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Establishment and Maintenance of Cell Suspension Cultures

Jaideep Mathur and Csaba Koncz

1. Introduction

Cell suspension cultures are rapidly dividing homogenous suspensions of cells grown in liquid nutrient media from which samples can be precisely removed *(1)*. Cell suspensions are used for generating large amounts of cells for quantitative or qualitative analysis of growth responses and metabolism of novel chemicals, as well as for studies of cell cycle under standard conditions *(2,3)*. In addition, cell suspensions serve as an ideal material for the isolation of protoplasts used in transient gene expression assays and *Agrobacterium*–mediated transformation *(4,5)*. The establishment of suspension cultures of *Arabidopsis thaliana* cells derived from leaf and hypocotyl calli has been reported *(6)*. In order to initiate *Arabidopsis* suspensions retaining a high regenerative potential, a procedure described by Ford *(7)* has been modified. Callus tissues derived from root or hypocotyl explants of *Arabidopsis* yield well proliferating cell suspensions capable of morphogenesis in liquid medium. The regeneration capability and genetic stability of suspension cells, however, decrease by the length of culture period. Therefore, it is recommended to use newly initiated cell suspensions when the applications require the regeneration of diploid fertile plants. The method described is used to establish morphogenic cell suspensions from the *Arabidopsis* ecotypes Columbia, C24, RLD, and Wassilewskija. Slight modification of the concentration of growth regulators (e.g., auxin) and/or the time of subcultures may be required for other ecotypes.

2. Materials

1. Dry seeds of *Arabidopsis thaliana.*
2. 10% Sodium hypochlorite solution containing 0.1% Triton X–100.

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3. Eppendorf tubes.
4. Sterilized double-distilled water.
5. Sieves of 850 μm and 500 μm.
6. Sterile pipets.
7. Pair of forceps.
8. Scissors or scalpel blades.
9. Microfuge.
10. Pipet pump.
11. Sterile 250-mL Erlenmeyer flasks with plugs.
12. Rotary shaker set at 120 rpm.
13. Aluminium foil.
14. Suspension culture medium (pH 5.8, 1 L): 4.33 g MS medium (*8*, Sigma, St. Louis, MO), 2X B5 vitamins (*9*, consisting of 2 mg nicotinic acid, 2 mg pyridoxin HCl, 20 mg thiamine–HCl, and 200 mg myo–inositol), 30 g sucrose, 0.5 mg 2,4–dichloro–phenoxyacetic acid (2,4–D), 2.0 mg indole3–acetic acid (IAA), 0.5 mg 6–(γ,γ–methylallylamino)–purine riboside (IPAR, *see* **Note 1**).

3. Methods

3.1. Initiation of Cell Suspensions from root explants

3.1.1. Initiation of Root Cultures

1. Surface sterilize 0.1 g (approx 5000) seeds in Eppendorf tubes by adding 1 mL of 10% (v/v) sodium hypochlorite solution containing 0.1% Triton X–100 as surfactant and shaking for about 15 min (*see* **Note 2**).
2. Collect the seeds by slow centrifugation in a microfuge for a few seconds and remove the supernatant. Wash the seeds five times with 1 mL of sterile water.
3. Germinate the seeds in Petri dishes containing 0.5X MS medium with 0.8% agar using 16 h light to 8 h dark period at 25°C.
4. Place 15–20 1-wk-old seedlings into 250-mL Erlenmeyer flasks containing 35 mL of liquid MS medium with 3% sucrose.
5. Place the flasks on a shaker set for 120 rpm at 25°C under 16 h light to 8 h dark period.
6. Harvest roots after 15–20 d (*see* **Note 3**).

3.1.2. Initiation of Cell Suspensions

1. Place the roots collected from an Erlenmeyer flask into a 9-cm Petri dish and remove all green tissue.
2. Using a scalpel blade or scissors, cut the roots into fine pieces (approx 2 mm long).
3. Remove the rest of liquid medium and resuspend the root explants in approx 50 mL of suspension medium in an Erlenmeyer flask (*see* **Note 4**).
4. Place the flask on the shaker and cover with aluminium foil (*see* **Note 5**).
5. Harvest the cells released from root calli after 21 d; using a 25-mL pipet and pass them through a 850 μm sieve (*see* **Notes 6** and **7**).
6. Allow the cells to settle for about 30 min before gently removing the liquid medium from the top. Add fresh suspension medium (up to 50 mL) and place the

flask back onto the shaker. The roots left in the sieve can be resuspended in fresh medium to generate more cells in suspensions (*see* **Note 6**).

3.2. Initiation of Cell Suspensions from Seed–Derived Calli

1. Sterilize seeds as described in **Subheading 3.1.1.** and place approx 1000 seeds in 50 mL liquid suspension medium in an Erlenmeyer flask (*see* **Note 8**).
2. Place the flask on the shaker and cover with aluminium foil.
3. Examine the cultures at periodic intervals after 21 d by taking out 0.5-mL aliquots from the flasks and observing under a microscope.
4. Once the cultures contain large number of single cells and proliferating cell clumps, sieve the cells sequentially through 850-μm and 500-μm sieves to obtain a homogenous starting material for suspension culture.
5. Allow the cells to settle and then replace the medium as described in **Subheading 3.1.2.** (*see* **Note 6**).

3.3. Maintenance of Cell Suspensions

1. Sieve and subculture the cell suspensions every 7 d until a homogenous proliferating cell population is established (*see* **Notes 9** and **10**).
2. After the fifth subculture, the suspensions can be usually divided into three portions at the time of subculture. To achieve this, approx 100 mL of fresh suspension medium is added to 50-mL cell suspension in the flask. After swirling the flask, 50-mL aliquots are dispensed into new flasks.

4. Notes

1. A 100X stock of B5 vitamin is prepared in distilled water and added to the medium before autoclaving. Growth regulator stocks of 1 mg/mL are filter-sterilized and added to cooled medium. All stocks are stored at 4°C.
2. Do not sterilize too many seeds in one Eppendorf tube because their drying may cause a problem. In case the seeds would clump, use a sterile toothpick to break the clumps before plating the seeds.
3. After 15–20 d approx 3–5 g of roots (fresh weight) should be available in each flask. The growth rate of roots is important for obtaining fine suspensions. The roots should be white, actively growing and not yellow–brown or green.
4. For the *Arabidopsis* RLD ecotype the application of 4.0 mg/L of α–naphtheleneacetic acid instead of other hormones in the suspension medium should also result in fast cell proliferation.
5. A dark incubator may be useful, since we have found considerable differences between the growth rates of cell cultures grown in the dark in comparison to those grown in the light. The slower growth rate in the light may be the result of photodegradation of IAA.
6. Great care has to be taken to ensure aseptic conditions. Flame the neck of flasks well before opening them. Constant swirling of the flask and moving the pipet is necessary to avoid clogging.

7. Occassionally check the cultures for bacterial and/or fungal contamination by plating aliquot of the supernatant on bacterial growth media and/or subculturing a small aliquot of cells in a rich medium containing 1% glucose added to the suspension medium.
8. In the case of certain *Arabidopsis* mutants, the root development may be inhibited, and it is thus difficult to obtain suitable amount of starting root material. By initiating seed derived calli, usually this problem can be successfully overcome.
9. At this stage, usually 3 g (fresh weight) of cell suspension is subcultured in 50 mL of medium and a three to fourfold increase in fresh weight is obtained during a growth period of 7 d. Such cultures reach their exponential phase between d 2.5 and 5.5. A prolongation of culture period for more than 8 d results in rapid browning and cell death.
10. Cultures at this stage are composed of single cells (approx 10–25 µm in length) and clumps of 16–64 cells.

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