Axenic culture of bryophytes in the home

Jonathan Sleath describes his domestic techniques for growing bryophytes in sterile culture

Axenic culture of bryophytes, where plants are grown on sterile media without contamination from other organisms, is an established technique used in developmental and taxonomic research. It has also been used as a method of *ex situ* conservation of endangered species. Although it usually requires laboratory facilities and reagents, a scaled down version is not impossible for the dedicated amateur, although inevitably it is not without its challenges.

Strictly speaking, true axenic culture is very difficult, particularly using the methods I will describe. There is frequently some growth of fungi, bacteria or algae. This is not always a problem. The late Harold Whitehouse, who pioneered and mastered the techniques for his work on tuberous mosses, used to say that the plants grew better with some degree of bacterial colonisation. It was thought that some bacterial metabolites might have been released which aided the growth of the plants.

My experience over the last 18 months has been chiefly with acrocarpous mosses. I have not attempted to grow pleurocarps or leafy liverworts, and success with thallose liverworts has been with ruderal species such as *Marchantia polymorpha* and *Lunularia cruciata*. Clearly there are issues around providing the correct conditions for the symbiotic fungi of liverworts. Background information about methods and techniques can be found in useful papers by Duckett *et al.* (2004) and Rowntree (2006).

Growing medium

Agar gel has a long history as a bacteriological growing medium and in the past this was the standard gelling agent for bryophyte culture. In recent years the use of gellan gel has become much more popular. This is supposed to have the advantage of being purer and more transparent. The literature refers to proprietary laboratory reagents such as Gelrite. This is a mucopolysaccharide derived from bacteria and, in part due to the demand created by its use in molecular gastronomy, it is readily available generically online as low acyl gellan gum. Various inorganic chemical solutions are used to provide nutrients, but these require a range of laboratory chemicals that are not always easy to obtain and can be quite complicated to prepare. The gellan also requires the presence of divalent cations to gel properly. Since I live in the eutrophicated English lowlands with a water supply derived from the chalk aquifers, I have just used tap water which seems to have enough calcium to set the gel and sufficient nitrogen and other nutrients to permit plant growth. I am now experimenting with a modified Knops solution (Reski *et. al.*, 1985) which contains more nutrients and is relatively simple to prepare. It is similar to what is used conventionally and contains sufficient cations to create a gel.

◁ Figure 1. Microwave oven used for sterilising. *All photographs Jonathan Sleath*

Surface sterilising the bryophyte

One of the biggest challenges is sterilising the material adequately without killing the plant. Often this is not achievable. Sodium hypochlorite solution, usually at 1.5%, was the method of choice in the past. More recently, NaDCC has become more popular since it is said to cause less damage to the gametophytic material. This is available in the form of water purification tablets. Two 8.5 mg tablets diluted in 3.4 ml of water will give a 0.5% solution which is the recommended concentration. The thicker the bryophyte material is, the more resistant it is to the effects of the chlorine and the longer it can survive in the solution. Undehisced capsules can be effectively sterilised for at least three minutes whilst retaining viable spores, leafy shoots can survive up to two minutes, but tubers attached to rhizoids are generally killed in less than a minute. Harold Whitehouse would dip shoots in 1.5% hypochlorite for a few seconds (Whitehouse, 1963).

Sterilising the vessels

Any object used in the process should be sterilised. I use 20 ml glass vials with plastic screwtop lids (available online) as the main culture vessels, but also glass petri dishes for thallose liverworts and a small pyrex jug for making up the gel. All can be effectively sterilised for up to 4 minutes in a domestic microwave oven, which can also be used for making up the gel (Fig. 1). Objects made of plastic or metal can be sterilised by spraying with 1% hypochlorite or 70% alcohol.

The clean environment

Few of us possess the resources to equip a home laboratory with a laminar air flow cabinet. I use the kitchen and something called a still air box (Fig. 2), which has been popularised by mushroom growers. This is essentially a transparent plastic storage box used upside down with two circular holes cut in the front to permit the hands to enter.

The interior is sprayed before use with either 1% hypochlorite (though this leaves white deposits and bleaches your clothing) or 70% alcohol. A lamp or torch placed on the top to illuminate the inside is useful. I wear a pair of disposable vinyl gloves as well.

▷ Figure 2. A still air box.

Preparing the gel

The gel should set at low concentrations, and I tend to use 0.3%. It does not dissolve easily. I place 100 ml of tap water in a small pyrex jug which I put in the microwave oven and heat until it boils. Then I sprinkle on 0.3 g of gellan gum and give it a vigorous stir before turning on the power again. It may be useful to know that 1 cm^3 of gellan powder weighs approximately 1.25 g. It may take several minutes of boiling and stirring before the gel dissolves and no particles can be seen, but I have never been able to obtain a completely clear solution. Since it reduces in volume during this process, I usually top it up with some deionised water. During this time, I also have the other glassware in the oven to sterilise, with the vial lids slightly unscrewed.

I then reach into the oven (wearing gloves), take off the vial lids, and pour in the gel solution until they are about a third filled. I then give everything another blast of microwaves until the gel in the tubes boils. At this point it is worth letting everything cool down for a while before screwing on the vial lids to reduce condensation. Then the lids are screwed back on, and the vials are left on their side at room temperature so that a slope is formed as they set.

I did use a filtrate of the suspension of the soil in which the metallophyte *Mielichhoferia elongata* was growing to make up the culture of this plant. The gel was very turbid but many leafy shoots grew, in contrast to the plain gel where there was no growth.

Making the cultures

The tips of leafy shoots are prepared under the stereo microscope and cleaned in sterile water to remove as much debris as possible. Individual tubers are impossible to cope with, but dissection of tubers attached to rhizoids can produce a tangle that can be picked up with fine forceps. Large gemmae such as *Marchantia* can be held within a tiny piece of lens tissue for transport and sterilisation. The material is then placed in a previously sterilised small petri dish in sterile water, and then the outside of the petri dish is surface sterilised before being transferred to the still air box, as is the other glassware and forceps etc. The NaDCC solution and sterile water are treated in the same way. I have sometimes used

△ Figure 3. *Bryum gemmilucens* growing in a 20 ml glass vial.

the inside of the microwave oven instead of the box for simple procedures.

I tend to loosen the tops of all the vials before starting so that they can be lifted off. It is a good idea to mark them beforehand with a permanent marker and write out a plan of what material is to go in which vial as it is easy to lose track. Leafy shoots and tangles of tuber-bearing rhizoids are transferred to the NaDCC for the appropriate length of time and then rinsed in sterile water before being placed on the surface of the gellan slope. Undehisced capsules can be transferred from the NaDCC to a drop of water on a sterile glass slide, broken open with forceps to release the spores, and then the water transferred with a sterile syringe or pipette to the slope.

Growing on

This, surprisingly, can be the most challenging part of the process. Even if the material has not been killed with chlorine, it may be too fastidious to grow (e.g. *Discelium nudum* or *Mielichhoferia elongata* on plain gel), but otherwise protonemal growth is usually visible within a couple of weeks. This gradually spreads throughout the gel. Germinating spores produce a mass of protonema on the surface of the slope, but it can be several months before leafy shoots start to appear. Cultures derived from gametophytic material generally produce leafy shoots earlier and then rhizoids on which tubers may form (Figs. 3, 4). One interesting observation is that bulbiliferous species of *Bryum* also produce bulbils on the protonema, often in abundance (Sleath, 2022; Fig. 5).

Without a professional growth chamber, it is almost impossible to provide the correct environmental conditions to permit normal growth of the plants, and the shoots are invariably etiolated, with leaves differing from those produced in nature. In order to boost light levels I moved the cultures outdoors in summer but unfortunately many were killed this year when they were inadvertently subjected to sunlight and became too hot (Fig. 6). The gel itself doesn't like this treatment either, and temperature fluctuations cause it to break down and liquify. I have kept some test gels inside in the dark over the last 18 months and they have remained perfectly stable. Harold Whitehouse kept his cultures in test tubes plugged with cotton wool and topped with foil on the windowsill of his office in the Cambridge Botany School. This faced southeast, and Chris Preston reminds me that he managed to achieve good results without elaborate technology by using a tea-towel for shading in sunny weather.

After a while, contaminating organisms can gain a foothold or the gel starts to break down and it is clear that the culture is going into a decline. It is possible then to subculture by using protonema or propagules growing below

△ Figure 4. Abundant tubers in an 18-month old *Bryum klinggraeffii* culture.

the surface of the gel. Often one needs to start a culture with fresh material, and of course many species have a short life cycle anyway.

Final observations

These methods provide an inexpensive and relatively simple way for those without laboratory facilities to try their hand at the *in vitro* culture of bryophytes. There is clearly scope for further experimentation and much trial and error is involved. It is most suited to those interested in the development of propagules on the protonema and rhizoids, and there is always the possibility of the discovery of such structures in species where they have not previously been recorded.

References

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△ Figure 5. Top. Bulbils on protonema of six-month old *Bryum gemmilucens* culture.
Figure 6. Above. 16-month old *Hennediella macrophylla* culture bleached by sunlight.

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