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Callus Culture and Regeneration

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1. Introduction

Regeneration of plants by micropropagation of in vitro cultures can be achieved from organ primordia existing in shoot tips and axillary bud explants. Alternatively, plants can be regenerated from unorganized callus tissues derived from different explants by dedifferentiation induced by exogenous growth regulators. Plant regeneration from calli is possible by *de novo* organogenesis or somatic embryogenesis. Callus cultures also facilitate the amplification of limiting plant material. In addition, plant regeneration from calli permits the isolation of rare somaclonal variants which result either from an existing genetic variability in somatic cells or from the induction of mutations, chromosome aberrations, and epigenetic changes by the in vitro applied environmental stimuli, including growth factors added to the cultured cells *(1–3)*.

In *Arabidopsis thaliana,* one of the earliest studies on callus formation was conducted by Loewenberg *(4)*, who grew seedlings in a medium containing kinetin and parachlorophenoxyacetic acid. Subsequent studies (for review, *see* **ref.** *5)* of cell culture and regeneration demonstrated that it is relatively easy to regenerate *Arabidopsis* from callus cultures by many approaches. However, the efficiency of regeneration and the proportion of somaclonal variants are considerably influenced by the ecotype and the source of explants, as well as by the medium and growth regulators employed. Hypocotyl and root explants thus provide excellent materials for callus initiation and regeneration in contrast to stem and leaf explants. The tissue culture protocols include callus induction in auxin containing media. Shoot regeneration is induced by lowering the auxin content and increasing the cytokinin levels in the media. The elongated shoots are usually transferred into root induction media, but even in the absence of roots, flowering and seed setting can take place in test tubes in vitro.

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The method described here utilizes root explants that can be cultured and efficiently regenerated in large quantities. The described technique can be applied equally well for the *Arabidopsis* ecotypes Columbia, C24, RLD, and Wassilewskija. Without changing the general protocol, some slight alterations may be required for other ecotypes or mutants of *Arabidopsis*.

2. Materials

1. Dry seeds of *Arabidopsis thaliana.*
2. 10% Sodium hypochlorite solution containing 0.1% Triton X–100.
3. Eppendorf tubes.
4. Sterilized double-distilled water.
5. Culture tubes.
6. 9 cm Petri dishes.
7. Pair of forceps.
8. Scissors or scalpel blades.
9. Sterile 250-mL Erlenmeyer flasks with plugs.
10. Rotary shaker set at 120 rpm.
11. Microfuge.
12. Basal medium (BM): Murashige and Skoog (MS) medium *(6)* or MS medium for *Arabidopsis* (MSAR) medium *(7)* with 3% sucrose (pH 5.8) can equivalently be used (*see* **Note 1**).
13. 0.5X BM: BM consisting of half concentration of MS macroelements and 0.5% sucrose.
14. MSAR I (Callus medium): Supplement BM with 0.5 mg/L 2,4–dichloro–phenoxyacetic acid (2,4–D), 2.0 mg/L indole–3–acetic acid (IAA), 0.5 mg/L 6–(γ,γ–dimethylallylamino)–purine riboside (IPAR), and gel using either 0.8% agar or 0.2% gelrite (*see* **Note 1**).
15. MSAR II (Shoot medium): Supplement BM with 2.0 mg/L 6–(γ,γ–dimethylallylamino)–purine riboside (IPAR), 0.05 mg/L α–naphtaleneacetic acid (NAA), and either 0.8% agar or 0.2% gelrite.
16. MSAR III (root inducing medium): Supplement BM with 1.0 mg/L IAA, 0.2 mg/L indole–3–butyric acid (IBA), 0.2 mg/L 6–furfuryl–aminopurine (kinetin), and either 0.8% agar or 0.2% gelrite.

3. Methods

1. Surface sterilize 0.1 g (approx 5000) seeds in Eppendorf tubes by adding a 1 mL of 10% (v/v) solution of sodium hypochlorite containing 0.1% Triton X–100 as surfactant and shaking for about 15 min (*see* **Note 2**).
2. Pellet 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 0.8% agar gelled 0.5X BM medium (BM medium containing half concentration of macroelements) using 16 h light and 8 h dark period at 25°C (*see* **Note 3**).
4. Place 15–20 1-wk-old seedlings into 250-mL Erlenmeyer flasks containing approx 35 mL of liquid BM medium with 3% sucrose.

5. Place the flasks on a rotary shaker at 120 rpm using 16 h light and 8 h dark period at 25°C.
6. Harvest the roots of these seedlings after 15–20 d of growth (*see* **Note 4**).
7. Place the roots into a Petri dish, remove all liquid and cut the roots into small pieces.
8. Transfer root explants onto MSAR I plates and either cover them with aluminium foil or place them in low light conditions.
9. After 3 wk, transfer the root-derived calli either to fresh MSAR I plates for maintenance or to MSAR II plates for shoot regeneration.
10. After 2 wk, pick the regenerating shoots from the calli and transfer them into glass jars with MSAR II medium for further elongational growth (*see* **Note 5**).
11. Shoots consisting of 4–6 leaves may be transferred further into MSAR III medium for 3–6 d to induce root development.
12. Transfer the elongated shoots (approx 2 cm) into culture tubes containing 0.5 BM agar medium with 0.5% sucrose for rooting, flowering, and setting seeds (*see* **Notes 6** and **7**).

4. Notes

1. Stocks of growth regulators are prepared at 1 mg/mL concentration, filter sterilized, and stored at 4°C. Growth regulators are added to autoclaved medium after it has cooled to about 60°C.
2. Do not sterilize too many seeds in one Eppendorf tube, as they may not be easily dried. In case seeds clump, use a sterile toothpick to break the clumps before plating the seeds.
3. Calli can also be obtained from germinating seeds that are placed on MSAR I plates after surface sterilization.
4. After 15–20 d of culture, 3–5 g (fresh weight) of roots should be produced in each flask. The roots should be white, actively growing, and not yellow–brown or green.
5. At this stage, it is possible to regenerate and amplify shoots from the regenerated leaves by cutting them into pieces and placing them on MSAR II Medium.
6. The culture tubes are capped with loose cotton to facilitate the aeration required for seed setting and maturation. 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 a low rate of fertilization.
7. Rooted plants can be transferred to soil after washing the roots with water containing a fungicide, such as Benomyl (0.02%). The plants are gradually acclimated by reducing the humidity of transfer chambers stepwise.

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