

I. Protocol for the preparation of metabolically competent protoplasts

Method for isolating large numbers of metabolically competent protoplasts from leaves of monocotyledons (grasses), dicotyledons (such as spinach and sunflower) (Ref. 1 - 3) or from hypocotyl tissue (e.g. Brassica napus) (Ref. 4).

1. Leaf slices of monocots and dicots are prepared by cutting the leaves with a sharp razor blade into segments 0.5 - 1 mm in size. In the case of dicots the epidermis can be scraped off before cutting by rubbing with fine carborundum powder or with a fine nylon brush.
2. Set up 50 ml of digestion medium (for 10 - 15 g of plant tissue) according to the recipe listed below (Solution A).
3. Incubate the leaf slices or pieces in a 19 cm-diameter dish containing the digestion medium for 3 hours at 25 °C, covered with a plastic film. It may be advantageous to replace the digestion medium at intervals of 1 hour, as the enzymes might become inactivated by substances released from broken cells.
4. After completion of the incubation the digestion medium is carefully removed and discarded. It normally contains very few protoplasts. The plant tissue is then washed 3 times by shaking gently with 20 ml wash medium (Solution B).
5. After each wash the tissue is collected by pouring through a tea strainer (0.5 to 1 mm pore size) and the combined washes are then filtered through nylon mesh (100 -200 µm pore size) to remove vascular tissue and undigested material.
6. The protoplasts are collected by centrifugation of the combined filtered washes for 3 minutes at 50 - 100 x g and the supernatant is aspirated and discarded.
7. This crude protoplast preparation also contains some cells and chloroplasts and it is important to purify the protoplasts to remove these contaminants. This can be done with solutions of sucrose and sorbitol of different densities.
8. The protoplast pellet is gently resuspended in 40 ml of Solution C and this suspension is divided among two 100 ml centrifuge tubes.
9. To each tube add slowly 5 ml of Solution D and then overlay this with 5 ml of wash medium (Solution B) to make a three step gradient.
10. Centrifuge at 300 g for 5 minutes.
11. The protoplasts now collect as a band at the interface between the 2 top layers. Carefully remove them with a pasteur pipet.
12. The protoplasts should be examined with a light microscope to ensure that the preparation is free of cells and chloroplasts.
13. When a large portion of the protoplasts is pelleted in this sucrose/sorbitol gradient the density of the 2 layers can be increased by adding 5 - 10 % Dextran (15,000 -20,000 M_r) or 10 - 20 % Ficoll to increase the percentage of the floating protoplasts.
14. The purified protoplasts can be concentrated by diluting with 10 ml of Solution B, centrifuging at 100 g for 3 minutes and then resuspending the pellet in a small amount of medium by gently shaking the tubes.
15. The protoplasts are stable for up to 24 h when stored on ice. Photosynthetic activity of the protoplasts can be determined by measuring co-dependent O₂ evolution with an oxygen electrode, provided rapid stirring is avoided as this will break some of the protoplasts. A suitable medium is listed as Solution E.
16. Protoplasts exhibit a relatively broad pH optimum but at more acidic pH values the bicarbonate concentrations should be lowered.

Solutions for Protocol I:

Solution A (Digestion medium)

Composition:		For 50 ml use:
500 mM	D-Sorbitol	4.56 g
1 mM	CaCl ₂	7.35 mg
5 mM	MES-KOH, pH 5.5	49.00 mg
2 %	Cellulase Onozuka R10	1.00 g
0.3 %	Macerozyme R10	0.15 g

The pH must be adjusted to 5.5 with KOH before adding the enzymes.

Solution B (Wash medium)

Composition		For 100 ml use:
500 mM	D-Sorbitol	9.11 g
1 mM	CaCl ₂	14.70 mg
5 mM	MES-KOH, pH 6.0	98.00 mg

The pH of the solution must be adjusted to 6.0 with KOH.

Solution C

Composition		For 100 ml use:
500 mM	Sucrose	8.56 g
1 mM	CaCl ₂	7.40 mg
5 mM	MES-KOH, pH 6.0	49.00 mg

The pH of the solution must be adjusted to 6.0 by adding KOH.

Solution D

Composition		For 100 ml use:
400 mM	Sucrose	6.80 g
100 mM	D-Sorbitol	0.90 g
1 mM	CaCl ₂	7.40 mg
5 mM	MES-KOH, pH 6.0	49.00 mg

The pH of the solution must be adjusted to 6.0 by adding KOH.

Solution E

Composition		For 100 ml use:
500 mM	D-Sorbitol	9.10 g
1 MM	CaCl ₂	15.00 mg
30 mM	Tricine-KOH	538.00 mg
5 mM	NaHCO ₃	42.0 mg

Reagents:

Calcium chloride dihydrate (Cat.No. 15587)
Cellulase Onozuka R10 (Cat.No. 16419)
Macerozyme R10 (Cat.No. 28302)
MES (Morpholinoethane sulfonic acid)
(Cat.No. 29834)
Sodium bicarbonate (NaHCO₃) (Cat.No. 30180)
D-Sorbitol (Cat.No. 35230)
Sucrose (Cat.No. 35579)
Tricine (N-Tris(hydroxymethyl)methylglycine)
(Cat.No. 37195)

Related Products:

Ficoll® 400 (Cat.No. 21373)
Gelrite® (Cat.No. 22168)
X-Gluc (Cat.No. 15248)

References:

- 1.) S.C. Huber and G.E. Edwards, *Physiol. Plant.* **35**, 203 (1975)
- 2.) S.P. Robinson et al., *Methodol. Surv. Biochem.* **9**, 13 (1979)
- 3.) G.E. Edwards et al., *Plant Physiol.* **62**, 313 (1978)
- 4.) T.L. Barsby et al., *Plant Cell Reports* **5**, 101 (1986)
- 5.) S.P. Robinson, *Methods Enzym.* **148**, 188 (1987)

II. Protocol for the preparation of regeneration competent protoplasts

Method for the isolation of *Brassica napus* protoplasts from mesophyll material derived from one single individual plant (greenhouse grown plants or in vitro cultured shoots) or from young in vitro grown hypocotyls when different individuals are acceptable.

When using leaf material a dark treatment (24 to 48 h) is recommended for achieving an optimal mesophyll protoplast isolation. To obtain sterile hypocotyls, seeds are surface sterilized with 7.5 % calcium hypochlorite for 1 h with continuous shaking, treated with 70 % ethanol for 1 min. and then washed twice in sterile distilled water. The seeds are germinated on MS-medium (Vamling et Glimelius, 1990).

Hypocotyl and mesophyll materials are treated identically for protoplast isolation; in case of modifications for the mesophyll treatment the values are given in brackets.

1. 4 to 5 days (mesophyll 30 days) old Hypocotyls are cut into 0.5 to 1 mm (1 to 2 mm) segments and incubated for 1 h in plasmolyse buffer (Vamling et Glimelius, 1990).
2. After plasmolysing the material is treated with 1 % Cellulase and 0.1 % Macerocyme in the medium K3 according to Nagy et Maliga, (1976), modified with the addition of 0.4 M sucrose. Hypocotyl material is incubated 16 to 18 h at 20 - 22 °C in the dark on a rocking tray with low speed. The mesophyll material is incubated under the same conditions, but without shaking and the time for enzyme treatment can be shortened to 4 - 5 h.
3. The enzyme treated material is filtered through a nylon mesh (50 µm pore size) to separate undegraded material. To the protoplasts containing enzyme solution an equal volume of the salt solution CPW16 (Banks et Evans, 1976) is added.
4. The mixed solutions are centrifuged in a swing-out rotor at 100 x g for 7 min.
5. Protoplasts with intact cell membranes float on the surface and can be removed with a Pasteur pipette.
6. The protoplast suspension is diluted about 10 times in the salt Solution W5 (Menczel et al. 1981).
7. Washing of the protoplasts in the salt Solution W5 is repeated once by flotation and replacing the medium.
8. The protoplasts are finally pelleted by centrifugation in culture medium KM8p, modified (Glimelius et al. 1986) and cultured at a density of 2.5 - 5.0 x 10⁴ /ml in culture medium.

References:

1. Banks M.S. and Evans P.K. (1976) *Plant Sci. Lett.* **7**, 409-416
2. Glimelius K. et al. (1986) *Plant Sci.* **45**, 133 - 144
3. Menczel L. et al. (1981) *Theor. Appl. Genet.* **59**, 191 - 195
4. Vamling K. et Glimelius K. (1990) *Legumes and Oilseed Crops I in Biotechnology in Agriculture and Forestry* **10**, Springer Verlag