



A Simple Method to Obtain Microbial-free *in vitro* Moss Cultures

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Abstract

Developing a suitable method for moss sterilization is a crucial step for successful *in vitro* culture. Different chemical and physical methods have been tried by many authors for more than a century but with low axenic culture percentages. An easy and economic (no use of chemicals or antibiotics) cultivation method is developed here, which proved high success with three moss species starting from gametophores.

Key words

Axenic cultivation, mosses, *Bryum*, *Philonotis*, sterilizing agents

Introduction

Bryophyta is one of the earliest inhabitants of terrestrial environments. They survived and tolerated for ages, various climatic conditions. However, harvesting large quantities of bryophytes for greening and different applications can seriously damage the natural habitat (Peck and Muir 2001). To avoid or at least minimize such a serious environmental problem an alternative solution exists in the *in vitro* culture technique which facilitates growing and regenerating plants. *In vitro* culture of plants including bryophytes furnishes, therefore a good solution for nature conservation. In addition it gives us opportunities to increase our knowledge about certain aspects of plant biology, development (Duckett *et al.* 2004, Victoria *et al.* 2011) and biotechnological research (Beike *et al.* 2010).

Trials for propagation of mosses in pure cultures started a long time ago by pioneer bryologists (e.g. Vöchting 1885, Becquerel 1906) and are still going on till now (e.g. Rowntree and Ramsay 2009, Rowntree *et al.* 2011, Carey *et al.* 2015), however the obtained results of survival percentages were poor or at least not satisfactory, particularly when the *in vitro* culture was started from gametophore tissues and not from spores.

Initiating axenic *in vitro* cultures from field grown plants requires surface-sterilization of plant part inocula to get rid of superficial contaminants (Basile 1972). As surface sterilization of mature capsules - before dehiscing- is effective, axenic cultures of mosses are most easily established from spores (Duckett *et al.* 2004, Lal 1984, Reski 1998, Hohe and Reski 2005). Unfortunately, starting cultures from spores is impractical for many species, especially dioicous taxa (*ca.* 60% of recorded moss taxa);

Hedenäs and Bisang 2011, which mostly never produce capsules (Glime 2013). Alternatively, gametophores have to be used for producing axenic cultures of the majority of mosses. However, surface sterilization of moss gametophore is usually ineffective in establishing axenic cultures. Gametophores are very delicate and the phylloids are in general tinny, mono-layered, inhabited by various xenic organisms like bacteria, algae, protozoa, fungi and others (Vujičić *et al.* 2009, Sabovljevic *et al.* 2012). As a result, vegetative fragments may be either killed by the sterilization procedure or by microbes (Duckett *et al.* 2004).

Generally, there are many previously described techniques for surface-sterilizing of small parts of moss gametophyte, as washing machine (Basile 1972, Basile and Basile 1988), ultrasound (Cano 1996), and chemicals (e.g. sodium dichloro-isocyanurate (SDICN) (Parkinson *et al.* 1996, Niedz and Bausher 20002, Sarasan *et al.* 2006, Liang *et al.* 2010). The survival percentages of sterilized gametophores were 2-11% with sodium hypochloride (NaOCl) and 37-66% with sodium dichloroisocyanurate (SDICN) (Sabovljevic *et al.* 2012) and 70% with combination between 70% ethanol for 5 sec. and 5% (NaOCl), 30 sec.

(Liang *et al.* 2010) [23]. Mercuric Chloride was also used for sterilization of protonema, however it was very harsh and the method has low survival percentage (Chen *et al.* 2009). Recently, an agar embedding method with antibiotic, significantly reduced the presence of bacterial contaminants in gametophyte tissue (Carey *et al.* 2015).

However, trials for the development of an easy, convenient, effective and, more successful method for obtaining axenic

cultures of different moss species starting from gametophores remain to be a current challenge. This work represents one of these trials, however distinguished because efficient sterilization is sought without using chemicals or antibiotics.

Materials and Methods

Fresh gametophores of three moss taxa collected from natural habitats in Cairo, Egypt, were used in this experiment. These taxa were *Bryum argenteum* Hedw. *Bryum subapiculatum* Hampe (Bryaceae) and *Philonotis hastata* (Duby) Wijk& Margad (Bartramiaceae). *B. argenteum* gametophores (given herbarium no. MF 03) were collected in October 2013, from Kobri El-Kobba, underground Metro station, ca. 30° 4' 58.10" N and 31° 17' 32.29" E, where they grow in shade, on a wet vertical side of a cemented pavement. *B. subapiculatum* gametophores (MF10) were collected in October 2013 from the Botanical Garden of the Faculty of Science, Ain shams University, ca. 30° 4' 40.75" N and 31° 16' 56.03" E, where they grew exposed to sun, on a wet cemented layer of a sewage drain. *Philonotis hastata* gametophores (MF14) were collected in November 2013, from Kobri El-Kobba, underground Metro Station, ca. 30° 5' 4.30" N and 31° 17' 42.71" E, where they grew exposed to sun, on a wet vertical side of a cemented pavement. Both *P. hastata* and *B. argenteum* carried easily detachable bulbils.

The present developed technique is modified from that of (Chen *et al.* 2009) . The following steps were carried out:

1. Freshly collected small moss samples were carefully cleaned mechanically from sticking soil particles and other impurities and rinsed with distilled

A Simple Method to Obtain Microbial-free *in vitro* Moss Cultures

- water and then vortexed three times for 10 minutes each.
- Individual gametophores were picked up under stereomicroscope (Novex model, Netherlands), then washed and rinsed with sterile distilled water (SD H₂O) three times for five minutes each.
 - Washed gametophores of each species were divided into two groups; the first group was propagated in 250 ml conicals, containing 100 ml SD H₂O, while the second one was propagated in 250 ml transparent plastic containers, containing a thin film (5 mm thick) of sterilized water-saturated soil (30 g soil; particles of the size $\leq 300 \mu\text{m}$).
 - The former group was kept on the lab bench under low light intensity for four to six weeks. The latter group was incubated in a plant growth chamber (Vision scientific plant growth chamber model V3-DM; Vision Scientific Company Ltd, Korea) for four to six weeks, under controlled conditions of adjusted light/ dark periods (16/8 hrs) using white illumination with intensity of 9200 Lux and fluctuant temperature of 25/18°C \pm 2 for light and dark periods, respectively.
 - The growth medium (water or soil) on which new gametophores were developed faster was considered more suitable for the corresponding species.
 - Newly developed gametophores of *B. argenteum* and *P. hastata* grew faster on water-saturated soil whereas those of *B. subapiculatum* grew faster in SD H₂O. All gametophores were carefully excised under aseptic conditions.
 - The excised gametophores were gathered, washed twice with SD H₂O while shaking, each time, for 5 min. they were then spread on dry sterile filter paper.
 - The gametophores of each species were then divided into six groups. The first 3 groups were cultured on 3 different solid media and the other 3 groups were cultured in 3 different liquid media as one gametophore per each jar in three replicates (i.e. a total of 54 jars for the three species). The composition of the three different solid media was as follows:
 - Nutrient free agar NFA (9 jars for the 3 species).
 - Half strength Murashige and Skoog mineral salts HsMS (1962) (9 jars for the three species).
 - Full strength MS mineral salts; FsMS (9 jars for the 3 species).The composition of the three different liquid media was as follows:
 - Nutrient free liquid medium; NFM(Strelized distilled water) (9 jars for the 3 species).
 - Half strength Murashige and Skoog mineral salts HsMS (9 jars for the three species).
 - Full strength MS mineral salts; FsMS (9 jars for the 3 species).
 - The 54 jars were incubated in the plant growth chamber under the previous described conditions (of illumination and temperature) and externally observed weekly for ten weeks. The protonemata and gametophores were examined microscopically afterwards i.e. after incubation for ten weeks.
 - The percentages of survived gametophores i.e. the axenic cultures (growth without any contamination) were calculated.

Observations and Discussion

Cultures in 45 jars out of 54 jars (i.e., 83.3 %) were axenic. The percentages of survival with the used technique were mostly higher in liquid media than on solid media (Table 1). The percentages of

Hanan Shaaban et al.

survival were 67-100% for *B. argenteum*, (33-100%) for *B. subapiculatum* and (33-100%) for *P. hastata* (Table 1).

These results show almost 100 % success for the axenic cultivation of gametophores in different types of liquid media (NFM, HsMS, FsMS) for the three mosses under investigation. These results are highly promising, compared with the percentages (5% -70%) of survival of gametophores reported by some authors e.g., (Sabovljevic *et al.* 2012, Liang *et al.* 2010) using different sterilizing agents. These results have been achieved, in the present work, due to the attention paid to two points during the *in vitro* cultivation: 1. Avoidance of sterilizing the cultured and the recultured gametophores by chemicals and 2. The use of repeatedly cleaned gametophyte tissues for inoculation on nutrient media. Cleaning was achieved by washing several times with SD H₂O which is considered a modification of the technique that has been used before by (Chen *et al.* 2009), who used chemicals for the sterilization of the re-cultured gametophores.

In the present work, when the new branches were excised and re-cultured on nutrient media, it was noticed that they grew more successfully than their progenitors. This agrees with results obtained by (Rowntree 2006) who reported that *Ditrichum plumbicola* Crundw. gametophyte grew significantly better when harvested from recultured non-axenic cultures than from the wild collected material. This might be interpreted on the basis that pre-culturing partially acclimatizes plants to culture conditions, and reduces the number and type of contaminants. Therefore 100% success is highly expected, using the present technique, if the re-culture step is repeated more than once.

Thus the technique used in the present work not only avoids probabilities of gametophores damage (by sterilizing agents and/or microbes) but also produces luxurious growth. Because contamination was absent in most jars, gametophores had full chance to obtain their required nutrients from the culture medium and grow to form large colonies establishing a good biomass (see figs. 1, 2, 3).

Table (1). Percentages of survival of *Bryum argenteum*, *Bryum subapiculatum* and *Philonotis hastata* in liquid and on solid media: nutrient free medium (NFM), half strength Murashig and skoog (HsMS) medium, full strength Murashig and Skoog (FsMS) medium and nutrient free Agar (NFA) medium.

Species	% of survival					
	Liquid media			Solid media		
	NFM	HsMS	FsMS	NFA	HsMS	FsMS
<i>B. argenteum</i>	100%	100%	100%	100%	67%	67%
<i>B. subapiculatum</i>	100%	100%	100%	33%	67%	67%
<i>P. hastata</i>	100%	100%	33%	100%	67%	100%

A Simple Method to Obtain Microbial-free *in vitro* Moss Cultures

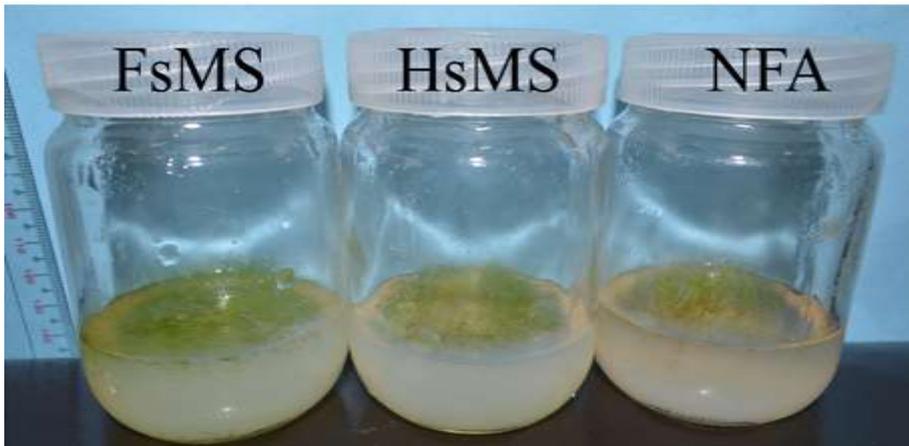


Fig. 1: Axenic cultures of *Bryum argentum* developed on solid media (NFA, HsMS & FsMS) after incubation for 10 weeks.

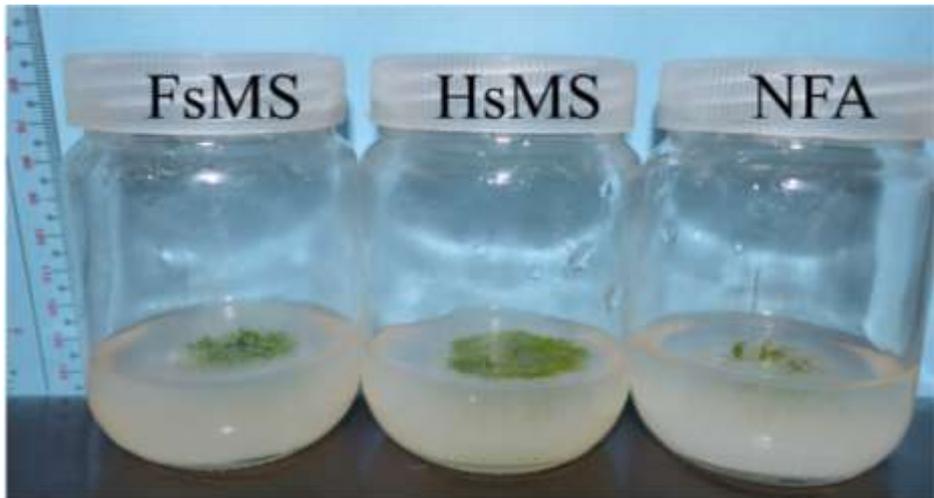


Fig. 2: Axenic cultures of *Bryum subapiculatum* developed on solid media (NFA, HsMS & FsMS) after incubation for 10 weeks.

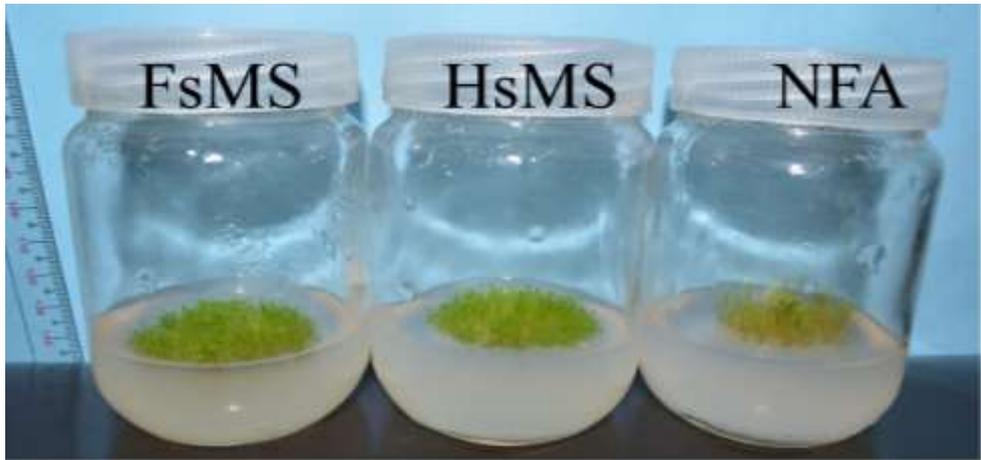


Fig. 3: Axenic cultures of *Philonotis hastata* developed on solid media (NFA, HsMS & FsMS) after 10 weeks incubation.

Although the changes from distended spore to filamentous protonema to gametophore buds can require increasingly more specialized conditions (Duckett *et al.* 2004, Glime 2007), yet the three species studied here exhibited normal development on all nutrient media as well as on NFM (solid or liquid) without any specified requirements. This might be attributed to the high light intensity used in the plant growth chamber (9200 lux). It may be mentioned, in this context, that high light intensity and temperature promoted branching of caulonema, proliferated potential bud sites and provided a thicker mat in *Hymenostylium*, *Campylopus* and *Physcomitrella* (Glime, B.C. Knoop 1986, Mehta 1988).

Gametophores were observed earlier on NFA solid medium than with the other nutrient media, especially in case of *B. argenteum*. This might be attributed to that developed protonemata were mainly caulonemata filaments in NFA solid medium. As caulonema grows more rapidly than chloronema (Cove *et al.*

2006), the caulonema allows the developing plant to colonize on available substrate more rapidly (Cumming and Cumming 2008).

Protonemata of both *B. argenteum* and *P. hastata* started to develop in the 1st week when cultured on HsMS and FsMS solid and liquid media, which agree with the results obtained for *Physcomitrella patens* (Hedw.) Bruch & Schimp. (Decker and Reski 2004), *B. argenteum* (Liang *et al.* 2010), and *Entosthodon hungaricus* (Boros) Loeske (Sabovljevic *et al.* 2012). The present results, therefore, reflect the success of the developed modified technique.

On the other hand, the development of *B. subapiculatum* protonema was in the 1st week only when cultured on HsMS solid medium but delayed to the 2nd week with HsMS liquid medium and on FsMS solid and liquid media. Comparing growth rates of the three mosses in response to various nutrient concentrations showed that HsMS solid and liquid media were better for the growth than other media. It is known that moss species prefer dilute

A Simple Method to Obtain Microbial-free *in vitro* Moss Cultures

culture solutions to concentrated ones (Awasthi *et al.* 2010). The concentrated media may even cause poor growth (Awasthi *et al.* 2012).

Thus, the modified technique developed in the present work for *in vitro* cultivation of *B. argenteum*, *B. subapiculatum* and *P. hastata* is an easy, inexpensive and successful technique which is worth extension for propagation, conservation and molecular studies of other species of mosses.

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A Simple Method to Obtain Microbial-free *in vitro* Moss Cultures

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