PROTOPLAST ISOLATION AND FUSION BETWEEN *BRASSICA CARINATA* BRAUN. AND *BRASSICA RAPA* L.

BERÁNEK M., BECHYNĚ M., KLÍMA M.

Abstract

Brassica carinata mesophyll and B. rapa hypocotyl protoplasts were used for fusing experiments using polyethylene glycol (PEG). Treatment with 30% PEG 6000 MW for 10 minutes was more effective, while combinations with 30% PEG 6000 MW/15 min, 30% PEG 4000 MW/15 min and 30% PEG 4000 MW for 10 minutes reduced viability of protoplasts and cell wall regeneration, cell division and production of microcolonies. Fusion frequency was about 25% with the treatment of 30% PEG 6000 MW/10 min. Cell colonies forming callus was observed after 14 days of cultivation on liquid B medium with addition of liquid C medium. Microcalli were subsequently transferred to solid E and F medium. Shoot regeneration from calli was induced via indirect organogenesis and somatic embryogenesis on MS hormon free medium. In total 58 calli were obtained from fusions between B. rapa 31/96 and B. carinata line 1; 14 calli produced shoots. According to the morfological diferencies half of shoots were heterocaryonicals. This observation approved the events from Flow cytometry. Approximately 40% of analyzed samples were only B. carinata plant type established via protoplasts fusion, 60% were of B. carinata and B. rapa.

Key words: PEG, protoplast, Brassica, fusion
Abbreviations: PEG, Polyethylene glycol; MS, Murashige and Skoog (1962)

INTRODUCTION

Genus *Brassica* is the most economically important one within the family *Brassicaceae* (syn. *Cruciferae*). Many species and types of Brassicas are fundamental oilseed crops, vegetables, forage crops, and are used for the production of spices, such as mustard. Among the *Brassica* crops, oilseeds have the highest economic value. *B. carinata* Br (2n = 17 BBCC), also known as Abyssinian mustard (Bajaj, Mohapatra, 1987) is a natural amphidiploid (Narasinhulu, Kirti, et al. 1992, Jourdan, Salazar, et al., 1992) with significant fungal resistance (Choudhari, Joshi, Ramarao, 2000) water stress (Narasinhulu, Kirti, 1992) and aphid attack tolerance (Yang, Jia, et al. 1990, Babic, Datla, et al., 1998). *B. carinata* plays an important role as an oilseed crop especially in tropical and subtropical regions but the quality of oil is lower in comparison with *B. rapa L*.

Protoplast fusion allows the creation of hybrid and cybrid combinations of species that are sexually incompatible, thus facilitating the transfer of genes from a related, but sexually incompatible species, to another one without genetic transformation. This technology has allowed not only intrageneric hybridizations, but the production of intergeneric hybrids and cybrids as well. Various desirable traits from the parents have been transferred to the hybrids and cybrids using this technology. In this study new pathway for breeding of *B. carinata* and *B. rapa* species was founded. Protoplast fusion was used as an effective tool for breeding brassicas. The target was to obtain optimal methods *in vitro* for breeding Brassicas. Considerable progress was accomplished in the cellular and molecular biology of Brassica species in the past few years. Plant regeneration has been increasingly optimized via organogenesis and somatic embryogenesis by means of various explants; with tissue culture improvements focused on factors such as age of the explant, genotype, and media additives. Somatic cell fusion has facilitated the development of interspecific and intergeneric hybrids in the sexually incompatible species of Brassica (Cardoza, Stewart, 2004).The aim of this study was to verify and optimize current methods for establishing protoplast culture in selected genotypes of genus *Brassica*. Consequently, fusion experiments by means of PEG solution between *B. rapa* and *B. carinata* have been made to obtain new genotype combinations for subsequent breeding processes.

MATERIALS AND METHODS

Four genotypes of *B. carinata* (lines 1, 2, 3, and yellow-seeded line 15H2901003) and nine genotypes of *B. rapa* (cultivars Reward, Tobin, Eldorado, Candle and lines P3/86, 33/96, 31/95, 31/96 and line 6 with quadruple pods) from the Czech National Gene Bank in the Crop Research Institute and yellow seeds from the Czech University of Agriculture Prague, Institute of Tropics and Subtropics were used as the donor plants for protoplast culture.

Donor plants

Seeds were surface sterilized in 70% ethanol (1 minute) and then in 30% NaOCl (commercial bleach SAVO) for 20 minutes. Then were rinsed three times in sterile distilled water and sown on MS medium without growth regulators (Hu, Andersen, Hansen, 1999). Plant material for mesophyll protoplasts was cultivated in a cultivation room under controlled environmental conditions (25^{0}C and 16/8h day/night photoperiod). Seedlings for etioled hypocotyl protoplasts were incubated in a thermostat at 25^{0}C in the dark.

Protoplast isolation and cultivation

Approx. 25 young leaves from 28 days old plant and 30 etiolated hypocotyls from 7 days old seedlings were segmented to 2mm slices and placed separately to 60mm plastic Petri dishes with 5 ml of an enzymatic solution (mixture of 1% Cellulase Onozuka R-10 and 0,25% Macerozyme in W5 medium (Mukhopadhyay, Arumugan, Pradhan, Murthy, Yadav, Sodhi, Pental, 1994), put into the thermostat and incubated at 25^0C for 18 hours in the dark.

Protoplast suspension was purified via repeated (three times) centrifugation in W5 medium (5 minutes at 100 G), supernatant removal, rewashing and subsequent centrifugation on sucrose gradient (Pelletier et al., 1993).

Protoplast fusion

Purified protoplasts were resuspended in 0,1 – 0,5 ml M+C medium, mesophyll and hypocotyl protoplasts were then mixed in the ratio of 1:1 and the density was adjusted to 10^5 – 10^6 protoplasts per 1 ml. 4 x 50 µl (4 drops) of protoplast suspension was added to the 35mm Petri dish and sedimented for 20 minutes. 50 µl of PEG 6000 or 4000 MW (Molecular weight) was gently added to each drop of sedimented protoplasts and incubated for 10 or 15 min. Then the PEG 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 B liquid cultivation medium. Petri dishes with protoplasts were transferred to the thermostat and cultivated at 25^0C in the dark for 3 days then cultivated according to Pelletier et al. (1993).

Plant regeneration

Cell wall was established spontaneously 7 days after protoplasts fusion according to microscope observation. Cell microcolonies were produced on liquid B medium with addition of liquid C medium after 14 days. Well developed microcalli were then transferred onto solid E medium. After 14 days of cultivation calli were placed onto solid F medium. Shoots, regenerated from calli via indirect organogenesis and somatic embryogenesis on F medium, were transplanted onto rooting MS hormone-free medium and were cultivated in a cultivation room under controlled environmental conditions (25^0C and 16/8h day/night photoperiod). Plantlets with well developed roots were transferred to *in vivo*.

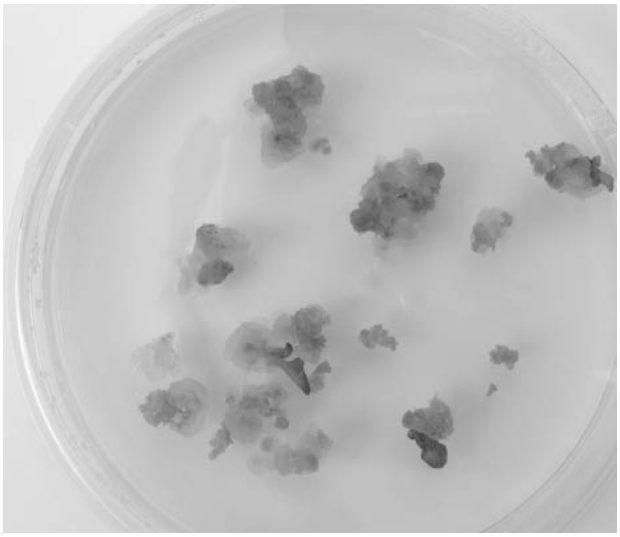


Fig. 1– callus culture and regeneration of *B. rapa* 31/96 and *B. carinata* line 1

Plant selection

Flow cytometry procedure using Otto I+II buffers

1. Fluorochrome (DAPI, 4 µg/ml or propidium iodide + RNase, both 50 µg/ml) were added to the selected buffer; ß-mercaptoethanol (2 µl/ml) might be used to avoid polyphenolics oxidation.

   (DAPI-staining: 25 ml Otto II buffer + 1 ml DAPI stock solution + 50 µl ß-mercaptoethanol; Propidium iodide-staining: 20 ml Otto II buffer + 1 ml PI stock solution + 1 ml RNase stock solution + 40 µl ß-mercaptoethanol).

2. Young intact leaf tissues (typically 1 cm^2) were chopped with a new razor blade in a Petri dish containing 1 ml of ice-cold Otto I buffer.

3. Filter the suspension through a 42 µm nylon mesh.

4. Analyse relative DNA content of isolated nuclei (Doležel, Binárová, Lucretti, 1989, Matzk, Meister, Schubert, 2000, Pfosser, Amon, Lelley, Heberle-Bors, 1985).

Otto Buffer I content in 200 ml solution: 4.2 g of 0.1 M citric acid monohydrate and 1 ml of 0.5% (v/v) Tween 20. Otto Buffer II content in 200 ml solution: 28.65 g of 0.4M Na_2HPO_4 .$12H_2O$ (Otto, 1990).

RESULTS AND DISCUSSION

Activity of PEG 6000 MW/10 min. *Brassica carinata.*

Genotype	Cell division	Microcolonies	Callus	Regeneration via indirect organogenesis	Regeneration via somatic embryogenesis
15H2901003	+	+	-	-	-
Line 1	+	+	+	+	+
Line 2	-	-	-	-	-
Line 3	+	+	+	-	-

+ = positive result
- = negative result

Activity of PEG 6000 MW/10 min. *Brassica rapa* subsp. *oleifera* f. *praecox*, f. *biennis.*

Genotype	Cell division	Microcolonies	Callus	Regeneration via indirect organogenesis	Regeneration via somatic embryogenesis
Reward	+	-	-	-	-
Tobin	+	+	+	-	-
Eldorado	+	-	-	-	-
Candle	+	+	+	-	-
P3/86	+	-	-	-	-
Line 33/96	+	+	-	-	-
Line 31/95	-	-	-	-	-
Line 31/96	+	+	+	+	+
Line 6	-	-	-	-	-

+ = positive result
- = negative result
Other results (activity of different MW of PEG and time of activity) are described bellow.

The development of protoplast technology for *B. rapa* and *B. carinata* has permitted the production of somatic hybrids between these cruciferous species and the formation novel cybrids and hybrids (Jaiswal, Hammat, Bhojwani, Cocking, Davey, 1990). 58 calli and 14 calli produced shoots by protoplasts fusion between *B. rapa* 31/96 and *B. carinata* line 1. Fusion frequency was about 25%. The differentiation process and shoot regeneration took 3-4 months; similar results were published before (Fahleson, Eriksson, Landgren, Stymmne, Glimelius, 1994b, Gerdemann-Knörck, Nielen, Tzscheetzsch, Iglisch, Schieder, 1995). Fusion and regeneration frequencies were almost the same as those obtained in hybridization experiments between more closely related species (Fahleson, Eriksson, Glimelius, 1993). The three parameters of protoplasts viability, yield and freqeuncy of cell division were used for arriving at the optimum MW of PEG and time of activity. This is more important for identification of suitable MW and time of activity influencing yield and viable protoplasts, for plant breeding Brassicas especially *B. carinata* Br. and *B. rapa* L. It is optimal for protoplast fusion, using 30% PEG 6000 MW per 10 min. 30% PEG 6000 MW/10 min solution was more effective, while combinations with 30% PEG 6000 MW/15 min, 30% PEG 4000 MW/15 min and 30% PEG 4000 MW for 10 minutes reduced viability of protoplasts and cell wall regeneration, cell division and production of microcolonies. These results conform to previously published work (Kirti, Prakash and Chopra, 1991, Kirti, Narasimhulu, Prakash and Chopra, 1992 a, b, Narasinhulu, Parkash, Chopra 1992). According to the morfological diferencies, one half of shoots were heterocaryonicals. One part of regenerated plantlets remined plants of *B. carinata* (from the aspect of size and form of leaves) and second part did not reminde *B. carinata* nor *B. rapa*. This observation approved the events from Flow cytometry. The obtained details proved that approximately 40% analyzed samples were only *B. carinata* plant established by via fusion of

protoplasts within this species and 60% of plantlets established via protoplast fusion between *B. carinata* and *B. rapa*. Flow cytometry analyses data are shown in Fig. 2, 3 and 4.Current results confirm that the successful and efficient utilization of tissue culture technique depends not only on species but even on variety (Power, Chapman, Wilson, 1985, Moreno-Ferrero, Nuez-Viňals, 1985, Li, Stoutjestijk, Larkin, 1999, Jain, Chowdhury, et al. 1988). According to our results, both *B. rapa* - 31/96 and *B. carinata* line 1 are suitable genotypes for further experiments in protoplast fusion. Successful plant regeneration from other varieties of *B. carinata* and *B. rapa* was not gained.

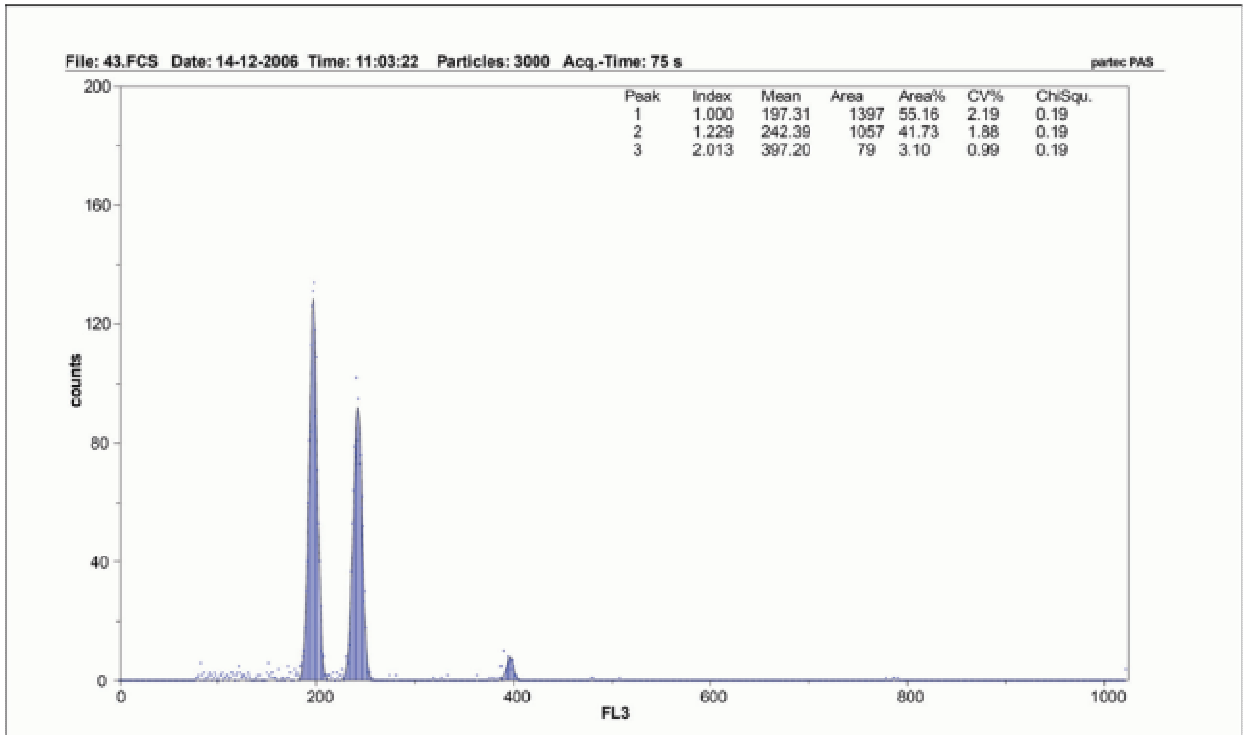


Fig. 2. : Histogam of Flow cytometry of *Brassica carinata* genotyp 1

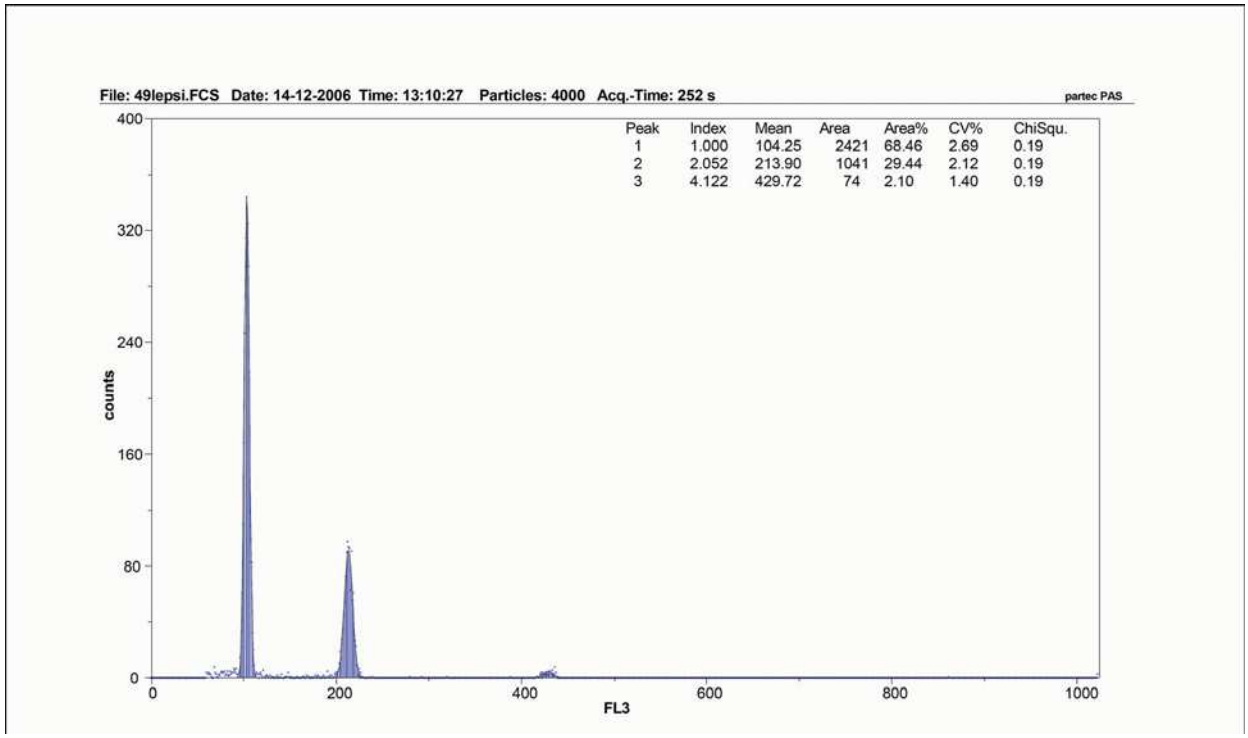


Fig. 3. : Histogam of Flow cytometry of *Brassica rapa* subsp. *oleifera* f. *praecox* 31/96

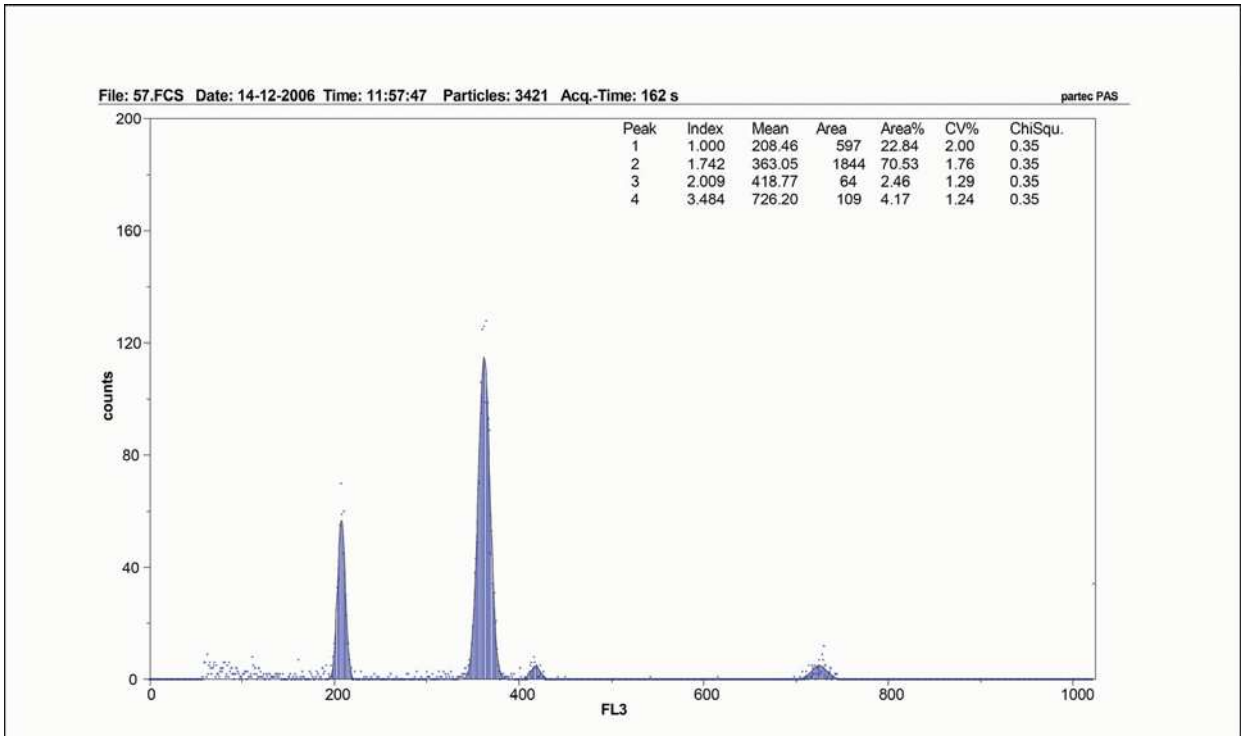


Fig. 4. : Histogam of Flow cytometry of *Brassica rapa* subsp. *oleifera* f. *praecox* 31/96 x *Brassica carinata* genotyp 1

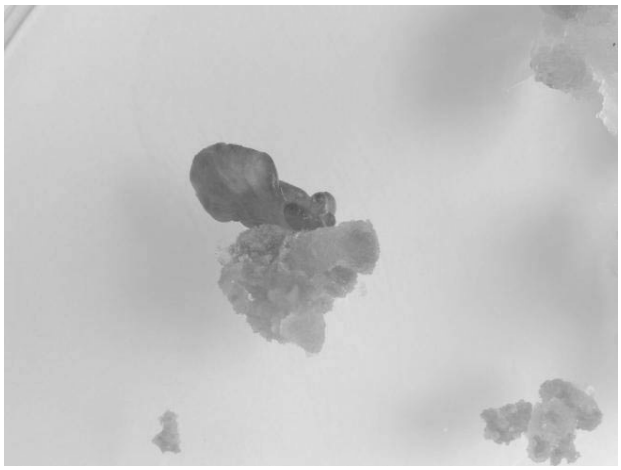
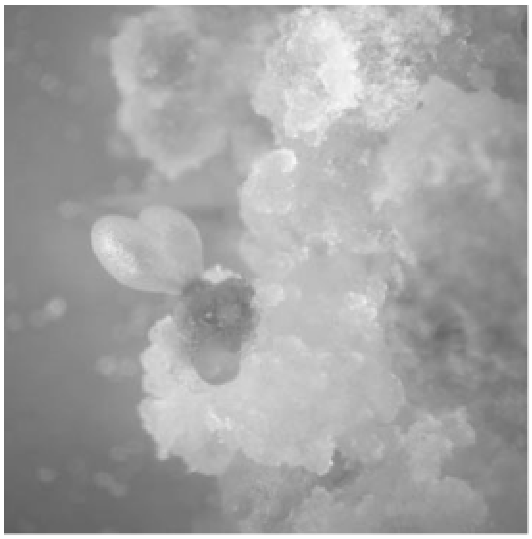


Fig. 5. : Regeneration genotypes B. r. 31/96 and B. c. 1



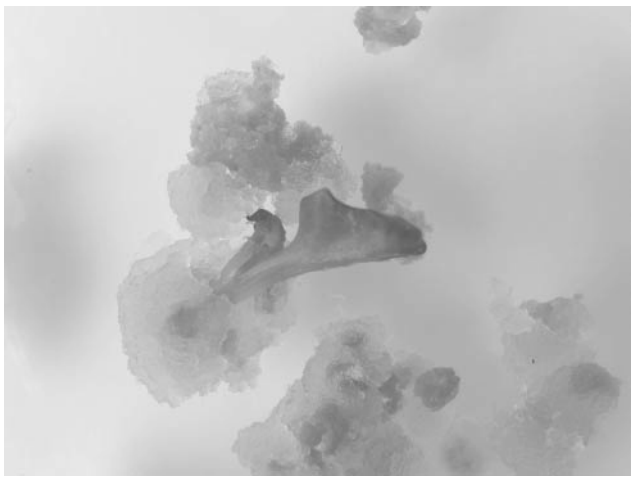


Fig. 6. : Regeneration genotypes B. r. 31/96 and B. c. 1 via indirect organogenesis

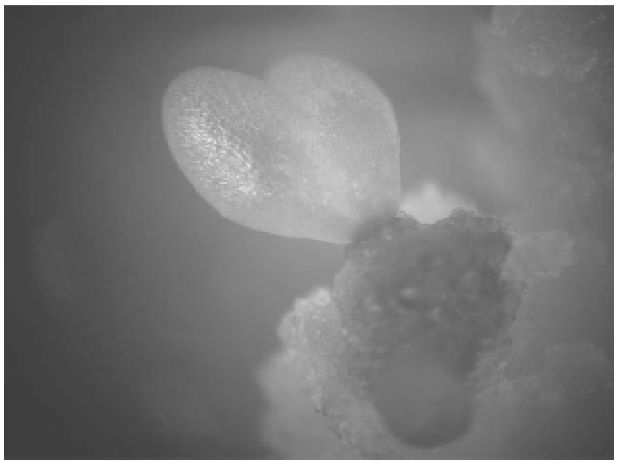


Fig. 7, 8. : Regeneration genotypes B. r. 31/96 and B. c. 1 via somatic embryogenesis

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Corresponding author:

Ing. Beránek M.
Czech University of Agriculture Prague,
Institute of Tropics and Subtropics,
Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic
martin.beranek@seznam.cz