PLANT REGENERATION FROM HYPOCOTYL PROTOPLASTS OF WINTER OILSEED RAPE (*BRASSICA NAPUS* L.)

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ABSTRACT

Protoplasts were isolated from hypocotyls of six breeding lines and two cultivars of winter oilseed rape (*B. napus* L.). Under presented culture conditions almost all of the protoplasts regenerated cell walls. Division frequency depended on the genotype and was from 50% to 64%. Shoot regeneration (also depended on the genotype) was induced with the frequency of 3.6% (for cv Bolko) on the medium containing IAA (0.1 $mg.dm^{-3}$), zeatin (0.5 $mg.dm^{-3}$) and BAP (0.5 $mg.dm^{-3}$). All shoots were rooted on MS basal medium supplemented with sucrose 30 $g.dm^{-3}$.

KEY WORDS: protoplasts, plant regeneration, winter oilseed rape, *Brassica napus* L.

INTRODUCTION

The possibilities of plant genetic modification with the use of protoplasts can only be realized in systems were methods of plant regeneration from protoplasts are available. There is already a huge literature about protoplast culture concerning different cells or tissues as a protoplast source, various methods and conditions of culture. For plants from the *Brassica* genus it has been shown that protoplasts from different parts of seedlings or plants can be regenerated into plants. Successful culture of cotyledon protoplasts was described by Lu et al. (1982), leaf protoplasts were cultured by Li and Kohlenbach (1982) and Millam et al. (1988) and hypocotyl protoplasts by Glimelius (1984). In spite of detailed information given by the cited authors, direct application of described conditions was impossible and usually needed an extra study. The main reason for this was that systems for protoplasts regeneration were very sensitive for such factors as genotype, physiological and developmental stage of original material as well as conditions of handling the cells and performing the culture.

In this article we present results concerning isolation, culture and plant regeneration from genotypes chosen by breeders.

MATERIALS AND METHODS

Polish breeding lines of double improved winter rapeseed: BOH 685, BOH 785, BOH 887, BOH 987, BOH 1087, BOH 1187 and cultivars Bolko and Leo were used. Seeds were kindly supplied by IHAR Experimental Station Borowo, Małyszyn and Department of Oil Crops IHAR Poznań. Seeds were sterilized in 0.1% $HgCl_2$ and germinated on hormone-free basal MS medium (Murashige and Skoog, 1962). Germination was performed at 25°C in the dark during 5-6 days.

Hypocotyls were cut into small pieces, preplasmolysed in 0.3M sorbitol with 0.05M $CaCl_2$, pH 5.7. After one hour this was replaced by enzyme solution containing Cellulysin 1% and 0.1% Macerase dissolved in K3 medium (Nagy and Maliga 1976). Incubation was performed overnight (17 hours) in the dark at 26°C with gentle mixing. The protoplast suspension was purified by passing through a sieve (100 µm mesh size), mixed with 1 volume of 1/10 CPW salts (Banks and Evans 1976) supplemented with 16% sucrose and centrifuged 15 minutes at 700 rpm. Floating protoplasts were harvested, washed with W5 (Menczel et al., 1981), centrifuged 5 minutes at 300 rpm and suspended in culture medium at a density of 2.5 x 10^4 protoplasts.cm^{-3} (ppts.cm^{-3})

Culture was initiated in 8p medium (Kao and Michayluk 1975) supplemented with 0.4M glucose, 2,4-D (2,4-dichlorophenoxy acetic acid) 1 $mg.dm^{-3}$, NAA (1-naphtaleneacetic acid) 0.1 $mg.dm^{-3}$, BAP (6-benzylaminopurine) 0.1 $mg.dm^{-3}$ and was performed in the dark at 26°C. Cell wall formation was estimated under phase contrast microscope on the second day of culture. Cell divisions was counted on the third day and expressed as a percentage of all plated protoplasts. When most of the cells completed divisions, culture medium was supplemented with 3 volumes of hormone-free 8p 0.4M glucose medium. Cell colonies obtained after 10-14 days were transferred on one of the following plating media: K3 – mineral and organic compounds according to Nagy and Maliga (1976) supplemented with 0.1M sucrose, 2,4-D 0.25 $mg.dm^{-3}$, NAA 0.025 $mg.dm^{-3}$ and BAP 0.025 $mg.dm^{-3}$; K6 – all components as above plus sucrose 0.06M; MS – with growth regulators as in K3. All media were solidified with 0.4% agarose. Culture was performed at 26°C, under white fluorescent light (15 $umol.m^{-2}.s^{-1}$) with 16 hour photoperiod. Minicalli of about 1-2 mm in diameter were transferred on the regeneration medium with various growth substances combinations

and cultured in conditions described above. All experiments were repeated 3-5 times. Regenerated shoots were cut off the calli and transferred on MS basal medium containing 30 $g.dm^{-3}$ sucrose and solidified with agar.

RESULTS AND DISCUSSION

Protoplasts were easily isolated from hypocotyls of all tested genotypes (Fig. A). Almost all of the protoplast (up to 95%) regenerated cell wall. First divisions were visible already on the second day of culture and most of the cells divided first time within 3-4 days (Fig. B). Frequency of cell division counted on the 4th day is presented in Table 1.

TABLE 1. Division frequency of protoplast derived cells after four days of culture

Genotype	BOH 685	BOH 785	BOH 887	BOH 987	BOH 108	BOH 118	Bolko	Leo
DF	50%	61%	64%	50%	54%	57%	58%	54%

Division frequency of different genotypes varied from 50% to 64% and was comparable to these reported by other authors: 65% (Glimelius 1984), 50% (Millam et al. 1988), 60% (Kirti 1988). It suggested that ability to regenerate cell wall and to start divisions had not been dependent on the genotype. This feature was related rather to cell type and its physiological stage.

At this stage of culture it was very important to add fresh, hormone-free medium. Too late feeding of protoplasts resulted in accelerated aging of cells. After 14-20 days of culture cell colonies were consisted of at least several cells and had characteristic star-like shape with protruding elongated, single cells from globular colony core (Fig. C). Further growth was performed on agarose-solidified plating medium. Out of three tested media the best growth was observed on K3. Growth on K6 or MS was clearly poorer and this reaction was independent on genotype.

To promote divisions and avoid browning some authors (Choung et al. 1985, Klimaszewska and Keller 1987 and Millam et al.1988) supplemented culture medium with Ficoll. We also noticed tendency of cells to become brown and expel dark precipitate but in our case in was relatively easy to avoid this reaction by proper leading the culture. Protoplast feeding should be done right after first divisions and plating the colonies as soon as they reached star-like structures.

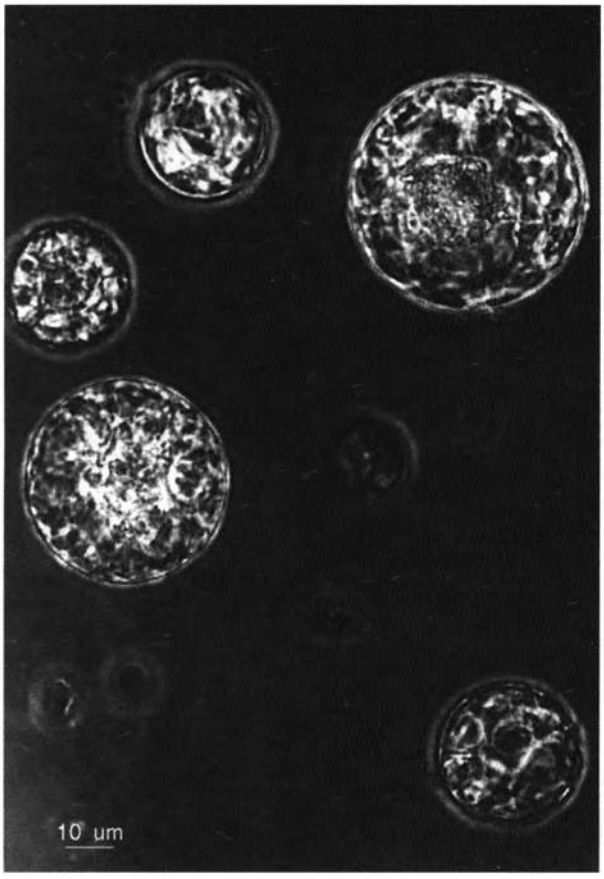
Within one week some colonies reached the size of about 1 mm – big enough to be transferred on fresh K3 medium. Successive removing the biggest colonies visible promoted growth of the remaining cells. Efficiency of shoot regeneration is presented in Table 2.

Regeneration of the first shoots occurred after two weeks of culture but most of the shoots emerged after 6 to 10 weeks. In almost all cases there was only one shoot per callus (Fig. E, F). Longer (more than 10 weeks) culture resulted in very seldom shoot regeneration. At this stage most of the calluses underwent very quick process of aging and dying. Out of eight genotypes, three did not regenerate shoots. Regeneration of the remaining five varied from 0.5% to 3.6% and was the highest for cultivar Bolko. All shoots after being transferred on MS basal medium developed roots within 2-3 weeks and after fur-

TABLE 2. Frequency of shoot regeneration. Percentage [%] of calluses regenerating at least one shoot on K3 basal medium supplemented with 0.03M sucrose and 0.4% agarose

	Genotype / Medium [$mg.dm^{-3}$]	BOH 685	BOH 785	BOH 887	BOH 987	BOH 1087	BOH 1187	Bolko	Leo
A	(0.1 IAA, 2.0 zeatin)	0	0.8	0.7	0	0.5	0	–	–
B	(0.1 IAA, 0.5 zeatin, 0.5 BAP)	0	2.2	2.5	0	1.9	0	3.6	2.3
C	(0.1 IAA, 1.0 zeatin, 1.0 BAP)	0	0.6	0.4	0	0	0	0.5	–
D	(0.25 IBA, 0.5 zeatin, 0.5 BAP)	–	–	–	–	–	–	1.0	0
E	(0.25 IBA, 1.0 zeatin, 1.0 BAP)	–	–	–	–	–	–	0.7	1.8
F	(0.25 IBA, 2.0 BAP)	–	–	–	–	–	–	0	0
G	(0.5 IAA, 0.5 zeatin, 0.5 BAP)	–	–	–	–	–	–	0.6	0.6
H	(0.5 NAA, 0.5 zeatin, 0.5 BAP)	–	–	–	–	–	–	0	0.5

BAA – 6-benzylaminopurine
IAA – indole-3-acetic acid
IBA – indole-3-butyric acid
NAA – 1-naphtaleneacetic acid



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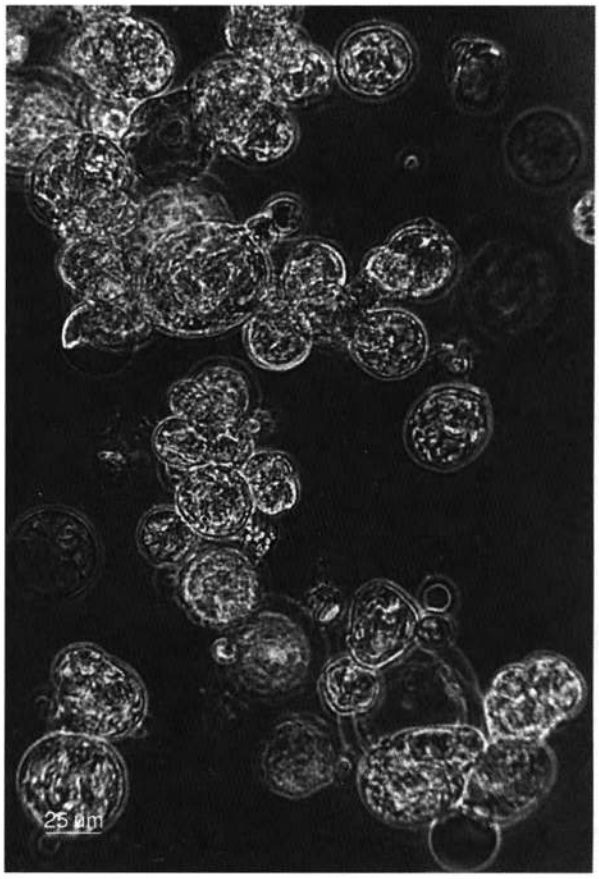

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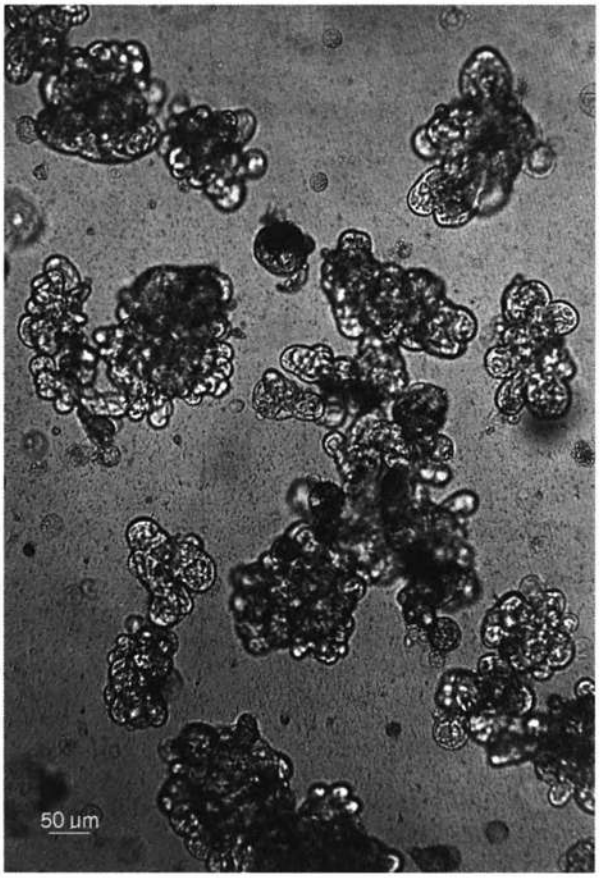

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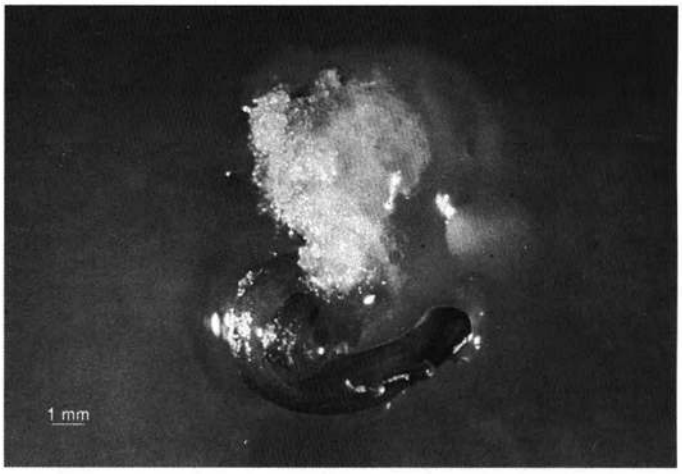

D

Fig. 1 – A-F. Regeneration of plants from protoplasts of winter oilseed rape cv Bolko.
A – Freshly isolated protoplasts from etiolated hypocotyls.
B – Mitotic divisions of protoplast derived cells after 3 days of culture.
C – Cell aggregates – early stage of star-like colonies after 10 days of culture.
D – Shoot regeneration after 6 weeks of culture on K3 medium containing 0.1 mg.dm^{-3} IAA, 0.5 mg.dm^{-3} zeatin, 0.5 mg.dm^{-3} BAP.

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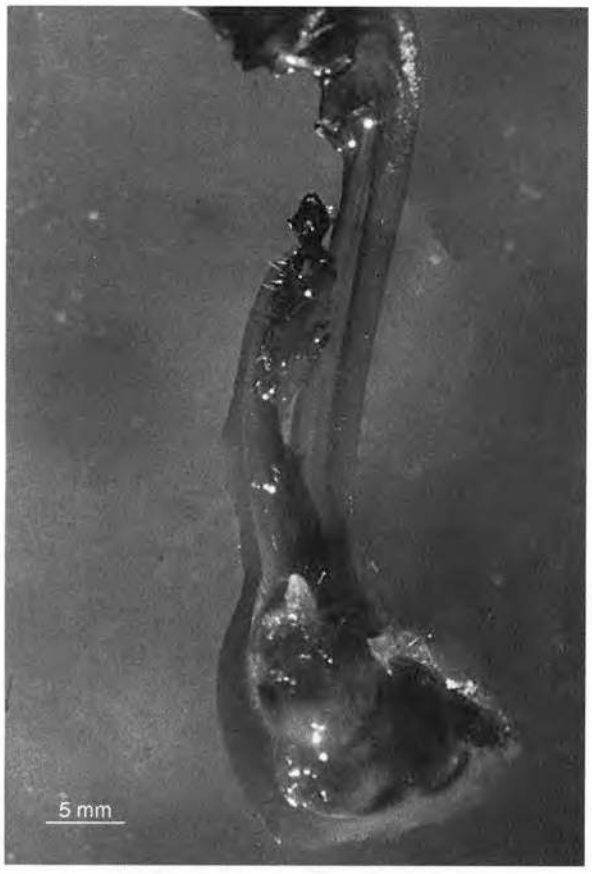

E

F

Fig. 1 — cont.
E – Shoot regeneration after 6 weeks of culture on K3 medium containing 0.1 mg.dm^{-3} IAA, 0.5 mg.dm^{-3} zeatin, 0.5 mg.dm^{-3} BAP.
F – Plants regenerated from protoplasts.

ther 2 weeks culture on the same medium were transferred to soil (Fig. D.).

Medium K3 containing growth substances as in B comparing with other six combinations induced the highest number of shoots. This was the case for all regenerating genotypes. Results presented here and concerning regeneration on IAA (0.1 mg.dm^{-3}), zeatin (0.5 mg.dm^{-3}) and BAP (0.5 mg.dm^{-3}) containing medium were consistent with those obtained by Glimelius (1984). In case of her research regeneration frequency on medium with the same growth regulators was as high as 70%. Regeneration reported by other authors varied from 3% (Choung et al. 1985) to 16% (Kirti 1988) and 20% (Xu et al. 1982, Klimaszewska and Keller 1987, Kirti 1988).

These reports however concerned culture of spring rapeseed. Culture of winter genotypes was presented only in few articles and regeneration frequency was much lower. Some papers reported only the first stages of culture without plant regeneration (Millam et al. 1988). Spangenberg et al. (1986) presenting conditions for culture of single protoplasts reported that callus formation from winter rapeseed was dramatically reduced comparing with spring cultivars. Kirti (1988) induced shoots from winter oilseed rape almost at the same level (3.8%) as in our work while the same author reported regeneration for spring type on the level of 20%.

Procedure described in this work gives repeatable shoot regeneration on the level high enough to apply it to protoplast fusion and regeneration of hybrids.

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LITERATURE CITED

BANKS M.S., EVANS P.K., 1976. A comparison of the isolation and culture of mesophyll protoplasts from several *Nicotiana* species and their hybrids. Plant Sci. Lett. 7: 409-416.

CHOUNG P.V., PAULS K.P., BEVERSDORF W.D., 1985. A simple culture method for *Brassica* hypocotyl protoplasts. Plant Cell Reports 4: 4-6.

GLIMELIUS K.,1984. High growth rate and regeneration capacity of hypocotyl protoplasts in some *Brassicaceae*. Physiol. Plant. 61: 38-44.

KAO K.N., MICHAYLUK M.R., 1975. Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126: 105-110.

KIRTI P.B., 1988. Somatic embryogenesis in hypocotyl protoplast culture of rapeseed (*Brassica napus* L.). Plant Breeding 100: 222-224.

KLIMASZEWSKA K., KELLER W.A., 1987. Plant regeneration from stem cortex protoplasts of *Brassica napus*. Plant Cell Tissue and Organ Culture 8: 225-233.

LI L., KOCHLENBACH H.W., 1982. Somatic embryogenesis in quite a direct way in cultures of mesophyll protoplasts of *Brassica napus* L. Plant Cell Reports 1: 209-211.

LU D.Y., PENTAL D., COCKING E.C., 1982. Plant regeneration from seedling cotyledon protoplasts. Z. Pflanzenphysiol. 107: 59-63.

MENCZEL L., NAGY I., KISS Z.R., MALIGA P., 1981. Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids. Theor. Appl. Genet. 59: 191-195.

MILLAM S.R.,BURNS A.T.H., HOCKING T.J., CATTELL K.J.,1988. Effects of a Ficoll-agarose system on early division and development of mesophyll protoplasts of a winter oilseed rape (*Brassica napus* L.). Plant Cell, Tissue and Organ Culture 12: 285-290.

MURASHIGE T., SKOOG F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15: 473-497.

NAGY J.J., MALIGA P., 1976. Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. Z. Pflanzenphysiol. 78: 453-455.

SPANGENBERG G., KOOP H.-U., LICHTER R., SCHWEIGER H.G., 1986. Microculture of single protoplasts of *Brassica napus*. Physiol. Plant. 66: 1-8.

XU Z.H., DAVEY M.R., COCKING E.C.,1982. Plant regeneration from root protoplasts of *Brassica*. Plant Sci. Lett. 24: 117-121.

REGENERACJA ROŚLIN Z PROTOPLASTÓW IZOLOWANYCH Z HIPOKOTYLI RZEPAKU OZIMEGO (*BRASSICA NAPUS* L.)

STRESZCZENIE

Protoplasty izolowano z hipokotyli sześciu linii hodowlanych i dwóch odmian rzepaku ozimego (*Brassica napus* L.). W przedstawionych warunkach kultury obserwowano wysoką zdolność do regeneracji ścian, podziałów komórkowych i tworzenia kolonii. Przebieg pierwszego etapu kultury był podobny we wszystkich badanych genotypach. Regeneracja pędów zależała od genotypu i najwyższa była u cv Bolko (3.6%) na pożywce zawierającej IAA (0.1 $mg.dm^{-3}$), zeatynę (0.5 $mg.dm^{-3}$) i BAP (0.5 $mg.dm^{-3}$). Wszystkie zregenerowane pędy ukorzeniano na pożywce podstawowej MS z dodatkiem sacharozy 30 $g.dm^{-3}$.

SŁOWA KLUCZOWE: protoplasty, regeneracja roślin, rzepak ozimy, *Brassica napus* L.