Culturing the Moss *Physcomitrella patens*

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**INTRODUCTION**

This article includes a series of methods for culturing the moss *Physcomitrella patens* at all stages of its lifecycle. Gametophytes are axenically cultured on solid agar-based media and in shaken liquid cultures. For long-term storage of gametophytes, cultures are maintained on solid medium at 10°C in a very short day. Cryopreservation may also be used. Finally, sporophytes are generated by self-fertilization and sexual crossing.

**RELATED INFORMATION**

The methods for growth of gametophytes on solid medium were adapted from Grimsley et al. (1977). The method for cryopreservation was adapted from Grimsley and Withers (1983). The method for production of sporophytes and isolation of spores was adapted from Ashton and Cove (1977). For more information about *P. patens* as a model organism, see *The Moss Physcomitrella patens: A Novel Model System for Plant Development and Genomic Studies* (Cove et al. 2009).

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**MATERIALS**

**CAUTIONS AND RECIPES:** Please see Appendices for appropriate handling of materials marked with <!>, and recipes for reagents marked with <R>.

**Reagents**

- **<R>:** BCD medium (liquid and solid) containing common moss media supplements as necessary
  - A common supplement is diammonium tartrate, which is added to BCD medium at a final concentration of 5 mM. For cryopreservation, also include mannitol (500 mM) in the appropriately supplemented liquid BCD medium (see Step 18).

- **<R>:** DMSO-glucose solution
  - Ethanol (70%)
  - <R> <!> Nitrogen-free medium containing 400 µM KNO₃

- *P. patens*, somatic tissue or spores
  - To establish a new culture, a fragment of tissue 1-2 mm in diameter is sufficient. For routine subculture, it is best to use protonemal tissue from a vigorously growing culture that is no more than 20 d old, which will be composed mostly of chloronemal tissue. Chloronemal tissue, the growth of which is enhanced when ammonium is provided as nitrogen source, is easiest to subculture. Tissue other than chloronemata (e.g., leaf cells) may take a long time to regenerate. For sexual crosses, it is best that at least one of the two strains to be crossed is self-sterile. Strains containing vitamin auxotrophies (e.g., for thiamine, p-aminobenzoic acid, or nico- tinic acid) are normally self-sterile but cross-fertile (Courtice and Cove 1978).

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METHOD

Procedure I: Growth of Gametophytes

**Using Petri Dishes Containing Solid Medium**

1. Inoculate a Petri dish containing appropriately supplemented solid BCD medium as follows:
   
i. For spores, use standard microbiological procedures to spread them on the agar. If individual plants are required, add ~100-200 spores per 90-mm-diameter Petri dish.
   
ii. For somatic tissue, place a clump of gametophyte tissue (usually 1-2 mm in diameter) on the agar. The size of the clump is not critical. However, for growth tests, the clumps should be as uniform as possible, and uniformity is best achieved by picking the tissue while looking through a microscope.

2. Seal the Petri dishes with Micropore surgical tape.
   
   *This reduces the risk of contamination without affecting the growth or development of the cultures. Do not seal cultures with Parafilm; this slows growth and prevents regeneration.*

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**Equipment**

Blender (e.g., Fisher Scientific PowerGen Model 125 Homogenizer)

*Different tissue blenders are used in different laboratories, and some have been made specifically for blending moss tissue. Provided the blending assembly can be sterilized, it appears that there is little difference among blenders in the end result—a tissue inoculum that will grow rapidly.*

Cellophane disks (type 325P), sterile (AA Packaging Ltd.)

*These are most conveniently sterilized dry, by autoclaving interleaved with disks of filter paper.*

Ethanol bath (controlled, low temperature)

Flasks, Erlenmeyer (1-L, sterile)

Forceps (fine)

Incubators pre-set to 10°C, 15°C, 20°C, and 25°C, with white light at intensities between 5 and 20 W/m² (e.g., Percival Scientific Model CU-36L5)

*Because of the radiant heat from light sources, it may be necessary to keep the air temperature below the desired culture temperature. In this protocol, the temperatures given are those of the medium in which the cultures are grown.*

<!> Liquid nitrogen

Liquid nitrogen storage facility

Microcentrifuge (optional; see Step 41)

Microscope, dissecting (optional; see Step 1)

Parafilm (optional; see Step 16)

Petri dishes (sterile, 90-mm)

*Most work uses 90-mm-diameter, pre-sterilized disposable plastic Petri dishes, but glass Petri dishes are also suitable. Ideally, the dishes should be vent-free to slow evaporation and limit contamination.*

Pipettes

Shaker (platform)

Spatula (sterile)

Spreaders

Tape, Micropore surgical (3M, 1530-0)

Test tubes (25 x 150 mm, sterile) with caps, or Magenta jars (see Steps 26, 27)

Tubes (microcentrifuge, 1.5-ml, sterile)

Tubes (plastic, 2-ml), containing 1.5 mL of agar medium, with caps (see Step 13)

*If space is not at a premium, 15-ml tubes containing 10 mL of medium provide a better resource for long-term storage.*

Vials (plastic, 2-ml, sterile)

Water bath pre-set to 30°C
3. Incubate the cultures in an incubator at 25°C with constant white light at intensities between 5 and 20 W/m². Depending on the purpose of the procedure, growth under these conditions may range from a few days (e.g., to score antibiotic resistance) to several weeks (e.g., to score the requirements of some vitamins).

**Using Petri Dishes Containing Solid Medium Overlaid with Cellophane**

4. Overlay a Petri dish containing appropriately supplemented solid BCD medium with a sterilized cellophane disk. Allow the dish to stand for at least 10 min to allow the cellophane to hydrate, and then, if necessary, straighten the disc while maintaining sterility.

5. Obtain one dish of protonemal tissue from plants that have developed for ~10 d from tissue-clump inocula growing on appropriately supplemented BCD medium (see Steps 1-3). Harvest the tissue with a spatula as shown in Figure 1A.

6. Add the tissue to 10 mL of H₂O₂, and blend it for ~2 min. The exact procedure will depend on the type of blender used. Blending should result in an easily pipettable suspension, but it should still consist of tissue clumps containing 20-50 cells. Overblending leads to poor regeneration.

7. Pipette 1-2 mL of the protonemal suspension from Step 6 onto each Petri dish from Step 4. Spread the suspension evenly. There should be sufficient tissue to inoculate five to 10 Petri dishes.

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**FIGURE 1.** (A) Easy harvesting, with a spatula, of *P. patens* protonemata grown on solid media overlaid with cellophane. (B) Six-day-old *P. patens* protonemata grown on cellophane over solid BCD medium supplemented with diammmonium tartrate.
8. Incubate the culture for 7 d at 25°C under a 16-h light/8-h dark cycle (with white light at intensities between 5 and 20 W/m²).

For an image of 6-d-old protonemata grown under these conditions, see Figure 1B. For a wild-type culture, each 90-mm-diameter Petri dish will yield ~200 mg (fresh weight) of vigorously growing protonemal tissue, consisting mainly of chloronemata.

9. Harvest the tissue by scraping it from the cellophane using a sterile spatula.

Once tissue is growing on cellophane, it is convenient to use this tissue (instead of the tissue described in Step 5) for further inocula by repeating Steps 6-9. However, do not repeat this cycle more than three times.

Using Liquid Medium

Growth rates in shaken liquid cultures are not as great as those obtained on solid media or in bioreactors (Boyd et al. 1988), especially if these are supplied with CO₂. However, to obtain large quantities of tissue for biochemical studies, to accumulate secreted products from tissue for characterization, or for tracer studies, liquid culture may be preferred.

10. Add tissue from one cellophane-overlay plate (see Steps 4-9) to 10 mL of H₂O and blend as described in Step 6.

11. Inoculate 200 mL of appropriately supplemented liquid BCD medium (in a 1-L Erlenmeyer flask) with 2 mL of the tissue suspension from Step 10.

Other quantities of the liquid BCD medium and/or the tissue suspension may be used instead.

12. Shake the cultures on a platform shaker at 25°C under continuous white light.

Vigorous agitation is not necessary for growth. In these conditions, tissue weight doubles in 3-4 d.

Procedure II: Long-Term Storage of Gametophyte Tissue

Storage on Solid Medium

13. Place a clump of gametophyte tissue (usually 1-2 mm in diameter) in each 2-mL plastic tube containing 1.5 mL of agar medium (usually, appropriately supplemented solid BCD medium).

14. Make sure that the lids are not tightly sealed. For screw-cap lids, tighten the cap and then release it about one-quarter of a turn.

15. Grow the cultures for ~3 wk in an incubator at 25°C with continuous white light at intensities between 5 and 20 W/m².

16. After 3 wk, tightly seal each tube. If the tube has an air-tight seal, this is sufficient; if it does not, seal the tube with Parafilm.

17. Transfer the tubes to an incubator at 10°C with a 2-h light/22-h dark cycle (with white light at intensities between 5 and 20 W/m²) for long-term storage.

Cultures can be kept in a healthy state for at least 3 yr under these conditions.

Cryopreservation

18. For each strain to be preserved, grow gametophyte tissue as described in Steps 4-8. After 7 d of incubation, transfer 100 mg of tissue onto a Petri dish overlaid with fresh cellophane. Pipette 1 mL of appropriately supplemented liquid BCD containing 500 mM of mannitol onto the surface of the tissue.

19. Incubate the culture for an additional 7 d at 25°C under a 16-h light/8-h dark cycle (with white light at intensities between 5 and 20 W/m²).

20. To each of ten 2-mL sterile plastic vials, add 1.5 mL of DMSO-glucose solution. Then add one-tenth of the tissue on the plate to each vial. Incubate the vials for 1 h at 20°C.

21. Freeze the vials at a rate of 1°C per minute to −35°C using a controlled low-temperature ethanol bath.

22. Place the vials in liquid nitrogen for storage.

It is advisable to freeze multiple aliquots of tissue. Recovery from cryopreservation is usually good for vigorous strains, but some mutant strains recover poorly. To thaw the cultures, proceed with Steps 23-25. Successful recovery has been achieved after 10 yr of storage; longer storage periods may be possible.
23. Retrieve the vials from the liquid nitrogen, and place them in a water bath at 30°C until thawed.

24. After thawing, add tissue from one vial to 10 mL of H₂O. Allow the mixture to stand for 30 min at room temperature.

25. Inoculate the tissue suspension from Step 24 onto appropriate solid medium as described in Steps 1-3 (use 1-2 mL of the tissue suspension per plate).

Procedure III: Production of Sporophytes and Isolation of Spores

26. Prepare test tubes (25 × 150 mm) with between 15 and 20 mL of solid nitrogen-free medium containing 400 µM KNO₃. Alternatively, fill Magenta jars two-thirds full with the same medium.

27. Inoculate the medium with protonemata by placing a clump of tissue (usually 1-2 mm in diameter) on the agar.
   i. If sporophytes resulting from self-fertilization are required, place a single inoculum into a test tube. Alternatively, place four inocula into a Magenta jar.
   ii. To establish a sexual cross, place one or more inocula of each strain into a tube (or a Magenta jar).
      
      Harvest and test the sporophytes produced from sexual crosses individually because they may result from either self- or cross-fertilization. Strains that have been kept for prolonged periods in vegetative culture may lose fertility.

28. Place the cap on the tube, but do not completely tighten it.

29. Culture for ~4 wk at 25°C in continuous white light at intensities between 5 and 20 W/m². Continuous white light accelerates sporophyte production somewhat, but sporophyte yield may be reduced.

30. Transfer the culture for an additional 3 wk to 15°C under an 8-h light/16-h dark cycle.

31. Irrigate the culture with H₂O to facilitate fertilization. Make sure to thoroughly wet but not submerge the culture. Allow it to stand for 24 h.

32. After 24 h, decant excess H₂O. Place the culture for 1 wk at 15°C under an 8-h light/16-h dark cycle.

33. Repeat the irrigation procedure as described in Step 31.

34. After 24 h, decant excess H₂O. Place the culture for 2-5 wk at 15°C under an 8-h light/16-h dark cycle.
   After this incubation period, sporophytes will have developed.

35. Use fine forceps to harvest pale brown sporophytes by separating them from the gametophytic material. Do not take the green or yellow sporophytes (these are immature) or the dark brown sporophytes (these burst easily).

36. Pinch the base of the seta, which can be identified by a zone of pigmentation, to release the spore capsule.
   Each capsule of a mature sporophyte contains 1 × 10³ to 4 × 10³ viable, haploid, uninucleate spores.

37. Place one or more sporophytes in a sterile 1.5-mL microcentrifuge tube. Sterilize the sporophytes by adding 1 mL of 70% ethanol and incubating for 4 min at room temperature.

38. Remove the ethanol. Gently rinse the sporophytes three times with 1 mL of H₂O at room temperature.

39. Add 1 mL of H₂O, and place the tube for 7 d in the dark at 4°C. This step increases spore germination, but some spores will germinate if this step is omitted.

40. Crush the sporophyte capsules and mix to produce a spore suspension.
   Any residual sporophytic tissue will not regenerate and can be ignored. The spore suspension may be kept for several weeks at 4°C.
41. If desired, dry the spores by removing as much H$_2$O as possible after centrifugation in a micro-centrifuge at 100g for 4 min and then allowing the excess H$_2$O to evaporate at room temperature under sterile conditions.

*Dry spores can be stored for several years.*

**REFERENCES**


