A simple, versatile feeder layer system for *Brassica oleracea* protoplast culture

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**Abstract.** Protoplasts from cauliflower (*Brassica oleracea* ssp. *botrytis*) and broccoli (ssp. *italica*) leaves and hypocotyls were successfully cultured on membrane filters over a feeder layer of cells from a *B. campestris* suspension culture. Cells from rice, tomato and tobacco suspensions were not as effective as the *B. campestris* cells. Plants were recovered from protoplasts of previously recalcitrant *Brassica* genotypes. Protoplasts cultured in low numbers (10-100) on the feeder layer divided and formed colonies capable of plant regeneration, as did fused protoplasts.

**Abbreviations:** BA: 6-benzylaminopurine; 2,4-D: 2,4-dichlorophenoxyacetic acid; IAA: indole-3-acetic acid; NAA: naphthaleneacetic acid; PCV: packed cell volume

**Introduction**

There are numerous published reports of plant regeneration from *Brassica oleracea* protoplasts. These include descriptions of protoplast regeneration from leaves (Robertson and Earle, 1986; Fu et al., 1985; Nishio et al., 1987; Yamashita and Shimamoto, 1989), cotyledons (Robertson et al., 1988), etiolated hypocotyls (Glimelius, 1984; Yamashita and Shimamoto, 1989; Lillo and Olsen, 1989), roots (Xu et al., 1982) and stems (Pua, 1987). Protoplasts were often cultured in liquid media (e.g. Robertson and Earle, 1986; Glimelius, 1984; Pua, 1987; Lillo and Olsen, 1989), but there are also reports of successful culture within agarose beads (Yamashita and Shimamoto, 1989), in a layer of agarose-solidified medium (Barsby et al., 1986), floating over a ficoll-enriched medium (Chuong et al., 1985; Klimaszewska and Keller, 1987), and in a liquid medium plated over an agarose-solidified one (Klimaszewska and Keller, 1987).

Protoplast culture in *B. oleracea* remains limited by genotypic differences in regenerability (Jourdan and Earle, 1989). In addition, the high protoplast density required for culture (10^5 to 10^6 protoplasts/ml) complicates culture of low numbers of protoplasts, such as microinjected or micromanipulated protoplasts. Sundberg and Glimelius (1986) cultured low numbers of micromanipulated *Brassica* leaf-hypocotyl fusion products (100 protoplasts in 10 µl medium), but recovered very few plants from them. Their culture system worked best for hypocotyl protoplasts, but many seeds are needed to isolate enough hypocotyl protoplasts for most gene transfer or protoplast fusion experiments, increasing the chances of contamination. Spangengberg et al. (1985) describe culture of individual *B. napus* protoplasts in microdroplets, but this method is laborious and requires a long time (150-200 days) to regenerate plants (Schweiger et al., 1987).

Feeder layers have been employed in numerous species to culture protoplasts of recalcitrant genotypes, or to reduce the minimum number of protoplasts needed for culture. The first successful culture and regeneration of maize protoplasts was achieved by plating protoplasts on a membrane filter over a cell suspension feeder layer (Rhoades et al., 1988). A petunia cell suspension feeder layer reduced 100-fold the minimum number for culture of petunia cells and protoplasts (Shneyour et al., 1984). Grape protoplast colony formation was enhanced and the minimum number of protoplasts for culture was reduced by using a feeder layer of grape suspension cells (Yamakawa et al., 1985).

This paper describes a simple, versatile method for culturing broccoli and cauliflower leaf and hypocotyl protoplasts using a *B. campestris* cell suspension as a feeder layer. We have successfully used this method to recover plants from previously recalcitrant genotypes, and to culture low numbers of protoplasts and fusion products.

**Materials and Methods**

*Plant Materials.* Protoplasts were isolated from cauliflower (*Brassica oleracea* ssp. *botrytis*) and broccoli (ssp. *italica*) lines provided by M. H. Dickson, New York State Agricultural Experiment Station, Geneva NY. NY IR 9941 is an insect-resistant broccoli inbred. NY 7642A is a cauliflower inbred carrying the *oga* cytoplasm from *Raphanus* (Dickson, 1985). NY 3317 is derived from a protoplast regenerant from the fertile maintainer line for NY 7642A (Jourdan et al., 1988). 852007 is derived from a regenerant of a Green Comet hybrid broccoli hypocotyl explant; protoplasts from this line generally regenerate well in the liquid culture system of Robertson and Earle (1986).

*Cell Suspensions.* A cell suspension derived from hypocotyl tissue of rapid-cycling *Brassica campestris* ('RCS'®; Lentini et al., 1986) was
Effect of the Feeder Layer

At 7 day intervals, 5 ml of this suspension was subcultured into 35 ml fresh medium [Linsmaier and Skoog (1965) medium with 3% sucrose, 200 mg/l casein hydrolysate (Sigma C-0626), 1 mg/l NAA, 5 mg/l BA, and 0.1 mg/l 2,4-D] in a 250 ml flask. A suspension derived from Lycopersicon esculentum Mill cv "VFNT cherry" tomato (DuPont et al., 1985) was maintained on medium containing Murashige and Skoog (1962) salts, Nitsch and Nitsch (1969) vitamins, 0.5 mg/l 2,4-D, 5 mg/l IAA, 0.3 mg/l kinetin and 3% sucrose. A rice (Oryza sativa L.) cell suspension from the laboratory of Ray Wu, Cornell University, was maintained in AA medium (Abdullah et al., 1986). A tobacco (Nicotiana tabacum L.) suspension from the laboratory of June Nassallah, Cornell University, was maintained in medium containing Murashige and Skoog (1962) salts, B5 vitamins (Gamborg et al., 1968), 4 mg/l p-chlorophenoxyacetic acid, 5 mg/l kinetin, and 15 g/l sucrose. BCS suspensions were maintained in dim light on a shaker at 150 rpm; other suspensions were maintained at 100 rpm.

Feeder Layer Plates. Feeder layers were prepared by pipetting 1.5 ml of a 9-12 day old suspension onto medium B (protoplast culture medium of Pellietier et al., 1983, without Tween 80, solidified with 2.2 g/l Gelrite (Scott labs)) in 10 cm petri plates. For some experiments, cell density was adjusted before plating: PCV was measured by centrifuging 10 ml suspension for 10 minutes at 100g. The ratio between PCV and total volume was then adjusted to 0.55 (the typical ratio for a 9-12 day old BCS suspension) by adding fresh medium or removing some of the supernatant before re-suspending the cells and plating on medium B. A membrane filter (usually Millipore type AABG mixed cellulose acetate/nitrate membrane; 0.8 μm pore size, black, gridded) was placed over the plated BCS cells.

Protoplast Isolation and Culture. Isolation of protoplasts from leaf and hypocotyl explants was as described by Robertson and Earle (1986). In most experiments, several 100 μl aliquots or one 500 μl aliquot of protoplasts with 5-10 x 10^4 protoplasts/ml (containing 5,000-50,000 protoplasts/aliquot) were plated onto the membrane filter with a wide-mouth pipetting device, taking care to limit the aliquots to the smallest number of membrane grids possible. To culture low numbers of protoplasts, 10 or 100 protoplasts were isolated in approximately 5 μl medium B with a micromanipulator and transferred to membranes on feeder layers.

Plates were cultured at 25°C in the dark for 10-14 days. The membranes and protoplasts were then transferred to fresh feeder layers in plates of Gelrite-solidified medium C (Pellietier et al., 1983), solidified with 2.2 g/l Gelrite or 10 g/l agarose (Type I, Sigma) without feeder layers. Once colonies were 0.5-2 mm in diameter, they were transferred to plates of medium E [Pelletier et al., 1983, without Tween 80, solidified with 2.2 g/l Gelrite or 10 g/l agarose (Type I, Sigma)] without feeder layers. When colonies were visibly to the naked eye (usually 20-30 days after plating), membranes were transferred to plates of medium E [Pellietier et al., 1983, solidified with 2.2 g/l Gelrite or 10 g/l agarose (Type I, Sigma)] without feeder layers. Once colonies were 0.5-2 mm in diameter, they were counted to estimate plating efficiency and were individually transferred to fresh plates of medium E for shoot formation and plant regeneration (Robertson and Earle, 1986). Plating Efficiency (PE) was calculated as 100% x (colonies counted/protoplasts plated).

Alternatively, protoplasts were cultured in liquid medium as described by Robertson and Earle (1986). Protoplasts were plated at 2.5 x 10^4 protoplasts/0.5 ml medium B in wells of a 24-well multiwell plate. Six, 8, and 10 days after isolation, 4 drops of medium C were added to each well. On day 12, 0.5 ml from each well was removed to another well. Three more four-drop aliquots of medium C were added to each well 16, 18, and 20 days after protoplast isolation. The contents of the wells were transferred to plates of medium E when colonies were clearly visible to the naked eye; their further culture was identical to that of colonies recovered with feeder layers.

Protoplast Fusion. Leaf protoplasts of NY 7642A and fluorescein diacetate-stained hypocotyl protoplasts of NY 3317 were fused according to the procedure of Thomzik and Hain (1988). Fusion products were identified by dual red (chlorophyll) and yellow-green (fluorescein diacetate) fluorescence after fusion and resuspension in medium B.

Results

Effect of the Feeder Layer

Tiny bumps on the membrane, about 0.1 mm in diameter, could be seen under the dissecting microscope beginning 5 days after protoplast isolation (Figure 1a, b). These bumps always preceded colony formation and presumably represented protoplasts which elongated and/or began to divide. Small (0.2-0.4 mm) colonies with radically extending "arms" could be seen 14-21 days after isolation. Colonies were easily visible to the naked eye 20-30 days after isolation (Fig 1c, d). No bumps or colonies formed in the absence of the feeder layer (Table 1). The feeder effect was not due to conditioned medium. Colonies grew from broccoli protoplasts plated onto membranes over a BCS feeder layer on medium B but not when the feeder layer was replaced with conditioned medium (Table 1). High feeder cell density was essential. In two experiments, a BCS cell suspension was adjusted to a PCV:volume ratio of 0.55; samples of the suspension were either undiluted or diluted with one or three volumes fresh BCS medium before plating 1.5 ml on medium B. Protoplast colony

Table 1. Effect of Feeder Layer and Conditioned Medium on Protoplast Plating Efficiency^a

<table>
<thead>
<tr>
<th>Condition</th>
<th>Experiment 1^b</th>
<th>Experiment 2^c</th>
</tr>
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<tbody>
<tr>
<td>BCS feeder</td>
<td>0.8 ± 0.2</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td>conditioned medium</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>no feeder</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.2</td>
</tr>
</tbody>
</table>

^a Plating Efficiency = 100 x (# visible colonies/# protoplasts plated). Values are means and standard errors for three-100 μl aliquots (each containing 5000 protoplasts) on each of three plates.
^b leaf protoplasts from broccoli inbred 852007
^c leaf protoplasts from broccoli inbred NY IR 9941
^d 1.5 ml of a 9-12 day old BCS suspension
^e 1.5 ml cell-free supernatant from a 9-12 day old BCS suspension

Fig.1. Brassica protoplast growth on the feeder layer. Bumps forming on the membrane 14 days after isolation of a) leaf and b) hypocotyl protoplasts (2x; width of area shown is 1 mm). c) Colonies on membrane 21 days after protoplast isolation (5x; grid lines are 3 mm apart) d) Feeder plate with membrane and colonies ready for transfer to regeneration medium. e) Plant regenerated from protoplasts of previously recalcitrant broccoli inbred NY IR 9941 (cup diameter is 75 mm).