INDIVIDUAL SELECTION, CULTURE AND ELECTROFUSION OF DEFINED PAIRS OF PROTOPLASTS AND REGENERATION TO PLANTS OF BRASSICA NAPUS

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Introduction

Individual cell culture represents a powerful tool for studies on the physiology of different cell types, analysis of differentiation programs, cell to cell interactions and genetic manipulations of plant cells. By individually culturing and manipulating defined cells it is possible to overcome the problems arising from the heterogeneity of mass cultures which represent populations of protoplasts and cells with different functional, developmental and cell cycle stages.

Microculture of Brassica napus protoplasts

The microculture system, developed for Nicotiana tabacum protoplasts (Koop and Schweiger, 1985a) was further automatized (Spangenberg, 1986; Schweiger et al., 1987) and adapted to the individual culture of rapeseed protoplasts in microdroplets of fully synthetic and unconditioned medium (Spangenberg et al., 1986). Individually selected protoplasts isolated from hypocotyls and petioles of rapeseed cultured in microdroplets of 30-100 nl modified Kao medium were capable of dividing and forming cell colonies with a morphology similar to those obtained by mass culture.

The frequency of viable hypocotyl protoplasts was up to 80% after 8 days in microculture and the frequency of division increased to 60% or more by that time.

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Microcallus formation of hypocotyl protoplasts was routinely observed in 30 to 40% of the individually cultured cells and reached values up to 50%. Similar results were observed for two different cell types selected from populations of petiole protoplasts of B. napus.

Tab. 1 Microculture of hypocotyl and petiole protoplasts

Protoplasts	number	day
individually selected	100	0
survivors	80	8
single division	60	8
multiple division	15	8
microcalli transferred onto solid medium	2	60-90
calli exhibiting morphogenetic activity	1	150-260

The microculture system proved useful for the analysis of cell to cell interactions at the single cell level between different protoplast types of B. napus (Spangenberg et al., 1985). Two small, unvacuolated protoplasts, when co-cultured, do not affect each other. No stimulation of growth could either be detected when a large, highly vacuolated protoplast was co-cultured with another large one. The situation, however, was completely different when a large and a small protoplast were co-cultured in a microdroplet. Under these conditions the readiness to divide of the small cell was increased at least by a factor of three.

Electrofusion of defined pairs of protoplasts and subprotoplasts of Brassica napus

Among the different techniques suitable for manipulating cells of higher plants, fusion plays an important role. Hereby it is of particular interest to fuse defined types and numbers of cells under controlled conditions (Koop et al., 1983; Koop and Schweiger, 1985b; Spangenberg and Schweiger, 1986).

Different combinations of subprotoplasts and protoplast types of B. napus were electrofused resulting in cell reconstitution (karyoplast-cytoplast fusion), nuclear transplantation (karyoplast-protoplast fusion) and organelle transfer (cytoplast-protoplast fusion). Electric field induced fusion of such subcellular fragments and protoplasts was achieved with a relatively high frequency (Tab. 2) compared with efficiencies of fusions performed at the populational level. The efficiency of electric field induced fusion of hypocotyl protoplasts was routinely in the range of 30-45% and exceptionally up to 75%.

Tab. 2 Electrofusion of defined pairs of individually selected protoplasts and subprotoplasts isolated from hypocotyls of B. napus

Fusion partners		
a	b	yield (%)
protoplast	protoplast	45 ± 18
karyoplast	protoplast	32 ± 9
cytoplast	protoplast	34 ± 3
karyoplast	cytoplast	30 ± 11

About 70% of fusion products from selected pairs of different protoplast types survived the manipulation step itself and the transfer from the electrofusion to the microculture chamber. More than 30% of the microcultured fusion products had divided at least once by the fourth day of culture. Routinely 20-30% and up to 50% of the microcultured fusion products formed microcalli consisting of 40-50 cells each. No significant differences in behavior during microculture were found between fusion products from different protoplast types, subprotoplast-protoplast fusions and reconstituted cells by fusion of a karyoplast and a cytoplast compared with control unfused hypocotyl and petiole protoplasts. After about 30 days of culture cell colonies of 0.2-0.5 mm in diameter were transferred to 1 µl wells and 500 nl culture media were added every week until calli grew to 1 mm diameter. Calli were transferred then onto 10 µl solidified medium. The transfer from liquid to solidified medium proved to be a critical step in the individual culture of rapeseed protoplasts until callus formation. As it could be shown for microcultured petiole protoplasts, a successful transfer of the obtained calli onto solidified medium was facilitated by the culture on liquid medium of the calli up to a size of 3-4 mm in diameter. Besides this, the growth rate of microcalli was higher when these were grown in microsuspension on a rotary shaker than on solidified medium. After about 150 days in culture calli were individually transferred onto 1 ml regeneration medium into Costar 24 well dishes for induction of morphogenesis.

The morphogenetic potential of 10 callus lines derived from individually cultured hypocotyl and petiole protoplasts of (Raphanus sativus) alloplasmatic cms B. napus, 6 callus lines from (B. campestris atrazine resistant biotype) alloplasmatic B.napus as well as 11 hybrid callus lines from different fusion products (5 derived from proto-

plast-protoplast, 2 from karyoplast-protoplast, 3 from cytoplast-protoplast fusions and 1 from cell reconstitution was analysed.

Root formation was observed in a total of 14 callus lines while shooting only occurred in 4 callus lines including one hybrid cell clone.

Rooting and further development of the shoots was achieved by transferring them onto MS medium containing 2.0 mg/l BA and various concentrations of NAA (1-40 mg/l) and activated charcoal (1 g/l). MS medium containing 2.0 mg/l BA and 0.1 mg/l NAA resulted in shoot regeneration rate of 3% of calli derived from mesophyll and petiole protoplasts. In this respect some differences could be observed depending on the genotype. The optimization of the shooting and rooting medium was performed with calli derived from mass-cultured protoplasts. The optimal conditions could be successfully applied to calli derived from individually microcultured protoplasts and fusion products. Data on the analysis and further characterization of the established cell clones and regenerated plants will be presented elsewhere (Spangenberg et al., in preparation).

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