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# Axenically culturing the bryophytes: establishment and propagation of the moss *Hypnum cupressiforme* Hedw. (Bryophyta, Hypnaceae) in *in vitro* conditions

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**ABSTRACT:** The study gives the first report of *in vitro* culture of the pleurocarpous moss *Hypnum cupressiforme* and on the problems of axenicaly culturing this bryophyte and the conditions for establishment and propagation. 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 MS-sugar free medium irrelevant of day length. This moss is a counterpart of some rare and endangered mosses from the same genus and data presented should be taken into account of conservation and propagation of its counterparts as well. Its propagation is valuable for horticultural, pharmaceutical and bioindication purposes, as well.

**Key words**: moss, *Hypnum cupressiforme, in vitro*, development, propagation

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## INTRODUCTION

Bryophytes (comprising hornworts, liverworts and mosses) 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 (Crum 2001) species worldwide. 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 (e.g. 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; Cvetić *et al.* 2005).

Besides, developing of methodology in axenical cultivation and propagation of bryophytes are significant in rare species conservation both for *ex situ* and reintroduction (e.g. Batra *et al.* 2003; Bijelović *et al.* 2004; Sabovljević *et al.* 2005; Rowntree & Ramsay 2005, 2009; Gonzalez

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*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; Rowntree *et al*. 2007, 2010). This is especially valuable for the species like bryophytes, many of which are dioecious and possibly long-last naturally in sterile condition 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 attempt. 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 vascular plants, and the number of cases achieved still does not satisfy sufficiently the demands of various research fields (e.g. Felix 1994).

Like other members from the bryophytes, the mosses are haploid-dominant plants with ca. 84% of the total bryophyte families (Goffinet *et al*. 2001) and ca. 98% of the total 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 applied for axenically culturing the bryophytes.

Recently, bryophytes received a lot attention in their chemistry research as a source of newly and/or bioactive compounds (Sabovljević & Sabovljević 2008). However, the problem for analyzing and/or certain identified substance production in larger amount is often inadequate axenical material, i.e. impossibility to have clean material in enough amount neither 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. Besides, axenically culturing bryophytes enable us to get enough bio-material for chemical and pharmaceutical investigations, and developing horticultural values of the landscape (e.g. Japanese gardens). Some of the species used as bioindicators are never tested in controlled conditions. Also, stable axenic culture is the first step towards commercial productions for the various purpose. The economic value is not close to the economic value of the flower plants but the economic value of bryophytes increase with the defining more and more potentials in the market (e.g. Welch 1948; Thieret 1956; Nelson & Carpenter 1965; Glime & Saxena 1991; Arrocha 1996; Gomez & Wolf 2001; Lara et al. 2006).

In this study, we have focused to pleurocarpous moss *Hypnum cupressiforme* Hedw. (cypress-leaved plait-moss) common and widespread species of moss belonging to the genus *Hypnum*. It is found in all continents except Antarctica and occurs in a wide variety of habitats and climatic zones. It typically grows on tree trunks, logs, walls, rocks and other surfaces. It prefers acidic environments and is quite tolerant of pollution. It was formerly used as a filling for pillows and mattresses - the association with sleep is the origin of the genus name *Hypnum*.

The genus comprise over 200 species world-wide (Smith 2004) and 18 in Europe (Hill *et al*. 2006). *H. cupressiforme* includes 6 varieties in Europe (Hill *et al*. 2006).

*H. cupressiforme* is a small to medium-sized moss about 2-10 cm long. It is pleurocarpous, having prostrate, creeping stems which form smooth, dense mats. The stems are branched and covered in overlapping leaves giving the impression of a cypress tree. The stem leaves are long and thin measuring 1.0-2.1mm by 0.3-0.6mm. They are concave and sickle-shaped, tapering towards the tip. The branch leaves are smaller and narrower than those on the stems. The moss produces short, cylindrical and slightly curved capsules which contain the spores. The capsules are 1.7-2.4mm long and have a lid-like operculum measuring 0.6-0.9mm. They are borne on reddish-brown stalks which are 1-2.5cm long. The moss is dioicous, having separate male and female plants. *H. cupressiforme* is a highly variable species and numerous varieties have been described.

The aim of the present study was to establish stable *in vitro* culture of this species and examine its development under axenic conditions.

Also, the protocol adapted to some of the *H. cupressiforme* culturing here can be used for culturing various *Hypnum* species widely used in gardening, decoration especially before Christmas in the Western World. There is a high pressure to native populations worldwide for market purposes not only to targeted species since harvesting also affect local populations of these mosses and entails the accidental removal of rare or endangered species. (Nelson & Carpenter 1965; Glime & Saxena 1991; Arrocha 1996; Gomez & Wolf 2001; Lara *et al*. 2006; Glime 2007).

The true challenge was to establish the axenic culture of this moss, having in mind that it represents micro-habitat itself for many organisms and so is a reservoir of xenic organisms problematic to dispose of. Also, the tin films of water between phylloids and phylloids and stems represent micro-habitats enabling survival for many organisms in harsh environment surrounding the moss.

## MATERIAL AND METHODS

The start material of the moss *H. cupressiforme* (both sterile and fertile) was collected in Petnica near the town of Valjevo, W Serbia, March 2003 (Fig. 1, 2). The voucher specimen was deposited in the Belgrade University Herbarium Bryophyte Collection (BEOU SN). Besides, it has separate sexes and its sporophytes are not easy to find in proper stage in nature.

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).

**Fig. 1.** *H. cupressiforme*, sterile plants

**Fig. 2.** *H. cupressiforme*, fertile plants with sporophytes

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/m2s. 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 *H. cupressiforme* were cultivated.

## RESULTS AND DISCUSSION

The attempts to establish the axenic culture from gametophytes i.e. 10mm 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 overgrow the xenic organisms, for the purpose of the use

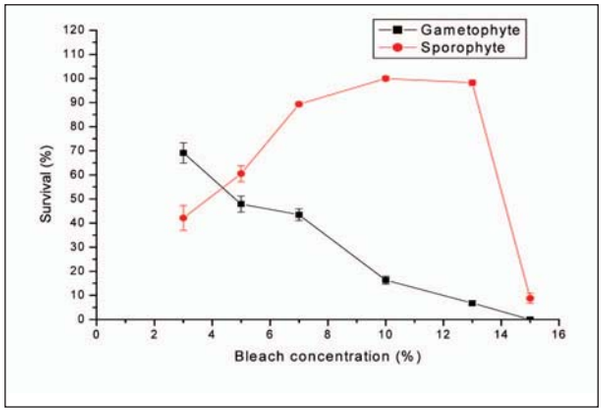


**Fig. 3.** Survival of *H. cupressiforme* gametophytes and sporophytes after 5 minute treatment with different concentration of commercial bleach.

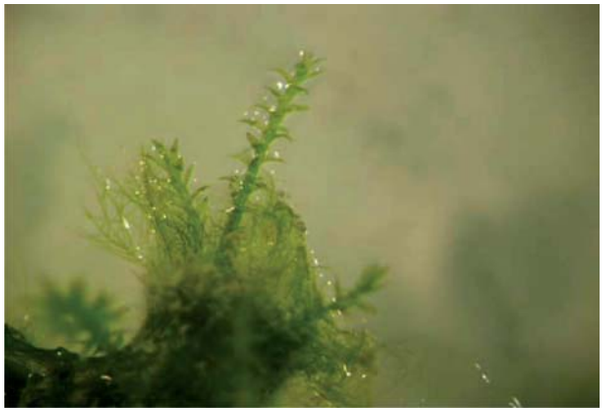

**Fig. 5.** Gametophytes and caulonema of *H. cupressiforme* on media enriched with sugars

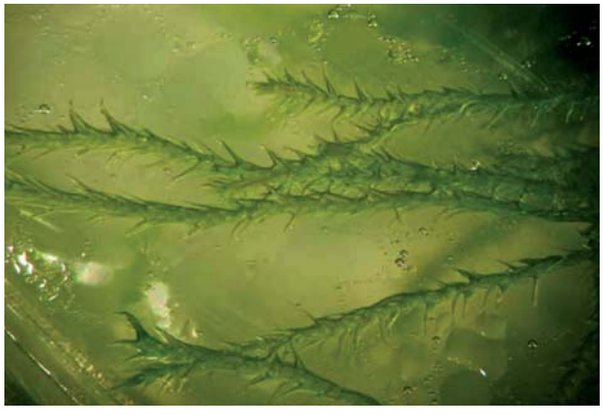

**Fig. 4.** Tender, elongated creeping branches of *H. cupressiforme*

**Fig. 6.** Fully developed branched *H. cupressiforme* gametophyte plant

of newly grown tips, remained useless (Fig. 3, 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 (e.g. Sabovljevic *et al,* 2003, Vujicic *et al,* 2009, 2010, Sabovljevic *et al*. 2010)

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 nearly 100% at 10% and 13% bleach for 5 minutes. In higher concentration the sterilization percentage remain high but the bleach started to harm the spores quantified by extensive spore germination 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%). On the MS medium enriched with sucrose or half sucrose, the phase of primary protonema pass to tender, elongated creeping branches (Fig. 4). In the subcultured condition to fresh medium they remained at the same developmental stage.

In all cultured conditions on various types of media *H. cupressiforme* formed secondary protonema and some kind of gametophytes. The protonema did not make callous or protonemal balls under any tested condition like some other bryophyte previously tested (Sabovljevic *et al. in press*).

Even with the variation of light-length and temperature condition, *H. cupressiforme* kept its growth and developed the gametophytes to certain extent.

A set of various combination of light length, temperature and mineral salts were used to achieve natural look of gametophyte development since the bud induction appear spontaneously in all applied conditions.

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 besides gametophyte developed also caulonema appeared (Fig. 5). 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, slightly difference in gametophyte development was achieved when sucrose was put by (MS1, MS4).

Bopp (1952) explained that in native conditions protonema have to achieved 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. Since, in all media tested, including MS7 that contained citokinin secondary protonema remained small (not larger than few millimeters), it can be made an assumption that *H. cupressiforme* produce quite enough kinetin-like growth regulators, and is sensitive to small amount of such a substances.

Buds developed rapidly into a stem which again branched and continue growing achieving full-size and normal leaf shapes of natural plants but not the plant shape (Figs. 6). A rather to very humid air condition of the growth-dishes can be a reason for this.

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 MS sugar free media at temperature of 18°C or 20±2°C, at both day length. In the temperature of 25±2°C the plants were more pale, slowly growing 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.

The phenolics released into the growth medium were noticed only when the sublucture period was longer than a month and not as much as in some other tested mosses (e.g. Sabovljevic *et al. in press*).

Axenically culturing *H. cupressiforme* shown that different developmental stage of this moss species can be stimulated or decreased by various combination of mineral nutrition, light and temperature. The different growth condition should be taken into account for different *Hypnum* counterpart species conservation and propagation. The results obtained here can be used for developing system for propagation and *ex situ* conservation of other rare (e.g. *H. imponens* Hedw., *H. lindbergii* Mitten, H. fertile Sendtn. ) and/or threatened *Hypnum* species (e.g. *H. pratense* (Rabenh.) W. Koch ex Spruce (EN in Ohio), *H. andoi* Smith (VU in Serbia), *H. revolutum* (Mitt.) Lindb. (EN in UK), *Hypnum vaucheri* Lesq. (VU in UK)).

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**REZIME**

# Aksenična kultura briofita: uspostavljanje i propagacija mahovine *Hypnum cupressiforme* (Bryophyta, Hypnaceae) u *in vitro* uslovima

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U radu je po prvi put predstavljeno uspostavljanje in vitro kulture pleurokarpne mahovine *Hypnum cupressiforme*, kao i problemi gajenja ove vrste u aseptičnim uslovima, te uslovi potrebni za propagaciju navedene vrste. Takođe, diskutovano je i o problemima sterilizacije sporofita, odnosno gametofita potrebnim za uspostavljanje *in vitro* kulture. Proučavan je uticaj hranljivih materija, dužine dana i različitih temperatura na razvojne procese *H. cupressiforme*. Pokazano je da su optimalni uslovi za mikropropagaciju iz izdanaka temperature u opsegu 18-20°C, MS (Murashige i Skoog) hranljiva podloga bez dodatka šećera, te da dužina dana nije bitna. Ova vrsta je bliska nekim retkim i ugroženim mahovinama istog roda (*Hypnum* sp.) i trebalo bi je takodje razmatrati u problemima konzervacije i propagacije. Propagacija *H. cupressiforme* je veoma važna u hortikulturi, farmaciji kao i za upotrebu kao bioindikatora.

**Ključne reči**: mahovina, *Hypnum cupressiforme, in vitro*, razviće, propagacija.