

## ***In vitro* micropropagation of *Bryum argenteum* Hedw.**

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**Abstract** – *Bryum argenteum* Hedw., a cosmopolitan moss, is now considered a greening material suitable for courtyards and golf courses in countries like China. Here we describe the development of new micropropagation procedures as a key step in the commercial development of *B. argenteum* in greening applications. The protonemata were grown in liquid culture and introduced onto solid media for gametophore development. The derived gametophores were broken into pieces by a blender and inoculated onto a sterilized substrate: a mixture of vermiculite and peat (6:1). A moss mat formed after a month. Effects of different media and phytohormones (IAA, 6-BA, GA<sub>3</sub>) on protonema multiplication and gametophore induction were investigated. The results showed that (1) protonemata were most easily propagated in Knop solution, compared with other liquid media (BCD and Beneke); (2) protonemata grown on Knop agar produced more gametophores than those cultivated on other solid media; (3) 0.1μM GA<sub>3</sub> added to the Knop medium reduced the time for gametophore emergence.

**Gametophore production/ greening materials / liquid culture / phytohormones / protonema / sterilization protocols / *Bryum argenteum***

### INTRODUCTION

Bryophytes occur in nearly every ecosystem on earth and play a major role in the recycling of carbon and nutrients through growth and decomposition (Vanderpoorten & Goffinet, 2009). Features like high temperature tolerance, desiccation-resistance, high water holding capacity, and easy maintenance (Lai, 2003; Vanderpoorten & Goffinet, 2009) suggest bryophytes as highly suitable materials for environmental recovery and greening. However, harvesting large quantities of moss populations from nature for greening applications may seriously damage natural ecosystems and the recovery of the harvested moss mats can take a long time (Peck & Muir, 2001). Thus, there is now a pressing need for the development of effective methods for large scale propagation.

The cosmopolitan moss, *Bryum argenteum*, easily recognized by its silvery green colour, can form a “beautiful, short and dense sod” and is appropriate for the greening of golf courses and courtyards (Lai, 2003; Glime, 2007). Jones and Rosentreter (2006) tried fragment the populations of *B. argenteum* collected from the field and let the pieces reproduce under controlled conditions. Effects of added sugars and phytohormones on the morphogenesis of *B. argenteum in vitro* had also been reported (Bijelovic *et al.*,

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2004; Sabovljevic *et al.*, 2005). However, methods for using protonemata to mass-produce leafy shoots of *B. argenteum* have not been investigated.

In this study, we investigated the effects of culture methods, media and phytohormones on the growth of *B. argenteum*, and also the propagation of *in vitro* cultured materials on natural substrates. A new method for micropropagation from *in vitro* culture is presented below; this provides a technological foundation for the future production of *B. argenteum* for commercial purpose.

## MATERIAL AND METHODS

### Culturing of axenic material and growth conditions

*Bryum argenteum* was collected from a natural population on the campus of East China Normal University, Shanghai, China. The pretreatment was as follows: remove the leaves and cut out 5 mm of the green tips of the stems. The green tips were disinfected with solutions of sodium hypochlorite (5% active chlorine), ethanol and sodium hypochlorite or 0.1% HgCl<sub>2</sub> and rinsed three times in sterile water, and then cultured on Knop (solid) medium under 20 ± 1°C and continuous (irradiance of 40lx/s) light. Protonemata developed after one month.

### Generation and multiplication of protonemata

500 ml flasks with 200 ml Knop solution were inoculated with the cultured protonemata. The protonemata were fragmented with an Ultra-Turrax running at 19000 rpm for 60 s and stirred with a marine impeller running at 400 rpm. Further fragmentation was carried out weekly using an Ultra-Turrax running at 19000 rpm for 60 s.

### Culture medium selection for protonemal multiplication and gametophore induction

Media used: BCD (Ashton & Cove, 1977), Beneke (Basile, 1975), Knop (Reski & Abel, 1985); all have a pH value 5.8 before autoclaving.

To produce the protonemata, dry weight was determined first by drying the materials of two samples of 10 ml at 105°C for 2 h at the start of the study then after 30 and 45 days. Protonemata were transferred to media solidified with 5g/l agar and the effects of different media on gametophore production were quantified as follows: the number of days needed for gametophore emergence, number of gametophores produced, and the biomass in each dish after 30 days.

Different phytohormones (IAA, 6-BA, GA<sub>3</sub>) were also tested by adding the individual hormonal compound to the Knop solid medium. The phytohormone concentrations were: 0.01 µM, 0.1 µM, 1 µM and 10 µM.

### Production of bryophyte mats on natural substrates

Gametophores, produced *in vitro*, were dried at room temperature, disintegrated by a blender (Philips HR 1707) into a fine powder, and sown into pots, containing a mixture of vermiculite and peat (6:1). Wild materials collected from the campus were treated in the same way.

## Statistical analyses

Results were expressed as the mean  $\pm$  standard error. A one-way ANOVA was used to demonstrate the significance of the different treatments. All the data were analyzed by using a software statistical package (SPSS, version 13.0).

## RESULTS

### Sterilization protocols

The sterilization efficiency of NaClO (0.1%, 0.5%) was low. All the samples were contaminated with fungal growth and died (Table 1, Nos. 1-3). When NaClO was used combining with 70% ethanol, we got much better results (Table 1, Nos. 4-6), and all the treatments had some sterile survivors, especially in the No. 4 treatment (70% ethanol 5s, followed by 5% NaClO 30 s) where the survival rate reached 70%. The recommended time for primary sterilization by 70% ethanol is 5 s because longer exposures damage the plants. Sterilizing with 0.1% HgCl<sub>2</sub> for 30 s or 60 s was also effective (Table 1, Nos. 7-8).

### Protonemal multiplication

The protonemata of *B. argenteum* appeared around the gametophores after two days in culture. Weekly fragmentation of the protonemata prevented the development of gametophores as found by Decker & Reski (2004) in *Physcomitrella patens*.

Table 1. Influences of disinfectant and disinfection time on explants of *B. argenteum*

Treatment number	Sterilization methods	Time (s)	Results (survival rate %)
1	0.1% NaClO	120	0% (contaminated and dead)
2	0.5% NaClO	60	0% (contaminated and dead)
3	5% NaClO	5	0% (contaminated and dead)
4	70% ethanol 5s + 5% NaClO	30	70%
5	70% ethanol 10s + 0.1% NaClO	90	10%
6	70% ethanol 30s + 0.5% NaClO	60	10%
7	0.1% HgCl <sub>2</sub>	30	60%
8	0.1% HgCl <sub>2</sub>	60	10%

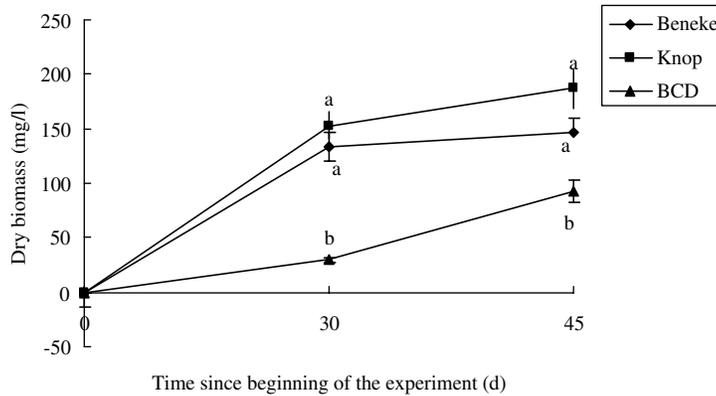


Fig. 1. Protonemal biomass change of *B. argenteum* in Knop, Beneke and BCD media in different periods from the primary dry biomass of ca. 0.1 mg/l. Error bars represent standard Error. Different small letters within rows are significantly different ( $p < 0.05$ ) ( $n = 5$ ).

### Culture medium selection for protonemal multiplication and gametophore induction

Knop was the best medium for protonemal multiplication (Fig. 1). In liquid culture, the biomass can reach 187 mg/l from ca. 0.1 mg/l after 45 days. The same was also true for gametophore production (Figs 2A, 2B).

On Knop, protonemata were clearly visible after one week but grew more slowly on BCD. They turned brown after two weeks on Beneke medium. After the protonemata were cultured on the solid media, gametophores began to appear after 21 days on Knop compared to 28 and 26 days on BCD and Beneke (Fig. 2C).

### Phytohormone selection on gametophore induction

The 0.01 $\mu$ M 6-BA stimulated gametophore productions (Fig. 3A). However, higher level of 6-BA (0.1-10  $\mu$ M) induced abnormal buds, which eventually failed to develop into normal gametophores (Fig. 3B). 0.1 $\mu$ M GA<sub>3</sub> reduced the time for gametophore production to 16 days (Fig. 3A), whilst IAA had no effect on gametophore induction. None of the three phytohormones had significantly affected the number of gametophores produced.

### Propagation on a natural substrate

Materials from *in vitro* cultures of protonema produced new shoots after one week from transplanting onto the vermiculite and peat mixture, while those from the wild were unchanged. After a month, there was more vigorous growth and more gametophores in the colonies initiated from the aseptic cultures (Fig. 4A) than from the wild (Fig. 4B). New gametophores were observed to derive from both protonemata and the original shoots in pot cultures.

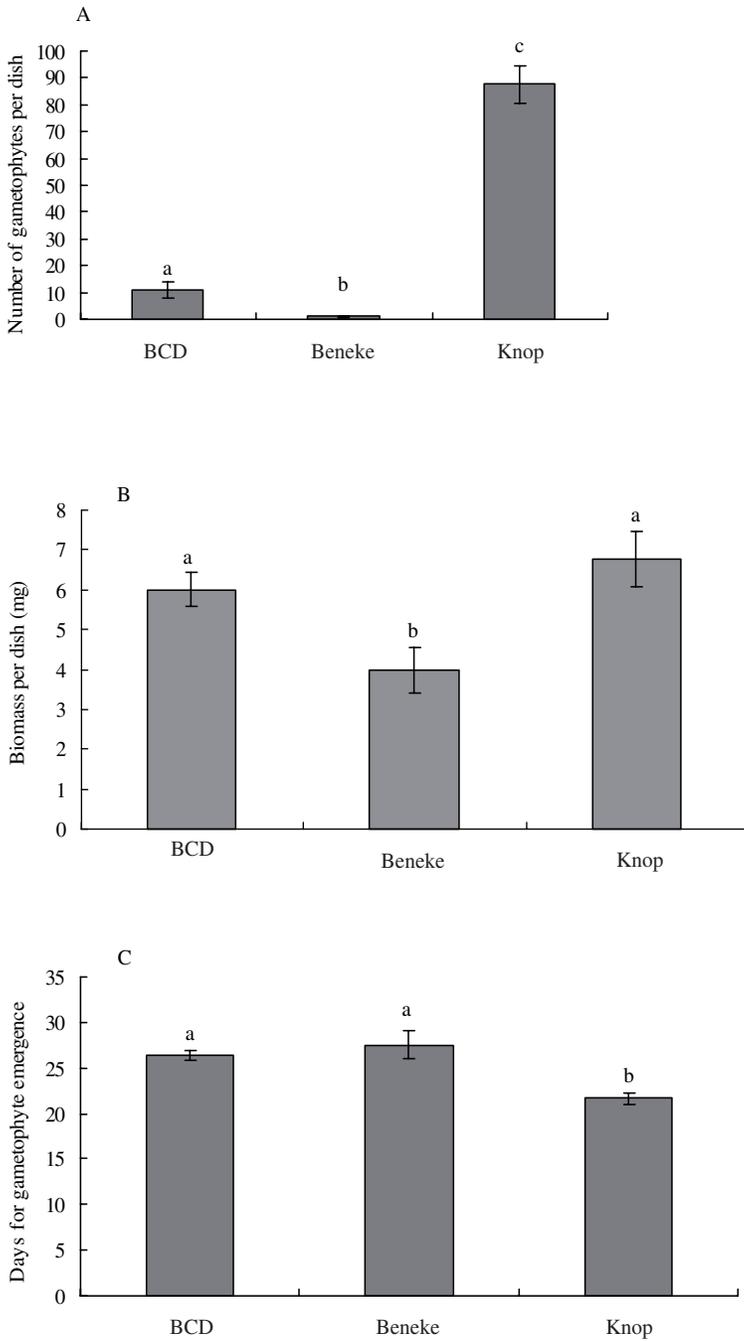


Fig. 2. Effects of different media on gametophore initiation in *B. argenteum* after 30 days cultivation (number of gametophores per dish (A), biomass per dish (B), days for gametophore emergence (C)). Error bars represent standard Error. Different small letters within rows are significantly different ( $p < 0.05$ ) ( $n = 12$ ).

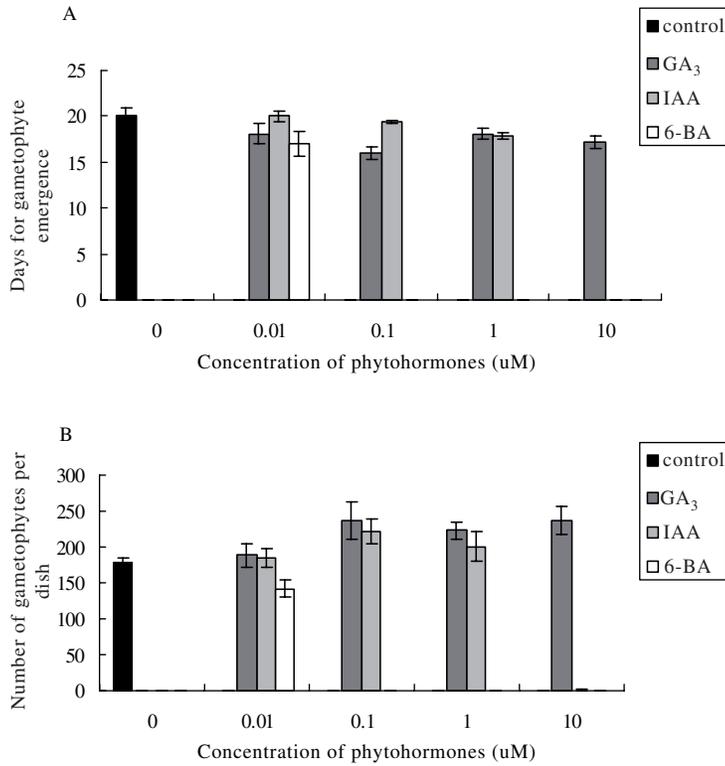


Fig. 3. Effects of GA<sub>3</sub>, IAA, 6-BA on days taken for gametophore emergence (A) and number of gametophores per dish (B) of *B. argenteum* after 30 days cultivation. Error bars represent standard Error. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  ( $n = 10$ ).

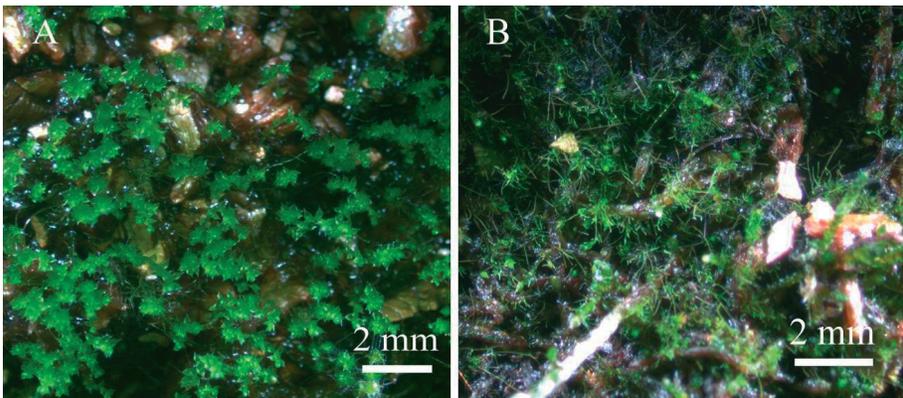


Fig. 4. Growth of moss fragments after one month from vitro culture (A) and from the wild (B). Note the much more prolific production of gametophores in A compared to the mixture of protonemata and much younger and more scattered gametophores in B.

## DISCUSSION

This study demonstrates that protonemata of *B. argenteum* can be bulked up by regular fragmentation in liquid cultures and that material grown *in vitro* is a better inoculum for initiating colonies on soil than fragmented wild-collected plants. The micropropagation system described here may assist future work requiring the large-scale production of moss materials.

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