

Biotechnology I

Plant cell tissue culture

Method for isolating large numbers of metabolically competent protoplasts from leaves of dicotyledons (cucumber seedlings) (Ref. 1 - 3)

Protoplasts are cells which have had their cell wall removed, usually by digestion with enzymes. Cellulase enzymes digest the cellulose in plant cell walls while pectinase enzymes break down the pectin holding cells together. Once the cell wall has been removed the resulting protoplast is spherical in shape.

1. Prepare Leaf slices by cutting the leaves with a sharp razor blade into segments 0.5 - 1 mm in size.
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.

Protoplasts exhibit a relatively broad pH optimum but at more acidic pH values the bicarbonate concentration should be lowered.

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

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

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)

Report format - This is to be a short report to summarize the work that you and your group have accomplished. This should be **typed up individually**.

- **Title** – Brief title of the experiment
- **Intro** – At least two (long) paragraphs protoplast production and the oxygen electrode..
- **Methods** – A short annotated flow chart of **each** procedure.

- **Results** – Include the data of each of the groups. Don't forget about figure legends and proper labeling of the figures.
- **Discussion** – One or two paragraphs discussing the results shown above. Include the results from each group in your discussion.
- **Conclusion** – what did you determine about protoplast production, *fully explain your findings and relate them back to the structure of the plant cell wall.* **The report should be four pages at the least.**