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AXENICALLY CULTURING THE BRYOPHYTES: ESTABLISHMENT AND PROPAGATION OF THE PLEUROCARPOUS MOSS *THAMNOBRYUM ALOPECURUM* NIEUWLAND EX GANGULEE (BRYOPHYTA, NECKERACEAE) IN *IN VITRO* CONDITIONS

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Abstract

The study gives the first report of *In vitro* culture of the moss *Thamnobryum alopecurum* and the insight into the problems of axenicaly culturing this bryophyte and the conditions for establishment and propagation. This moss is a counterpart of many rare and endangered mosses from the same genus and date presented should be taken into account of conservation and propagation of its counterparts as well. Problems of surface sterilization are elaborated regarding sporophyte vs. gametophyte. The influence of nutrient, light length and temperature on different developmental stages is discussed. The best conditions for micro-propagation from shoots are slightly lower temperature (18-20°C), on BCD medium irrelevant of day length.

Introduction

Bryophytes (comprising mosses, liverworts, hornworts) are the second largest group of higher plants after flowering plants, with estimated 15,000 (Hallingbäck & Hodgetts, 2000; Gradstein *et al*., 2001) to 25,000 species worldwide (Crum, 2001). Bryophytes, although the second largest group of terrestrial plants, received much less attention in conservation and protection and in comparison to vascular plants and higher animals much less are known on their biology. They comprise very diverse plant groups e.g., peat-mosses, latern-mosses, leafy liverworts with quite diverse biological characteristics i.e., structure, size, ecology, reproduction, survival, etc.

Although culturing plant tissues and organs under axenic conditions was first established and profitably employed in bryophytes, especially mosses (Servettaz, 1913), bryophytes did not retain for long their rightful place as a highly favored research object; therefore most studies of plant morphogenesis are now being done on vascular plants. Besides the problems with bryophyte establishment in axenic culture, it is often problem of material availability, genetic variability of material, disposal of axenic organisms leaving on bryophytes and low level of species biology knowledge (Duckett *et al*., 2004). Apart from economic considerations of experimental work with bryophytes, many fundamental and applicative physiological, genetical, morphogenetic, ecological and evolutionary, as well as other problems could be studied more easily in bryophytes rather than in vascular plants (Sabovljević *et al*., 2003). Bryophytes are useful objects for the elucidation of comlex biological processes such as apogamy, apospory, stress-induced cellular responses in plants, and the fusion and growth of protoplast, etc., (Lal, 1984; Cove *et al*., 1997; Oliver & Wood, 1997; Shumaker & Dietrich, 1998; Reski, 1998; Wood *et al*., 2000; Bogdanović *et al*., 2009; Cvetić *et al*., 2005; 2009; Sabovljević *et al*., 2010a,b; Vujičić *et al*., 2010).

Besides, axenical cultivation of bryophytes as well as developing of methodology in propagation of bryophytes are significant in rare species conservation both for *ex situ* and reintroduction (Batra *et al*., 2003; Bijelović *et al*., 2004; Sabovljević *et al*., 2005; Rowntree & Ramsay, 2005; 2009; Gonzalez *et al*., 2006; Mallon *et al*., 2006; 2007; Rowntree, 2006; Cvetić *et al*., 2005, 2007; Bazeanu *et al*., 2008; Chen *et al*., 2009; Vujičić *et al*., 2009; 2011; Rowntree *et al*., 2011) . This is especially valuable for the bryophyte species many of which are dioecious and possibly long-lastingly in sterile condition naturally or for the species with low reproductive effort and/or vegetative reproduction.

Axenic culturing of bryophytes seems to be so complicated that many investigators gave up the attempts. However, due to possible interaction with other organisms in non axenic conditions, sterile culturing is necessary for certain experimental procedures. Progress in bryophyte tissue culture has not gone as fast as in culture of the cells of other vascular plants, and the number of cases achieved still does not satisfy sufficiently the demands of various research fields (Felix, 1994).

Like other members of the bryophytes, the mosses are haploid-dominant plants comprising ca. 84% of bryophyte families (Goffinet *et al*., 2001) and ca. 98% of the species (Glime, 2007). This class is unquestionably the most diverse and the largest class of Bryophyta (*sensu stricto*, excluding peat-mosses, lantern mosses, hair-mosses and alies).

Mosses have a variety of body types and are divided generally in two artificial groups according to the position of sexual organs and sporophytes: acrocarpous and pleurocarpous. The phylloids are in general tinny and mono-layered disposed on the cauloid so to offer the living space for various xenic inhabitants: bacteria, algae, protozoas, fungi and others. These features complicate the standard methodology for surface sterilization of vascular plants when used for axenically culturing the bryophytes.

Recently, bryophytes received a lot attention in chemistry research as a source of newly and/or bioactive compounds (Sabovljević & Sabovljević, 2008). However, the problem for analyzing and/or certain substance production in larger amount is often inadequate axenical material, i.e. impossibility to have clean material in enough quantity to establish bryophyte monoculture fields. One of solution, even it seems the problematic one, is to establish *In vitro* culture, to find the proper developmental conditions to propagate it for the wanted purpose.

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In this study, we have focused to pleurocarpous moss *Thamnobryum alopecurum* Nieuwland ex Gangulee widely but scatter distributed in the Northern Hemisphere growing on vertically surfaces of wet shaded rocks and tree stems. The genus *Thamnobyrum* comprise 42 species, distributed world-wide (Crosby *et al*., 2000) and 7 occur in Europe (Hill *et al*., 2006).

The aim of the present study was to establish stable *In vitro* culture of *T. alopecurum* and examine its development under axenic conditions, with a goal to develop *Thamnobryum* model system for *ex situ* conservation of related and threatened counterpart species: viz., *T. angustifolium* (Holt) Nieuwl. (critically endangered in the World Red List of Bryophytes, endemic to UK) and *T. fernandesii* (Sergio) Ochyra (endangered in the World Red List of Bryophytes, endemic to Madeira), *T. cataractum* Hodgetts & Blockeel (vulnerable in Europe (ECCB, 1995), endemic to UK), *T. rudolphianum* Mastracci (endemic to the Azores), *T. subseriatum* (Mitt.) ex Sande Lac.) Tan (endemic to Japan), *T. capense* (Broth. & Dix.) Enroth (endemic to South Africa), *T. corticola* (Kindb.) De Sloover (endemic to Bioco and Sao Tome), *T. malgachum* (Cardot) O'Shea (Madagascar), *T. speciosum* (Broth.) Hoe (endemic to Hawaii) or *T. maderense* (Kindb.) Hedenäs (subendemic to Macaronesia, with some isolated population in North Africa, Portugal, Spain and UK).

Also, the protocol adapted to some of the *Thamnobryum* culturing here can be use for culturing and not collecting the species from the wild like in case of North American *Thamnobryum alleghaniense* (C. Müll.) Nieuwl widely used in gardening.

The true challenge was to establish the axenic culture of this moss, having in mind that it grew in wet habitats and that every water drop from the moss material represent reservoir of xenic organisms problematic to dispose of, not to mention the thin films of water among phylloids and between phylloids and stems. Besides, it has separate sexes and its sporophytes are not easy to find in proper stage in nature.

Material and Methods

The material of the moss *T. alopecurum* was collected in Sauerland near Solingen, Germany, 03.12.2005 (Fig. 1). The voucher specimen was deposited in the Belgrade University Herbarium Bryophyte Collection (BEOU 4144).

Fig. 1. *Thamnobryum alopecurum* in *in situ* condition prior to collection for the *In vitro* establishment (Sauerland, Germany, *leg./photo* M. Sabovljevic, 03.12.2005).

After collection, the chosen plants were separated carefully from the mechanical impurity placed in glasses, covered with cheese cloth, and rinsed with tap water for 30 minutes. Sporophytes and apical parts of gametophytes were then disinfected for 5 minutes with a 3, 5, 7, 10, 13% or 15% solution of Sodium hypochlorite (commercial bleach, NaOCl). Finally, they were rinsed three times in sterile deionised water.

As a basal medium for establishment of *In vitro* culture, we used Murashige & Skoog (1962) (MS) medium containing Murashige and Skoog mineral salts and vitamins, 100 mg/l inositol, 0.70% (w/v) agar (Torlak purified, Belgrade), and 3% sucrose and BCD medium (see Sabovljević *et al*., 2009 for the media details).

Once, the establishment was done, and the plants produced, the *In vitro* developed plant segments (tips and protonema pieces) were used for further developmental experiments.

In order to observe the influence of sucrose and/or mineral salts on the morphogenesis of this species, the following medium composition combination were tested:

MS1: half strength of MS mineral salts, sugar free;
MS2: half strength of MS mineral salts, 1.5% sucrose;
MS3: half strength of MS mineral salts, 3% sucrose;
MS4: MS mineral salts, sugar free;
MS5: MS mineral salts, 1.5% sucrose;
MS6: MS mineral salts, 3% sucrose;
MS7: MS mineral salts, enriched with plant growth regulators (0.1μM IBA and 0.03μM BAP)
BCD1: BCD mineral salts, 1.5% sucrose;
BCD2: BCD mineral salts, 3% sucrose;
BCD3: BCD mineral salts, sugar free;

The pH of the media was adjusted to 5.8 before autoclaving at 114°C for 25 minutes.

The temperature and light duration varied in combined with sets of media:

Combination C1: 16/8 hours of light to darkness, at 25 ± 2°C.
Combination C2: 8/16 hours of light to darkness, at 20 ± 2°C.

Combination C3: 16/8 hours of light to darkness, at 20 ± 2°C.
Combination C4: 16/8 hours of light to darkness, at 18 ± 2°C.

Light was supplied by cool-white fluorescent tubes at a photon fluency rate of 47 µmol/m^{2}s. Cultures were subcultured for a period of 4-6 weeks. For analysis of condition set influence to development 10mm long apical segments (gametophyte), spores or protonema were transferred to various nutrient media. For each medium composition combined with light conditions, 40 transplants of *T. alopecurum* were cultivated.

The influence of tested environmental condition was quantified by measuring seep of plant gametophyte development and the morpho-anatomical similarity with nature plants estimated by the range -+, +- and ++, where ++ is the axenically developed plant looks the most like naturally developed plant (Table 1).

Tab. 1. The effects of selected substrata and light-temperature conditions on the development of *Thamnobryum alopecurum*. For the abbrevaiations see chapter Material and methods. (-+) – a bad growth of plantlets (+-) – a moderate growth of planlets, (++) – a very good growth of plantlets

	MS1	MS2	MS3	MS4	MS5	MS6	MS7	BCD1	BCD2	BCD3
C1	-+	-+	-+	-+	-+	-+	-+	-+	-+	-+
C2	+-	-+	-+	+-	-+	-+	+-	-+	-+	++
C3	+-	-+	-+	+-	-+	-+	+-	-+	-+	++
C4	+-	-+	-+	+-	-+	-+	+-	-+	-+	++

Results and Discussion

The attempts to establish the axenic culture from gametophytes i.e., 10 mm plant tips failed since the concentration for surface sterilizations killed the plant material or was not effective enough to kill the xenic organisms on the plants and not to harm the plants at the same time. So, even there where the plants survived the bleach surface sterilization and transferred to the mineral salts, it was overgrown quickly with fungi, algae and bacteria. The try outs to leave it until transferred plantlets overgrew the xenic organisms, for the purpose of the use of newly grown tips, remained useless (Fig. 2, note that the survival does not mean axenic as well). There is rather low probability percentage of good bleach surface sterilization of the moss tips, without harming plants, for establishment *In vitro* culture (Sabovljevic *et al*., 2003).

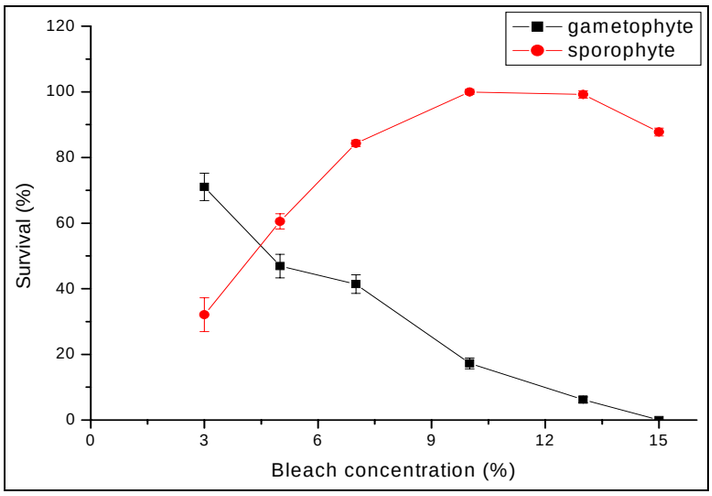

Fig. 2. The survival of the start moss material after bleach treatment.

Surface sterilization of the sporophytes was more successful since we choose the almost mature but unopened capsules and did the sterilization in various concentration of bleech for 5 minutes like for the gametophytes. The advantage of this process was that we did not need the capsules material itself (so we could harmed it lethally) but the spores from inside that should remain viable. Once, the surface of sporophytes was sterilized, the capsules were opened in sterile conditions and the spores were taken out with sterile needle to the mineral salt containing media. The success of this way starting culture concerning sterilization of start plant material was achieved with 100% at 10% bleach for 5 minutes. In higher concentration the sterilization percentage remain high but the bleach started to harm the spores quantified by spore germination slightly decrease.

Spores were germinated on MS medium enriched with sucrose (MS3). After releasing from the capsules, spores germinated in relatively high percentage (up to 100%). However, on the MS medium enriched with sucrose they remain in the phase of primary protonema. The subculturing to fresh medium was not a signal for plantlets to pass to the next developmental stage.

The protonema made callous rather than spreading far from spore or transferred protonemal peaces formed protonemal balls. On the mentioned medium even with the variation of light-length and temperature condition *T. alopecurum* remained in the phase of protonemal balls (Fig. 3.). A set of various combination of light length, temperature and mineral salts were tested to achieve the bud induction and gametophyte development.

It can be concluded that in the condition when medium contain the sugar (MS1, MS3, MS5, MS6, BCD1, BCD2) the spore germination is stimulated, but the gametophyte development stopped at protonemal eventually caulonemal stage. Schoefield (1981) stated that in most bryophytes, spores germinate 7-30 days after exposure of spores to good conditions. In our case, it was quicker when media contained sucrose (2-4 days) than the spore germination on sucrose free media. Interestingly, slight difference in gametophyte development was achieved when sucrose was put by (MS1, MS4).

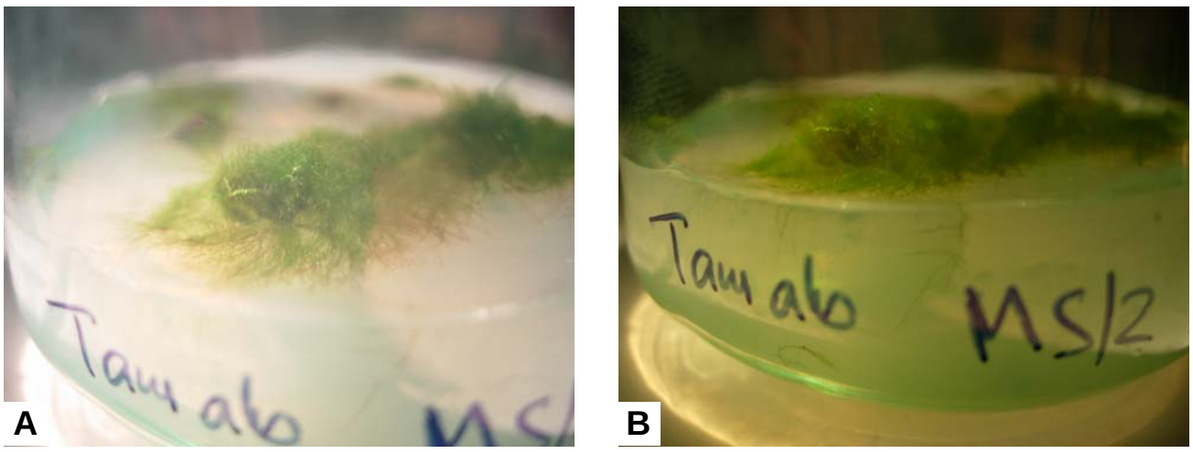

Fig. 3. The protonema ball of *Thamnobryum alopecurum* grown on MS half strength sucrose free medium. A. – one months old. B – three months old.

On BCD sugar free (BCD3), the bud formation was noticed after a month, similarly as on MS enriched with selected concentration of plant growth regulators (MS7). This is rather surprising since the last one already contained cytokinin, and in the previous medium the plants should produce it itself. Bopp (1952) explained that in native conditions protonema have to achieve the certain size which then produce enough amount of kinetin-like growth regulators released in substrate. This is a trigger for bud induction or passing from filamentous to meristemal growth.

Buds developed rapidly into a stem which again branched and continue growing achieving half-size and normal leaf shapes of natural plants but not the plant shape (Figs. 4 and 5). A very humid air condition of the growth-dishes can be a reason for this. Also, Birse (1957) showed that *Thamnium alopecurum* (Hedw.) B.S.G. (= *Thamnobryum alopecurum* (Hedw.) Nieuwl.) altered its growth form depending on its habitat condition, a feature was also demonstrated by Meusel (1935) and Schofield (1981).

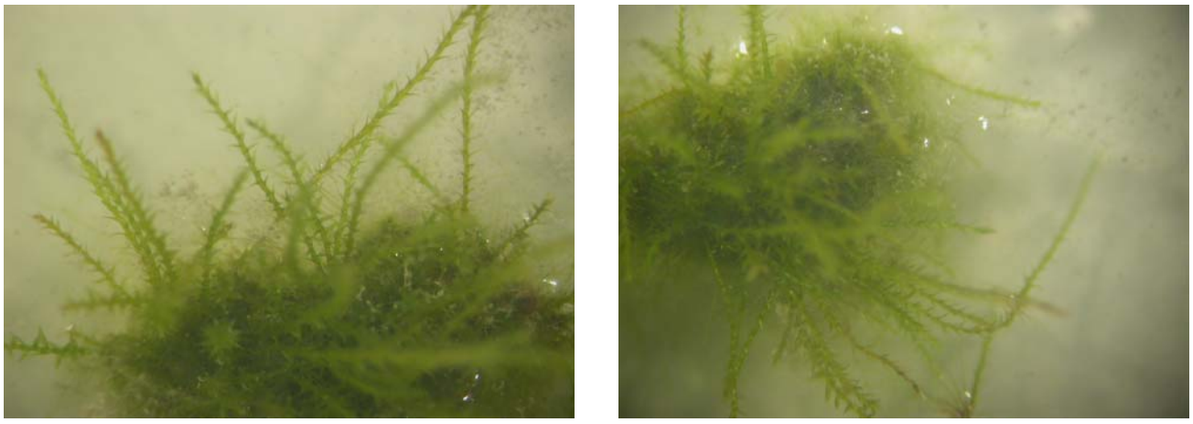

Fig. 4. The shoots developed on BCD3 combined with C4.

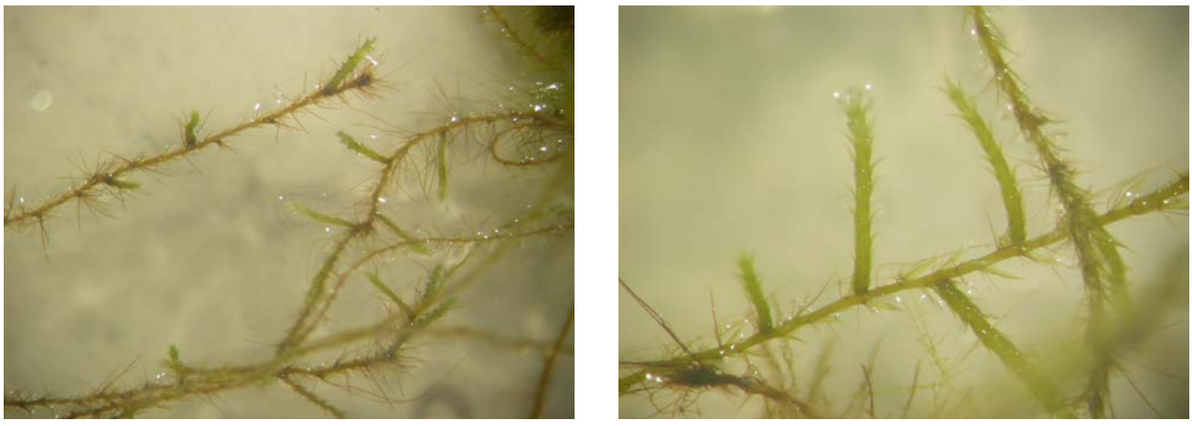

Fig. 5. Spreading on medium and new shoots developing on MS7 combined with C3.

The 10mm shoot and branch tips were used further for subculturing into new media combined with four combination of controlled conditions of day length and temperature.

The best developed and the most similar to the plants developed in nature were grown on BCD3 at temperature of 18°C or 20±2°C, at both day length. In the temperature of 25±2°C the plants were pale, long remaining in callous stage and/or produced significantly smaller, shorter, tinny, fragile and unbranched shoots. Spore germination was not effected by the day length and it was similar in all temperatures but at 25±2°C, spores germinate and stop from further developing of protonema.

When the plantlets tips were transferred to new media, they produced the secondary protonema, at first not far from the plantlets, but forming balls of protonema and then developing shot on them which spread further and branching depending on conditions of growth (Fig. 6). The protonemal balls as well as developed moss patches significantly produce phenolics which are released into the growth medium (Fig. 7) especially in higher temperatures. Phenolics can be explanation why the growth on certain combination of conditions tested is postponed. On the other hands, this can be also a great input for the production of natural phenolic compounds.

Axenically culturing *T. alopecurum* showed that different developmental stage of this moss species can be stimulated or stopped by various combination of mineral nutrition, light and temperature. The different growth condition should be taken into account for different *Thamnobryum* counterpart species conservation and propagation.

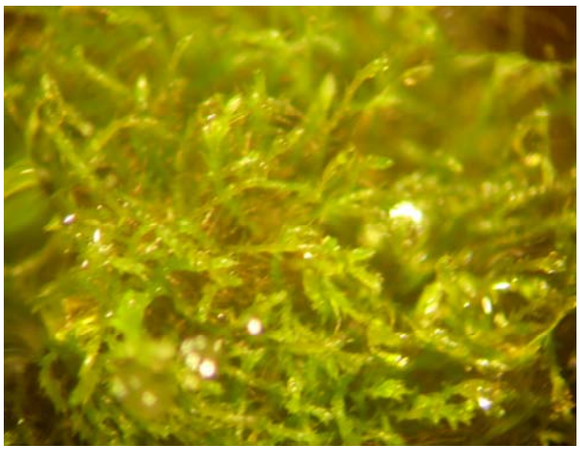

Fig. 6. A fully developed patch of *T. alopecurum* in axenic culture.

Fig. 7. *T. alopecurum* in *In vitro* culture. The color of the medium became brown-yellowish from plant released phenolic compounds (10 weeks old culture).

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