Moss Methods Sheet

CULTURE PROCEDURES - David Cove

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Temperature

Physcomitrella patens and *Ceratodon purpureus* grow on solid media in temperatures up to about 28°C and possibly somewhat higher in liquid medium. Little difference in growth rate is observed in the temperature range 20°C to 26°C. Temperatures between 24°C and 26°C are used for routine culture. Growth is slower but still satisfactory at 15°C and this has been used as the permissive temperature when temperature-sensitive mutants have been sought. Because of the radiant heat from the light source it will probably be necessary to keep the air temperature below the desired culture temperature. The temperatures given are those of the medium on/in which cultures are grown.

To induce gametangia production in *P. patens*, the temperature must be below 18°C and routinely a temperature of 15°C is used.

For long term culture, lower temperatures are used but light conditions are also critical (see Culture Maintenance, below)

Light

For routine culture the exact quality of light provided is not critical. I routinely use continuous light from fluorescent tubes at an intensity of between 5 and 20 Wm^{-2}. Intermittent light may have developmental effects but in general results only in slower development. The most commonly used light cycle is 16 h light + 8 h dark, usually chosen because of the availability of growth chambers set to this regime. Whichever light cycle you use, be aware that your results may affected.

A short day (8 h) light cycle encourages gametangia production in *Physcomitrella*, but is not essential.

Growth on solid and liquid media

Some Leeds strains contain mutant alleles leading to requirement for vitamins. Media for growing these strains will need to have the appropriate supplements added.

Routine culture on solid BCD medium.

Solid medium in Petri dishes may be inoculated either with spores which can be spread using standard microbiological procedures or with somatic tissue. For routine sub-culture it is best to use protonemal tissue from a vigorously growing culture. Chloronemal tissue, the growth of which is enhanced when ammonium is provided as nitrogen source, is easiest to sub-culture. A fragment of tissue 1 to 2 mm in diameter (containing about 100 to 200 cells) is sufficient to establish a new culture. Tissue other than chloronemata, *e.g.* leaf cells, may take longer to regenerate. For growth in test tubes, it is important not to use a lid which seals the tube tightly.

If contamination is a problem, Petri dishes may be taped with "Micropore" medical plaster strip, without affecting the growth or development of cultures. This is a porous material that slows evaporation from the dish slightly and reduces contamination principally because it limits air exchange during handling. Sealing cultures with Parafilm slows growth of *Physcomitrella* and prevents rapid regeneration, but has less effect of *Ceratodon*.

Growth on solid medium overlayed with cellophane

Protonemal tissue, free of agar, for the isolation of protoplasts or DNA/RNA/protein may be obtained by inoculating protonemal fragments onto cellophane overlaying solid medium in a Petri dish. Petri dishes containing solid BCD medium (+ 1 mM $CaCl_2$) and containing 5mM di-ammonium tartrate, are overlaid with sterilised cellophane discs.

Cellophane disks are best sterilised dry in a glass Petri dish, interleaved with filter papers to prevent sticking.

The discs we use are Type 325P, obtainable from AA Packaging Ltd., Liverpool Road, Walmer Bridge, Preston, Lancashire PR4 5 HY, England. The price of our last order (2003) was about £7.50/1000 for 80 mm discs (but we had to order 50,000 disks). AA Packaging unfortunately insist on payment at the time of ordering.

I avoid serial subculture with its risks of the accumulation of somatic mutations, by using the following regime to obtain tissue for protoplasting (the timings assume growth in continuous light at 25°, and need to be adjusted if other regimes are used:

days before tissue needed	action
20	inoculate 16 protonemal fragments onto solid BCD + ammonium
13	pick off the cultures with a minimum of agar and place in 4 ml sterile distilled water. Blend and inoculate 2ml onto two 90 mm plates of BCD + ammonium overlaid with cellophane. Place one drop on nutrient agar medium to test for contamination.
6	scrape off the tissue from one plate using a sterile spatula and place in 10 ml sterile distilled water. Blend. Inoculate 2ml onto five 90 mm plates of BCD + ammonium overlaid with cellophane. Place one drop on nutrient agar medium to test for contamination.
0	There should be about 500 mg fresh weight of tissue per Petri dish and this will be sufficient to isolate at least 1 million protoplasts.

We prepare suspensions of protonemal fragments by blending protonemal tissue in sterile distilled water. The exact procedure will depend on the type of blender used. We have routinely used small glass blending vessels with stainless steel blender blades designed for the MSE Atomix homogeniser (but this is no longer in production). We have had blending vessels made and can supply them (see resources section). Using these vessels, you need to blend for about 1 min at approximately 12,000 r.p.m. (a small craft drill will do this satisfactorily). More recently, the St. Louis lab has used a PowerGen 125 homogeniser (Fisher Scientific) with 110 mm disposable plastic dispersing tools. These tools are plastic, autoclavable and with care can be used many times. They blend volumes between 1 and 20 ml quickly and regeneration results are good. Other laboratories have used a variety of blenders and most, after fine tuning, give good results

Growth in liquid medium

Culture in liquid medium may be used for the production of large quantities of tissue, but it is usually simpler to obtain intermediate quantities of protonemal tissue using cellophane-overlay plates as described above.
For culture in shaken flasks, a tissue inoculum, prepared as for the inoculation of cellophane-overlay plates, may be used to inoculate liquid BCD medium. Vigorous agitation is not necessary for growth, but growth rates in shaken liquid cultures are not as great as those obtained on Petri dishes or in a fermenter supplied with CO_2 - enriched air.

Fermenter culture can be very successful but the maintenance of fermenter cultures free of contamination is difficult. For further details consult Boyd *et al.*, 1988b.

Culture Maintenance

For routine maintenance of cultures, growth on solid medium is easiest but prolonged growth always generates the risk of somatic mutation. For long term culture maintenance the following alternatives are used:

Cryopreservation (Grimsley & Withers, 1983)

Long term maintenance by cryopreservation is often unsuccessful and so many replicate cultures need to be prepared

Freezing

1. Grow tissue of the strains to be preserved for seven days (25° continuous light) on 90 mm cellophane-overlay plates of BCD medium plus ammonium.
2. transfer the tissue on the cellophane, to fresh BCD medium plus ammonium plus 500 mM mannitol.
3. Pipette 1 ml of liquid BCD plus ammonium plus 500 mM mannitol medium onto the surface of the tissue.
4. Incubate the culture is incubated for a further seven days (25° continuous light).
5. Pipette 2 ml of 5% v/v DMSO + 10% w/v glucose into a sterile plastic vial.
6. Add tissue (about 1/10th of plate per vial).
7. Incubate at 20° for 1 hour.
8. Freeze the vials at a rate of 1°C per minute to _35^{o}C
9. Place into liquid nitrogen for storage.

Thawing

Retrieve vial from liquid nitrogen and place in water bath at 30°C until thawed. Add contents of vial to 10 ml of sterile distilled water. Leave for 30 minute at room temperature. Rescue tissue and inoculate onto appropriate solid.

Stock maintenance in sterile distilled water

A simpler way of preservation that seems to be working well for *Physcomitrella* is to remove 7 day old tissue from a cellophane overlay plate, place it into about 10 ml of sterile distilled water in a vial and store in darkness in a refrigerator at 4°C. Such tissue remains viable for a considerable time (perhaps more than two years, the experiment is still in progress).

Stock maintenance at low temperature in short day conditions

Cultures of *Ceratodon* on solid medium, stored in a regime of 2h light 10° + 22h dark 8° (these are more or less the minimum temperatures for Percival incubators), appear to remain viable for more than two years (experiment still in progress). Although most strains show little or no growth, the tissue remains healthy and regenerates quickly upon subculture. The regime may not be as satisfactory for *Physcomitrell*a which may require somewhat higher temperatures, but we will keep this under review. This regime promises to make stock maintenance at least of *Ceratodon* much easier. All my *Ceratodon* stocks are now inoculated on 12 well plates (4 ml BCD + ammonium per well) and kept in this regime. Tissue from 24 month-old cultures (the oldest I have) regenerates vigorously. The light treatment is an essential part of this regime. Tissue stored at 4° in darkness, dies in about 6 months.