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Protoplast Isolation, Culture, and Regeneration

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1. Introduction

Protoplasts of *Arabidopsis thaliana* have been isolated from a variety of explant sources with varying degree of success *(1–3)*. Most workers have faced problems in achieving a high frequency of sustainable division of protoplasts in liquid culture. The problem can be alleviated to a certain extent by embedding mesophyll protoplasts in calcium alginate *(4)*. We have developed a technique allowing the culture of protoplasts derived from roots or cell suspensions in liquid medium and their regeneration to plantlets *(2)*. The yield of root-derived protoplasts capable of division and regeneration is considerably higher in comparison to protoplasts obtained from leaf mesophyll cells. The protoplast isolation requires a ready supply of root cultures maintained in auxin containing medium. Alternatively, a high yield of protoplasts, suitable for transient gene expression studies using direct DNA transformation, can also be obtained from cell suspension cultures, excluding any limitation of available starting material. Because the methods may have to be tailored to the needs of different experiments, here we describe protocols for the isolation and culture of protoplasts from leaf mesophyll tissue *(4)*, as well as from root and cell suspension cultures. These methods are equally effective for the *Arabidopsis* ecotypes Columbia, C24, RLD, and Wassiljewskija.

2. Materials

2.1. Materials and Equipment

1. Dry seeds of *Arabidopsis*.
2. Seed sterilizing solution: 10% sodium hypochlorite containing 0.1% Triton X–100.
3. Sterilized double-distilled water.

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4. 250 mL Sterile Erlenmeyer flasks.
5. 0.22 μm Filters for sterilization of solutions.
6. Clinical centrifuge (500*g*).
7. Scissors and razor blades.
8. Pair of forceps.
9. 25-, 50-, and 100-μm Sieves.
10. 12 mL Screw-capped glass centrifuge tubes.
11. 10 and 25 mL Sterile pipetes.
12. Pipet pump.
13. Pasteur pipets with cotton plugs and teats.
14. Hemocytometer.
15. Inverted microscope.
16. 55 and 90 mm Petri dishes.

All glassware should be sterilized prior to use and all operations are carried out using a sterile bench.

2.2. Media and Solutions

All media and solutions are adjusted to pH 5.8 with 1*M* KOH or 1*M* HCl. Protoplast medium (PM), and the enzyme solution are filter sterilized. Growth regulator stocks (1 mg/mL) are filter sterilized and added separately to sterilized media.

1. Basal medium (BM): MS medium *(5)* containing B5 vitamins *(6)* with and without gelling agents (0.8% agar or 0.2% gelrite), and 3% sucrose if not stated otherwise.
2. 0.5 BM medium: consisting of half concentration of MS macroelements *(5)*, B5 vitamin, and 3% sucrose with and without gelling agents (0.8% agar or 0.2% gelrite).
3. MSAR I medium *(7)*: BM medium containing 2.0 mg/L indole–3–acetic acid (IAA), 0.5 mg/L 2,4–dichloro–phenoxyacetic acid (2,4–D), 0.5 mg/L 6–(γ,γ–dimethylallylamino)purine riboside (IPAR).
4. MSAR II medium *(7)*: BM medium containing 2.0 mg/L IPAR, 0.05 mg/L a–naphtaleneacetic acid (NAA) with 0.2% gelrite.
5. MSAR III medium *(7)*: BM medium containing 1.0 mg/L (IAA), 0.2 mg/L indole–3–butyric acid (IBA), 0.2 mg/L 6–furfurylaminopurine (kinetin), and 0.2% gelrite.
6. Protoplast medium (PM): 0.5X BM medium containing MSAR I hormones with 0.45*M* sucrose or 0.45*M* mannitol.
7. Enzyme solution: 1.0% cellulase (Onozuka R–10; Serva, Heidelberg, Germany), 0.25% macerozyme (R10; Serva) dissolved in PM medium.
8. 0.45*M* Mannitol and 0.45*M* sucrose solutions.
9. Sodium alginate solution: 1% (w/v) solution in BM medium containing 0.45*M* sucrose.
10. Calcium agar plates: 20 m*M* calcium chloride, 0.45*M* sucrose, and 1% agar.

3. Methods

3.1. Isolation and Culture of Protoplasts

3.1.1. Leaf Mesophyll Protoplasts

1. Sterilize *Arabidopsis* seeds in an Eppendorf centrifuge tube for 15 min using 1 mL of seed sterilizing solution. Wash the seeds five times with sterile water (1 mL) and dry them by placing the open tubes in the sterile bench. Plate the seeds for germination on 0.5 BM agar plates.
2. To grow plants for mesophyll protoplast isolation, transfer 5–8 7-d-old seedlings into 250-mL jars containing BM medium and culture them at 25°C using 16 h light and 8 h dark period (*see* **Note 1**).
3. Use leaves from 3–5-wk-old plants (*see* **Note 2**).
4. Cut leaves in half through the mid vein and then into 4–6 pieces by cross–sectioning with a sharp blade (*see* **Note 3**).
5. Wash the cut leaves once with 0.45*M* sucrose solution. Transfer the leaf explants into a 9-cm Petri dish and add 15 mL of enzyme solution. Incubate the material for 12–16 h at room temperature in the dark (*see* **Note 4**).
6. Shake the Petri dishes gently to release the protoplasts and incubate them further for 30 min (*see* **Note 5**).
7. Collect the protoplast suspension using a broad-mouthed pipet using gentle suction, and filter the protoplast sequentially through 100 μm and 50 μm sieves (*see* **Note 6**).
8. Gently dispense the protoplast suspension into 12-mL screw-capped glass tubes and centrifuge at 50*g* for 5 min.
9. Remove the dark-green band of floating protoplasts concentrated at the top of the tubes and transfer them to a new glass tube (*see* **Note 7**).
10. Add 10 mL 0.45*M* mannitol solution and centrifuge the protoplasts at 60*g* for 5 min. Resuspend the protoplast pellet and wash the cells twice in a similar fashion with 0.45*M* mannitol solution.
11. Suspend the protoplast pellet obtained after the final wash in 1 mL of sodium alginate solution and count the protoplast yield using a hemocytometer. Adjust the protoplast density to 5–7 × 10^5 cells/mL by adding more alginate solution (*see* **Notes 8** and **9**).
12. Using a wide-bore pipet, take up the protoplasts dispersed in sodium alginate solution and create 250–500-μL drops on the surface of calcium–agar plates (*see* **Note 10**).
13. Allow the alginate to form a gel for 45 min. Transfer the individual droplets with a spatula into 55-mm Petri dishes containing approx 5 mL of PM medium and culture the protoplast in a growth chamber at 25°C under dim light.
14. Remove 2.5 mL of the PM medium and add 2.5 mL of fresh PM medium after 7 and 14 d (*see* **Note 11**).
15. Remove 1 mL of PM medium and add 1 mL of MSAR I medium to the protoplast cultures on d 21, 28, and 35 (*see* **Note 12**).

3.1.2. Root-Derived Protoplasts

1. Sterilize seeds for 15 min using 10% sodium hypochlorite solution and after washing and drying of the seeds, plate them on 0.5 × BM agar plates.
2. In order to obtain root cultures for protoplasts, transfer 15–20 7-d-old seedlings into 50 mL of BM in Erlenmeyer flasks and place the flasks on a rotary shaker at 120 rpm and 25°C using 16 h light and 8 h dark period (*see* **Note 13**).
3. After 10–14 d growth, remove plantlets from the flasks and separate the roots from the green tissue. Using a scalpel, cut the roots into small pieces (approx 2– 4 mm) and transfer the root segments into Petri dishes with MSAR I medium (*see* **Note 14**).
4. Place the Petri dishes on a shaker set at 100 rpm in the dark (or at low light conditions) for 7–12 d (*see* **Note 15**).
5. Remove the liquid medium from the Petri plates and wash the root explants once with 0.45*M* sucrose solution. Remove the washing solution and add approx 20 mL of enzyme solution (*see* **Note 4**).
6. Incubate the root explants for 12–16 h with occasional shaking (*see* **Note 16**).
7. Collect the protoplast with a broad-mouthed pipet using gentle suction and sieve them through 100-, 50-, and 25-µm sieves (*see* **Notes 6** and **17**).
8. Place the protoplast suspension in screw-capped 12 mL glass tubes and centrifuge for 5 min at 50*g*.
9. Collect the band of floating protoplasts concentrated at the top of the solution and transfer them into a new tube (*see* **Notes 7** and **18**).
10. Resuspend the protoplasts in 0.45*M* mannitol solution and pellet the cells at 60*g* for 5 min. Wash the protoplast pellet by repeating this step twice (*see* **Note 19**).
11. If the protoplasts are being cultured in alginate drops, suspend the cells in 1 mL of alginate solution. If the protoplasts are being cultured in liquid medium, resuspend the cells in 1 mL of PM medium.
12. Take 5 µL from the protoplast suspension and count the number of cells in a hemocytometer (*see* **Note 20**).
13. For embedding of the protoplasts, use a wide-bore pipet to take up the cells dispersed in sodium alginate solution (at a density of 3–5 × 10^5 cells/mL) and create 250–500-µL drops on calcium–agar plates (*see* **Notes 10** and **21**).
14. Transfer the drops of alginate gel-carrying protoplasts into 55-mm Petri dishes containing approx 5 mL of PM medium and culture the protoplasts in a growth chamber at 25°C under dim light.
15. For liquid culture, adjust the initial density of protoplasts to 1 × 10^6 cells/mL until cell divisions start and then dilute the protoplast suspension with PM medium to a density of 5 × 10^5 cells/mL (*see* **Note 22**).
16. Remove 2.5 mL of the spent medium and add 2.5 mL of fresh PM medium after 7 and 14 d (*see* **Note 11**).
17. Remove 1 mL of PM medium and add 1 mL of MSAR I medium on d 21, 28, and 35 (*see* **Note 12**).

3.1.3. Protoplasts from Cell Suspensions

1. Methods used for the initiation and maintenance of cell suspensions are described in Chapter 4.
2. Collect 50 mL of 2-d-old cell suspension in a sterile Falcon tube and allow the cells to settle for 5–7 min. Remove the supernatant.
3. Wash the cells once with 0.45*M* sucrose solution and centrifuge them at 50*g* for 5 min.
4. Remove the supernatant and resuspend the pelleted cells in approx 50 mL of protoplasting enzyme solution (*see* **Note 4**).
5. Dispense the cells in three, 90-mm Petri dishes, seal the plates with parafilm, and place them on a shaker at 50 rpm.
6. After 4 h of incubation, collect the protoplast suspension and filter the cells through 50- and 25-µm sieves. Dispense the protoplasts to 12-mL, screw-capped glass tubes (*see* **Note 17**).
7. Centrifuge the protoplasts for 5 min at 50*g*. Collect the band of floating protoplasts from the top of solution (*see* **Notes 7** and **18**).
8. Wash the protoplasts twice with 0.45*M* mannitol solution by centrifuging at 60*g* for 5 min and resuspending the pellet each time (*see* **Note 19**).
9. Resuspend the protoplasts in 1 mL of PM medium or alginate solution (as described in **Subheading 3.1.2.**) and count the cell number in a hemocytometer (*see* **Note 20**).
10. For liquid culture, adjust the density of protoplasts in PM medium to 1 × 10^6 cells/mL. When the protoplasts start dividing, dilute the suspension with PM medium to 5 × 10^5 cells/mL (*see* **Note 22**).
11. For embedding the protoplast into alginate, adjust the cell density to 3– 5 × 10^5 cells/mL and create alginate drops of 250– 500– µL on calcium–agar plates (*see* **Note 10**).
12. After 45 min, remove alginate drops carrying the embedded protoplasts and transfer them into 55-mm Petri dishes containing 5 mL of PM medium. Culture the protoplasts at 25°C under low light conditions (*see* **Note 21**).
13. Remove 2.5 mL of the spent medium and add 2.5 mL of fresh PM medium after 7 and 14 d (*see* **Note 11**).
14. Remove 1 mL of PM medium and add 1 mL of MSAR I medium on d 21, 28, and 35 (*see* **Note 12**).

3.2. Regeneration of Plants from Protoplast-Derived Calli

1. Remove the liquid medium using a pipet, and transfer the microcalli carried by the alginate beads, after sectioning the gel beads into four to five pieces, into MSAR II medium (*see* **Note 23**).
2. Place the Petri dishes in an illuminated culture room (3000 lx) using 16 light and 8 h dark period at 25°C.
3. Pick out green calli and regenerating shoots and transfer them into MSAR II medium until shoots attain a size of approx 2 cm (*see* **Note 24**).

4. Transfer the shoots into MSAR III medium to induce root formation for 3–6 d, then place the plantlets in culture tubes containing 0.5X BM agar medium with only 0.5% sucrose for flowering and seed setting (*see* **Note 25**).

4. Notes

1. Do not place the glass jars close to the light source because condensation inside the jars results in vitrification of the leaves.
2. The age and state of leaves is critical ***(8)***. Do not use pale, discolored, curled, or vitrified leaves. Do not use leaves from plants that are already flowering.
3. In order to decrease the amount of debris in the protoplast preparation, the cut should be clean and the leaf should not be crushed. Allow the weight of the blade and handle alone to do the cutting and change the blade between different dishes.
4. The activity of enzyme preparations may differ from batch to batch and thus the optimal time of incubation may vary. It is advisable to determine the requirements for optimal protoplasting and store a particular enzyme batch in bulk.
5. At this stage of enzyme digestion, the cell suspension should be green in color and numerous spherical protoplasts should be seen floating under the microscope. A dirty preparation will contain many broken protoplasts and may result in low viability of surviving protoplasts.
6. We use steel sieves of different pore size available from Fastnacht (Bonn, Germany), but plastic mesh with the right pore size can be sewn into a cone and put in a glass funnel fitted onto a 100-mL Erlenmeyer flask. The whole assembly can be wrapped by aluminium foil and sterilized by autoclaving.
7. Although the band of floating protoplasts is removed carefully, some protoplasts are invariably lost. An alternative way is to use a thin, sterile, glass capillary tube connected to a pipet pump with a narrow tubing. The capillary is inserted to the bottom of the centrifuge tube, and the debris pellet and the enzyme solution are gently removed without disturbing the band of floating protoplasts.
8. An optimal preparation from leaves of five plants yields about 3–4 × 10^6 protoplasts.
9. Gelrite solution (0.2% dissolved in 0.45*M* sucrose solution) can be used instead of sodium alginate solution in the same fashion. This obviates the need of releasing the microcalli from the embedding matrix at a later stage, which may be necessary when alginate is used as embedding matrix.
10. Larger drops may be prepared and later cut into smaller pieces. However, we find it easier to use small drops since it does not create problems in spreading. Also, individual and uniform drops carrying roughly the same number of protoplasts can be used to test a wide range of culture media and conditions.
11. After 14 d of culture, the dividing cells should form colonies of 4–16 cells. For leaf mesophyll protoplasts, this time may be extended by another 7 d.
12. By day 35, microcalli are visible that may differentiate to roots and somatic embryo–like structures.
13. Roots should be actively growing, white in color, and not yellow–brown or green. The plants grown in the flask should not flower. The quality of root cultures affect the yield of protoplasts and the time required for subsequent cell proliferation.

14. Divide the roots from one 250-mL flask into three portions and, after cutting them into smaller pieces, transfer each portion of roots into approx 20 mL of MSAR I medium in a 90-mm Petri dish. More root material in this volume may delay the cell proliferation response.
15. Root explants exhibit numerous regions of cell proliferation, particularly at the sites of lateral roots, and assume a bulging appearance at this stage. They should be white in color and not brown. The liquid medium at this stage shall contain a few single cells.
16. Protoplasts start to be released after 4 h, and a periodic check is beneficial to reach an optimal yield (1.5–3 × 10^6 cells/mL). Alternatively, digestion of roots with 2.0% cellulase (Onozuka R–10, Serva) releases protoplasts within 3–5 h.
17. A glass Pasteur pipet with the tip broken off serves well since the protoplasts are not subjected to the suction force in the narrow diameter of the pipet tip.
18. Collect the protoplast bands from three tubes into one tube to obtain a sufficient pellet size in the subsequent steps.
19. When changing the solutions, try to remove as much of the previous solution as possible without disturbing the protoplast pellet. The soft transition from one osmoticum to another results in less protoplast bursting and loss between the steps.
20. If the number of protoplasts exceeds 100/square of the hemocytometer, dilute the protoplast suspension.
21. Protoplasts embedded in alginate may be slower in dividing by 1 or 2 d.
22. Depending on the quality of protoplast preparation, up to 75% of protoplasts survive and 20–40% of cells will undergo divisions during the first 3–5 d of culture. In liquid medium, an initial high density may result in aggregation and collapse of protoplasts. Therefore, care should be taken to properly dilute the protoplast suspension. Aggregation of protoplasts may also be observed if there was too much debris and/or dead protoplasts in the preparation, which may be a consequence of either inadequate removal of the enzyme during the washes or sudden osmotic changes.
23. Alternative approaches involve a depolymerization of the alginate gel in 20 m*M* sodium citrate solution, using an appropriate osmoticum ***(4)***, or a removal of visible microcalli from the alginate gel and transferring them in the regeneration medium ***(9)***. We found that the regeneration of calli comprising of more than 64 cells is not hindered by alginate embedding.
24. Care must be taken to remove dead cells from the regenerating cultures.
25. The culture tubes are capped with loose cotton to facilitate a proper aeration necessary for seed setting in vitro. Take care not to place the tubes too close to the light source because this will cause moisture condensation inside the tubes and result in low efficiency of fertilization. Alternatively, rooted plants can be transferred to soil after washing the roots gently with a dilute fungicidal solution (e.g., 0.02% Benomyl, DuPont, Boston, MA). After proper hardening, the plants will flower and set seed.

References

1. Morris, P. C. and Altmann, T. (1994) Tissue culture and transformation, in *Arabidopsis*. (Meyerowitz, E. M. and Somerville, C. R. eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 173–222.

2. Mathur, J., Szabados, L. and Koncz, C. (1995) A simple method for isolation, liquid culture, transformation and regeneration of *Arabidopsis thaliana* protoplasts. *Plant Cell Rep.* **14**, 221–226.
3. Wenck, A. R. and Marton, L. (1995) Large–scale protoplast isolation and regeneration of *Arabidopsis thaliana*. *Bio/Techniques* **18**, 640–643.
4. Damm, B. and Willmitzer, L. (1988) Regeneration of fertile plants from protoplasts of different *Arabidopsis thaliana* genotypes. *Mol. Gen. & Genet.* **213**, 15–20.
5. Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.
6. Gamborg, O. L., Miller, R. A. and Ojima, K. (1968) Nutrient requirements of suspensions cultures of soybean root cells. *Exp. Cell Res.* **50**, 151–158.
7. Koncz, C., Schell, J. and Redei, G.P. (1992) T–DNA transformation and insertional mutagenesis, in *Methods in* Arabidopsis *Research*. (Koncz, C., Chua, N–H., and Schell, J., eds.), World Scientific, Singapore, pp. 224–273.
8. Mason, J. and Paszkowski, J. (1992) The culture response of *Arabidopsis thaliana* protoplasts is determined by the growth conditions of donor plants. *Plant J.* **2**, 829–833.
9. Altmann, T., Damm, B., Halfter, U., Willmitzer L., and Morris, P. C. (1992) Protoplast transformation and methods to create specific mutants in *Arabidopsis thaliana*, in *Methods in* Arabidopsis *Research*. (Koncz, C., Chua, N–H., and Schell, J., eds.), World Scientific, Singapore, pp. 310–330.