regeneration was developed. Isolated protoplasts from four-weeks-old *in vitro* shoot tip culture of *Brassica oleracea* var. *botrytis* cv. Siria F1 and *Brassica napus* doubled haploid of breeding line OP-1 were cultured at a density of $9.8–11.2 \times 10^4$ protoplasts/ml in darkness at 25°C in a modified medium containing 2% glucose, 0.25 mg/l 2,4-D, 1 mg/l BAP and 1 mg/l NAA. The first divisions of protoplasts were observed on the third day of culture in *B. oleracea* and on the fourth day in *B. napus*. The protoplast cultures were diluted with low osmotic medium on 7th and 11th day. The frequency of dividing cells was about 80% in *B. oleracea* and 50% in *B. napus*. After one month, the microcalli of approximately 0.5–1 mm in size were transferred into an induction medium with various combinations of growth regulators. Minimum duration of enzyme treatment time and extended dark period in the initial phase of culture increased the survival rate of protoplasts. Organogenesis started when the calli enlarged in size on an induction medium (1 mg/l NAA, 0.02 mg/l GA_3, 1 mg/l 2iP) with 2% sucrose and 0.8% agar. Regeneration frequency of calli was found to be 69–75% in *B. oleracea* and 2–3% in *B. napus*. Well-developed shoots were transferred for rooting to a half-strength MS medium without growth regulators. More than 100 *B. oleracea* regenerants were transferred into soil, and they produced normal heads and set seeds. This very simple procedure is efficient and suitable mainly for *B. oleracea* var. *botrytis* and represents a background for fusion experiments.

Keywords: *Brassica napus* L.; *Brassica oleracea* L.; organogenesis; protoplast culture; regeneration

The *Brassica* species being widely used as a vegetable and oilseed crops attracted great attention not only of breeders using conventional methods but also of those concerned with biotechnological methods. Whereas *in vitro* techniques developed in the last decades improved the quality of edible oil (double low cultivars of rapeseed), likewise new oilseed crop varieties useful for industrial purposes were developed (Fahleson *et al.* 1994;

Barro *et al.* 2003). Microspore and protoplast culture techniques are used the most frequently for manipulation of foreign genes to broaden the gene pool and in expanding genetic diversity. Protoplasts can be emphasized as good accessories used in crop improvement programmes, particularly in overcoming incompatibility barriers through protoplast fusion (Schenck & Röbbelen 1982; Heath & Earle 1996), development of

CMS lines (Pelletier *et al.* 1983; Barsby *et al.* 1987; Jourdan *et al.* 1989; Cardi & Earle 1997), genetic transformation through direct uptake of DNA (Spangenberg *et al.* 1986; Maley & Pauls 1985) and mutation breeding. Generation of intergeneric somatic hybrids is no exception, Toriyama *et al.* (1987), Fahleson *et al.* (1994) and Navrátilová *et al.* (1997) have done a great deal of work in this respect.

The regeneration frequencies being genotype dependent vary between species, genotypes and source materials (Bidney *et al.* 1983; Glimelius 1984; Vamling & Glimelius 1990). Plant regeneration has been reported from hypocotyl and leaf protoplasts of *B. oleracea* var. *botrytis* (Glimelius 1984; Jourdan *et al.* 1990; Walters & Earle 1990; Kirti *et al.* 2001) and hypocotyl, mesophyll and stem peel protoplasts (Li & Kohlenbach 1982; Glimelius 1984; Chuong *et al.* 1985, 1987) in *B. napus*. Culture procedures like protoplast embedding in an agarose solidified medium (Barsby *et al.* 1986), and feeder layer cultures (Walters & Earle 1990) used for a small number of protoplasts showed very low regeneration frequencies and were laborious for handling.

The high regeneration ability of protoplasts is a pre-requisite for protoplast utilization in crop improvement programmes. For obtaining a high regeneration frequency, it is necessary to optimize the conditions of protoplast isolation, culture density, composition of culture media and growth regulator concentrations, dark and light periods in the initial culture phase and the developing phase of calli capable of transfer to an induction medium.

The objective of the present work was to improve the protoplast culture and plant regeneration technique in *Brassica napus* and *Brassica oleracea* as a background for their further utilization in fusion experiments.

MATERIALS AND METHODS

Plant material

A pre-screening of plant material for its suitability for protoplast culture included i*n vitro* clones of *Brassica napus* microspore-derived doubled haploids, and commercial cultivars of *Brassica oleracea* var. *botrytis* (data not shown). *B. napus* doubled haploid derived from F1 hybrid with double low seed quality originating from the Research Institute of Oilseed Crops in Opava (Kučera *et al.* 2004) and commercial hybrid cultivar Siria F1 of *Brassica oleracea* var. *botrytis* with high embryogenic responsiveness in microspore cultures (Klíma *et al.* 2004) were then selected as model genotypes for detailed experiments. The seeds were germinated after sterilization for one minute in 70% ethanol followed by 30% commercial bleach Savo for twenty minutes on half-strength Murashige and Skoog (1962) medium (MS) without growth regulators in culture cabinets under controlled conditions (with a light intensity of 84 μmol/m^2/s, 16/8 h day/night photoperiod, 23 ± 2°C). Then shoot tips were subcultured continuously on the same medium as donor plant material for protoplast isolation.

Protoplast isolation

Leaves from one-month-old shoot culture were cut transversaly into 1–2 mm segments and treated with 1% cellulase Onozuka R 10 (Serva) and 0.25% macerozyme R 10 (Serva) in W5 salt solution (Menczel *et al.* 1981). The material was kept overnight in a thermostat at 25°C without shaking. Isolated protoplasts were filtered through a nylon mesh (60–72 μm) and transferred into 10 ml centrifuge tubes. W5 salt solution was added to the protoplast suspension and centrifuged at 100 g for 5 minutes. The supernatant was removed and 20% sucrose solution was mixed to the protoplast suspension and covered with W5 salt solution and centrifuged at the same parameters to form a thin ring of floating protoplasts. After centrifugation protoplasts were collected with Pasteur pipette and dispersed in W5 solution for the next two centrifugations. Finally the pellets were dispersed in a culture medium. The number of viable protoplasts was counted after fluorescein diacetate staining according to time intervals of 10–12 h and 16–18 h in the enzyme solution.

Protoplast culture

Protoplasts were cultured at a density of 9.8 to 11.2 × 10^4/ml in 30 mm Petri dishes in 1ml of modified liquid culture medium B (Pelletier *et al.* 1983) supplemented with 0.25 mg/l 2,4-D, 1 mg/l NAA, 1 mg/l BAP and 2% glucose. The cultures were kept at 25°C in dark. After 7 days of the initial culture, 0.5 ml of low osmotic medium C (Pelletier *et al.* 1983) without 2,4-D was added

to each Petri dish and the addition of medium was repeated on the 11th day of culture. Star shaped microcalli developed within 15 days of culture. After the development of microcalli visible by naked eye, the cultures were transferred to light. The plating efficiency defined as the ratio of the number of protoplasts undergoing division to the total number of protoplasts cultured was measured. After about one month when the calli attained sizes of about 0.5–1.0 mm in diameter, they were transferred to solid medium E (PELLETIER *et al.* 1983) at 23°C under dim fluorescent light (40 μmol per m^2/s) in a day/night regime 16/8 h in the culture cabinets. Various growth regulator combinations in medium E (2% sucrose, 1 mg/l NAA, 0.02 mg/l GA$_3$, 1 mg/l 2iP) were tested, referred to as E$_1$ (2% sucrose, 0.25 mg/l NAA, 0.02 mg/l GA$_3$, 1 mg/l 2iP, 100 ml/l coconut milk, 4 g/l agarose), E$_2$ (2% sucrose, 3.0 mg/l BAP, 0.1 mg/l GA$_3$, 4 g/l agarose) and E$_3$ (1 mg/l NAA, 0.02 GA$_3$, 1 mg/l BAP).

Plant regeneration

The percentage of callus formation frequency was calculated as the number of formed calli to the total number of protoplasts undergone division. The well developed calli with shoot primordia were transferred to regeneration media F (0.5 mg/l BAP, 0.1 mg/l NAA, 1% sucrose, 8 g/l agar) and MS without growth regulators with 1% sucrose and 10 g/l agar. Regeneration frequency was established as the ratio of the number of calli regenerating shoots to the number of calli developed in the set experiment.

Chromosome analysis

The ploidy level of regenerants was assessed according to the flower morphology, seed set and chromosome counts. Chromosomes were counted in root tip cells. When the roots were about one centimetre in length, they were treated with paradichlorobenzene for 3 h, hydrolyzed in 1N HCl (15 min at 60°C) and fixed in ethylalcohol and acetic acid (2:1). The roots were squashed in a drop of aceto-orcein and observed under 1000× magnification.

RESULTS

A marked quantitative difference of more than 19% in viability (Table 1) of protoplasts was observed between the two time regimes in the enzymatic solution (10–12 h, 16–18 h). Three types of protoplasts were found in the isolated cultures, i.e. small, medium and large ones (Figure 1). Divisions occurred in all the three types of protoplasts and started on the 3rd day of culture in *B. oleracea* and on the 4th day of culture in *B. napus* (Figure 2). On the 7th day of culture more than two mitotic divisions were observed in 60% of protoplasts in *B. oleracea,* but in *B. napus,* the frequency was low (30%). After the addition of medium C (PELLETIER *et al.* 1983), the division frequency increased and microcolonies of 8–16 cells were observed in 80% of protoplasts in *B. oleracea*, whereas in *B. napus* microcolony formation was found in less than 50% of protoplasts. The high division frequency after the addition of low osmotic solution indi-

Table 1. Effect of enzyme treatment duration on viability of protoplasts

Duration of enzyme treatment	No. of protoplasts (MFA)	No. of viable protoplasts	Viable protoplasts (%)
10–12 h	14.1	6.9	48.9
16–18 h	13.4	4.0	29.9

MFA – Microscopic Field Area

Table 2. Regeneration in a mesophyll protoplast culture of *B. oleracea* var. *botrytis* cv. Siria and *B. napus* breeding line OP-1

Species	Culture density	Plating efficiency (%)	Callus formation frequency (%)	Percentage of regeneration (%)
B. oleracea	11.2×10^4/ml	80.87 ± 4.96	47.99 ± 7.80	69.05 ± 7.45
B. napus	9.8×10^4/ml	47.94 ± 7.43	39.96 ± 5.31	2.72 ± 2.98

Data (means and standard deviation) show results from two independent experiments with each species
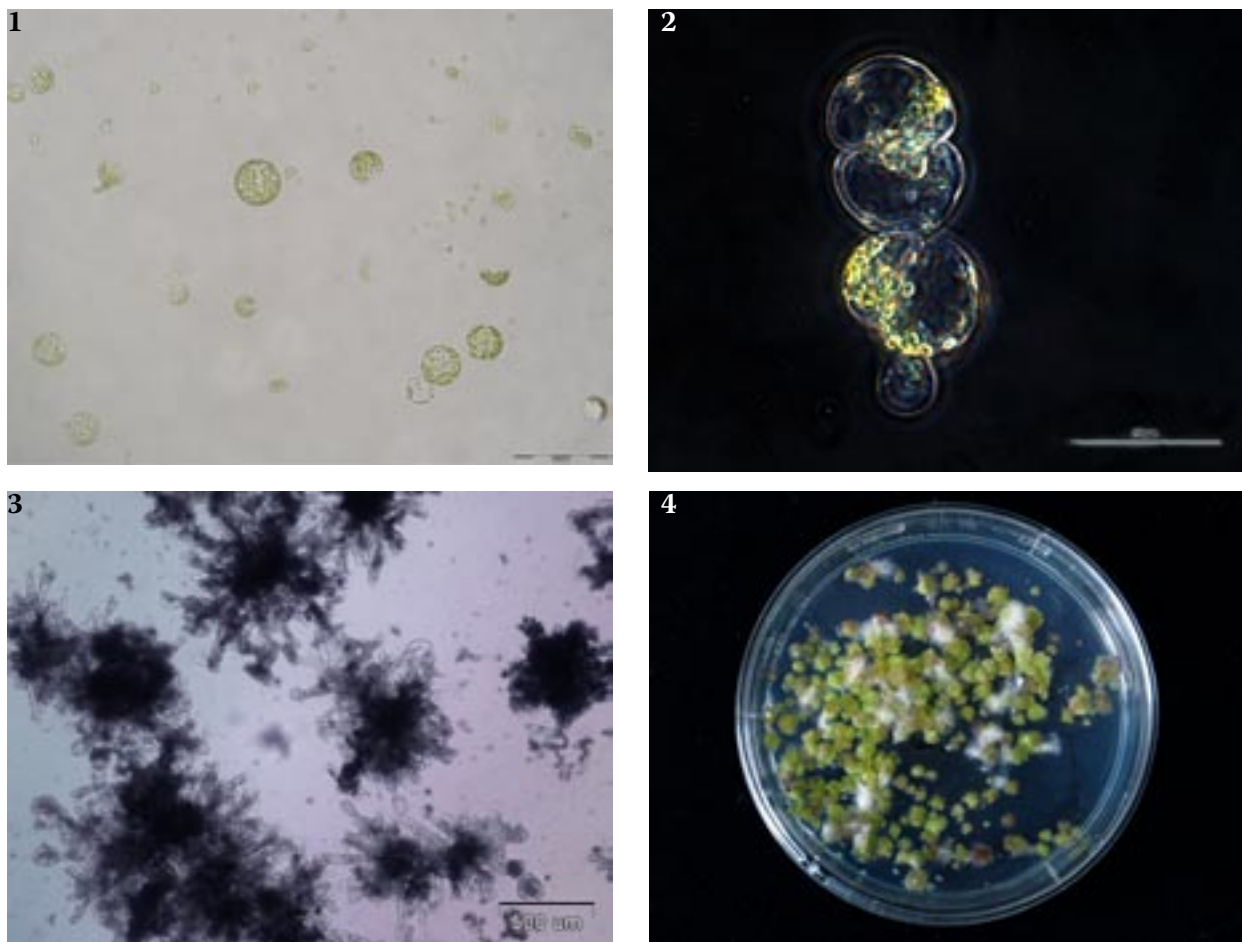
Figure 1. Freshly isolated protoplasts of *B. oleracea* (bar = 100 μm)

Figure 2. The first division after four days of protoplast culture in *B. napus* (bar = 50 μm)

Figure 3. Star shaped microcalli from *B. oleracea* protoplasts after 15 days in culture (bar = 500 μm)

Figure 4. Green calli after about three weeks in an induction medium (*B. oleracea*); the calli bear root hair-like outgrowths and shoot buds

cates that a higher concentration of osmoticum, beneficial in the initial cultures, inhibits enhanced cell division essential for proliferation of microcalli. The microcolonies enlarged and became star shaped (Figure 3) within 15 days of culture. Based on different experiments, it is concluded that the time used for isolation of protoplasts in the enzyme solution represented a crucial factor. The protoplasts isolated within 10–12 h showed higher viability than those isolated within 16–18 h. Temperature and light have a profound effect on division frequency. The cultures which were transferred after 7 days to light at 23°C showed very few divisions in comparison with those kept in dark for 15 days at 25°C. The developmental stage of calli in the liquid culture is important for

transfer onto the solid medium. In our experiments one-month-old calli reached the size of about 1 mm and after transferring to medium E, within 2–3 weeks they enlarged in size and showed a good regeneration ability. Hair-like root overgrowths appeared on calli very often and later shoot bud primordia originated on the same calli. In the initial developmental phase (1–2 weeks) the calli were cream-yellow in colour but after a period of two weeks they turned green (Figure 4). In *B. oleracea* the calli were of fragile consistency and contained embryo-like structures; when these structures were transferred to the regeneration medium, they regenerated shoots. The calli that did not regenerate on medium E readily regenerated shoots on medium F (Pelletier *et al.* 1983)

Figure 5. Plant regenerated from the protoplast derived callus in *B. oleracea* developing roots

or MS without growth regulators. Medium E is essential for the induction of shoot regeneration in developing calli. The shoots were transferred to half-strength MS medium for rooting and for prevention of vitrification (Figure 5). In *B. oleracea* all the developed shoots regenerated into whole plants whereas in *B. napus* the plant regeneration efficiency was 45–50%.

From various modifications of induction medium, media E and E1 produced regeneration frequencies of about 70% in *B. oleracea* but in the case of *B. napus* the regeneration frequency was very low (3%). Medium E2 induced stem-like structures

in the calli of *B. oleracea* and regenerated shoots after further transfer to the same medium. The higher concentration of BAP seemed to lead to the formation of embryo-like structures. The development of shoots took comparatively a longer time and regeneration frequency was about 31%. In *B. napus* the calli enlarged but no regeneration took place. In medium E3 calli regenerated shoots in *B. oleracea* but the percentage was lower than 15% (Table 3). Several independent experiments were performed for cauliflower cultivar Siria F1 and rapeseed breeding line OP-1. Table 2 details the development of cultured protoplasts of both species. Morphological and horticultural characteristics of about hundred regenerants of *B. oleracea* grown in the greenhouse were examined. The majority of the plants were normal, but some plants with morphological alterations such as absence of apical meristem or curly small leaves were recorded (Figure 6). All the plants developed normal white curds (Figure 7) except two that had violet streaks on their heads. Flowers were normal but large variation in the production of pollen was noted. Most of the plants had flowers with a good amount of pollen. All the examined *B. oleracea* regenerants had the expected $2n = 18$ chromosome number.

DISCUSSION

The regeneration of plants from protoplasts is a prerequisite for their utilization in crop improve-

Figure 6. *B. oleracea* abnormal plant bearing curly leaves

Figure 7. *B. oleracea* plant generated from protoplast with normal head

Table 3. Effect of different media on shoot regeneration from protoplast derived calli in *Brassica oleracea* var. *botrytis* cv. Siria

Medium**	No. of cultured calli	No. of survived calli	No. of calli with shoots	Regeneration (%)
E	327	327	225	68.80
E_1	280	280	177	63.20
E_2	202	193	59*	30.56
E_3	107	99	14	14.14

*The calli developed into stem-like structures, the small axes were subcultured and developed into shoots. A total of 59 plants were regenerated out of 193 calli

**E – 2% sucrose, 1 mg/l NAA, 0.02 mg/l GA3, 1mg/l 2iP; E_1 – 2% sucrose, 0.25 mg/l NAA, 0.02 mg/l GA3, 1 mg/l 2iP, 100 ml/l coconut milk, 4 g agarose; E_2 – 2% sucrose, 3.0 mg/l BAP, 0.1 mg/l GA3, 4 g agarose; E_3 – 1 mg/l NAA, 0.02 GA3, 1 mg/l BAP

ment programs. *Brassica oleracea* var. *botrytis* cv. Siria being an important vegetable and having higher protoplast division efficiency (80%) and regeneration ability (70%) can easily be used in crop improvement practices. Minimum duration of enzyme treatment time and extended dark period in the initial phase of culture increased the survival rate of protoplasts and hence the regeneration process. In the previous findings in *Brassica* vegetables, either the survival rate was low or the regeneration percentage decreased (Glimelius 1984; Kirti *et al.* 2001). The protocol proposed by us is simple, rapid and effective.

Regenerants from both *B. oleracea* and *B. napus* were successfully produced using the improved culture procedure. The isolation time of protoplasts in an enzyme solution greatly affects not only the viability of protoplasts but also the nature of plasmalemma (Pilet 1985), thus affecting the wall biosynthesis and hence the division process. Culture density has a profound effect on the plating efficiency of protoplasts. In concordance with the results of Chuong *et al.* (1985), it was found that with higher culture densities higher plating efficiency could be achieved. For mesophyll protoplasts, Vamling and Glimelius (1990) also recommended a higher concentration of protoplasts.

In the initial culture medium, an equal amount of BAP and NAA in the presence of a low amount of 2, 4-D was favourable in our experiments. Dietert *et al.* (1982) also pointed to the better growth of callus in the presence of a low concentration of 2,4-D. However, according to Glimelius (1984) and Kohlenbach *et al.* (1982) a high amount

of 2, 4-D is essential for cell division and callus proliferation. No such effect was evident in our experiments. It might be linked to the endogenous level of auxins in the used plant material.

A prolonged dark period is essential for the stability of protoplasts and hence for the formation of microcalli. In the presence of light, H^+ ion extrusion takes place, which increases the acidity of culture medium (Schubert & Matzke 1985). Cleland (1975) showed that H^+ ion extrusion was enhanced by the action of auxins. Under illumination the cultures turn brown, which affects the division efficiency of protoplasts. In our experiments not even after one month of culture was there a sign of browning in the cultures which were transferred to light after microcalli had been formed.

Differentiation or induction medium is a critical part of the protoplast culture protocol. Based on our results it can be concluded that cytokinin 2iP is more efficient than BAP in the induction of regeneration of calli. After one month on the induction medium, further transfer to regeneration medium F and MS (without growth regulators) showed no profound effect on regeneration frequency. The differentiation of organs was found to take place on medium E. Development of fragile calluses is in accordance with the reports by Kirti and Chopra (1990) in *B. juncea* and Kohlenbach *et al.* (1982) in *B. napus*. The absence of root development in almost 50% of regenerated shoots in *B. napus* is comparable to the results of Qiong Hu *et al.* (1999) and is genotype dependent. In our experiments, *B. oleracea* and *B. napus* showed a high division frequency in medium B in comparison with medium KM8p (Kao & Michayluk 1975),

which corresponded to protoplast division in some haploid lines generated from a microspore culture of *B. carinata*. In the present liquid culture system it is easy to handle the calli, and the medium described by Pelletier *et al.* (1983) proved to be quite suitable for *B. oleracea* protoplast culture. There are some variations in growth regulator concentration requirements for different subspecies (Robertson *et al.* 1988; Jourdan *et al.* 1990; Kirti *et al.* 2001).

Mesophyll protoplasts have several advantages over the hypocotyl ones. A large number of protoplasts can be obtained from just one plant, and further offspring can be regenerated from apical meristems in subsequent culture cycles and used for protoplast isolation. Further, mesophyll protoplasts are preferable for elite lines for which enough seed is not available, whereas for hypocotyls a huge amount of seeds with good germination is required. Mesophyll protoplasts can be used as a very effective material in overcoming incompatibility barriers through somatic hybridization, genetic manipulation, for studying cell metabolism and generation of genetic variation in a short time period.

Abbreviations

BAP	– 6-benzylamino purine
2,4-D	– 2,4-dichlorophenoxyacetic acid
GA_3	– gibberellic acid
2iP	– isopentenyl adenine
NAA	– 1-naphthalene acetic acid

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Corresponding author:

Narpal Deep Kaur, Česká zemědělská univerzita v Praze, Institut tropů a subtropů, Kamýcká 129, 165 21 Praha 6-Suchdol, Česká republika
tel.: + 420 233 901 220, e-mail: narpaldeep@gmail.com