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Original Article

Axenically culturing the bryophytes: a case study of the moss *Herzogiella seligeri* (Brid.) Z. Iwats. (Plagiotheciaceae)

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***Abstract*:**
Vujičić, M., Sabovljević, A., Sabovljević, M.: ***Axenically culturing the bryophytes: a case study of the moss Herzogiella seligeri (Brid.) Z. Iwats. (Plagiotheciaceae). Biologica Nyssana, 1 (1-2), December 2010: 77-82.***

A moss genus *Herzogiella*, from the pleurocarpous family Plagiotheciaceae contains only seven species world wide. It occurs in North, Central and South America, Europe and Asia. In Europe, only three species occurred, namely *H. seligeri, H. striatella* and *H. turfacea* of which, the last one is threatened. With aim to develop the methodology for protection, conservation and active propagation of *H. turfacea*, more commonly distributed counterpart, *H. seligeri*, were taken from the National Park Fruška Gora and axenically culture were established. The study gives overview into the problems of sterilization, *in vitro* establishing, development, propagation and biology of species, as well as indices applicable to threatened counterpart.

Key words: *Herzogiella seligeri*, *in vitro*

Introduction

Bryophytes (comprising mosses, liverworts, hornworts and alies) are the second largest group of higher plants after flowering plants, with estimated 15,000 species worldwide (Hallingbäck and Hodgetts, 2000). 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 etc).

Although culturing plant tissues and organs under axenic conditions was firstly 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 and Wood, 1997; Shumaker and Dietrich, 1998; Reski, 1998; Wood *et al*., 2000; Cvetić *et al*., 2005, 2009; Bogdanović *et al.,* 2009; 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 (e.g. Batra *et al*., 2003; Bijelović *et al*., 2004; Sabovljević *et al*., 2005; Rowntree and Ramsay, 2005; 2009; Gonzalez *et al*., 2006; Mallon *et al*., 2007; Rowntree, 2006; Cvetić *et al*., 2007; Brezeanu *et al*, 2008; Chen et al., 2009; Vujičić *et al*., 2009, Rowntree *et al*., *in press*). This is especially valuable for the species like bryophytes many of which are dioecious and possibly long-lastingly in sterile condition naturally.

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 (Felix, 1994).

Like other members of the bryophyta, the mosses are diverse haploid-dominant plants. Mosses did not received a lot attention in chemistry research as a source of newly and/or bioactive compounds (Sabovljević and 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 amount neither to establish bryophyte monoculture fields. One of solution, even it seems problematic one is to establish *in vitro* culture, to find the proper developmental conditions and to propagate it for the wanted purpose (Sabovljević *et al.,* 2010)

In this study, we have focused to pleurocarpous moss *Herzogiella seligeri*. The aim of the present study was to establish stable *in vitro* culture of this species and examine its development under axenic conditions. Since up to date any plagiothecioid moss were not cultivated axenically, the true challenge was to establish the axenic culture of this moss having in mind its tiny and tender morphology and anatomy.

Material and methods

A moss *H. seligeri* (Bridel) Z. Iwats. (syn. *Leskea seligeri* Bridel, Musc. Rec. 2(2): 47. 1801; *Dolichotheca seligeri* (Bridel) Loeske; *Isopterygium seligeri* (Bridel) C. Jensen; *Plagiothecium silesianum* (Weber & Mohr) Schimper; *Plagiothecium seligeri* (Bridel) Lindberg; *Sharpiella seligeri* (Bridel) Z. Iwatsuki) has been studied. It is wide spread in contrast to its rare and endangered counterpart *H. turfacea* (Ireland, 1992). *H. seligeri* is one of the first invading species on the rotten logs and tree bases of all kind of trees. The capsules are mature at the beginning of summer. This is a species distinctive by its wide-spreading leaves, appearing in several rows, and its long (2-3.5 mm), arcuate capsules.

Plants in thin mats, light- to yellowish green, glossy. Stems to 30 × 1.5-3 mm, prostrate to ascending, pseudoparaphyllia lacking. Leaves wide-spreading, ovate to ovate-lanceolate, smooth, nondecurrent or 1-3 cells indistinctly decurrent, 1-2.5 × 0.5-0.9 mm, margins serrulate to serrate; cell walls pitted at leaf base, indistinctly pitted distally, sometimes pits lacking; median cells 30-70 × 5-7 μm; alar cells quadrate to short-rectangular, sometimes rounded to oval and inflated, 17-48 × 12-26 μm. Sexual condition autoicous. Seta light brown to red, 1.5-2.5 cm. Capsule light brown to reddish brown, inclined, 2-3.5 × 0.5-0.8 mm, cylindric, strongly arcuate, when dry contracted below mouth; operculum conic, 0.4-0.6 mm. Spores 12-22 μm.

The materials for axenic culture were collected in Fruška Gora Mt in March 2007 on rotten log and the voucher specimen was deposited in the Bryophyte Collection of the Belgrade University Herbarium (BEOU 4421).

After collection, the plants were stored in plastic bags at +4°C, till the begging of the experimental work. In the laboratory conditions, the material were cleaned under dissecting microscope from the visible mechanical impurity. The sporophyte with mature almost ripen but unopened capsules were separated, and the gametophyte tips consisted of the leafy stems of ca. 10 mm longitude were separated carefully, placed in glasses, covered with cheese cloth, and rinsed with tap water for 30 minutes. Sporophytes and gametophyte parts were then disinfected for 5 minutes with 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 and 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;

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 *H. seligeri* were cultivated.

The influence of tested environmental condition was quantified after 30 days by visually estimation of the plant habitat marked as the best those of appearance like in nature.

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 survive the bleach surface sterilization and transferred to the mineral salts, it was overgrown quickly with fungi, algae and bacterias. The try outs to leave it until transferred plantlets overgrow the xenic organisms, for the purpose of the use of newly grown tips, remained useless (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. Sabovljević et al., 2003)

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.

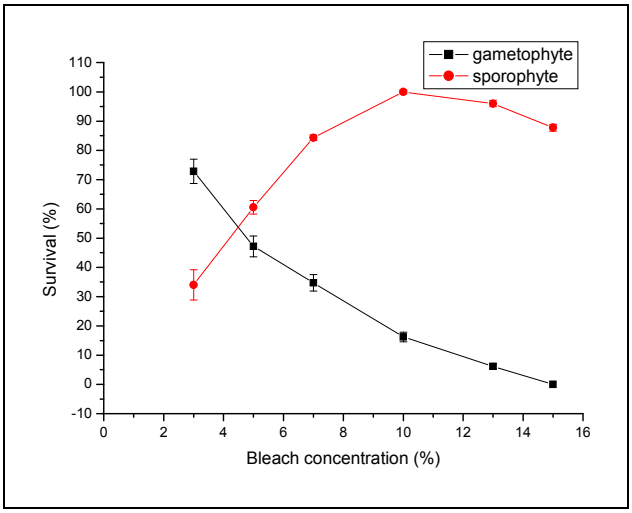
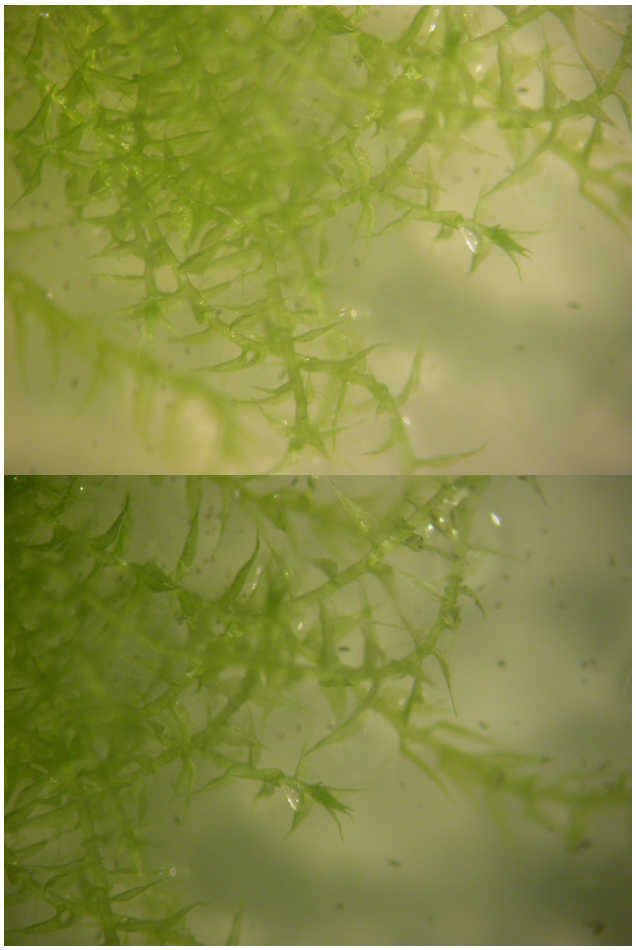


Fig 1. Percentage of surviving of gametophyte and sporophyte parts after bleach treatment.

The best bleach concentration for surface sterilizing of *H. seligeri* was 10%. The percentage of tested propagules, both of gametophyte tips and sporophytes survival, decreased with concentration increase (**Fig.1**). However, the bleach could not offer proper sterilization since propagules contained hardly disposal contamination afterwards with fungi, algae or bacteria, or the high concentration is lethal in high percentage (above 10%). The bleach concentration under 10% are functional since the propagules survived in high percentage, but not 100% of used material was axenical. Bleach concentrations above 7% for five minutes exposure

kill most of the propagules and are not appropriate for this moss gametophytes *in vitro* culture establishment.



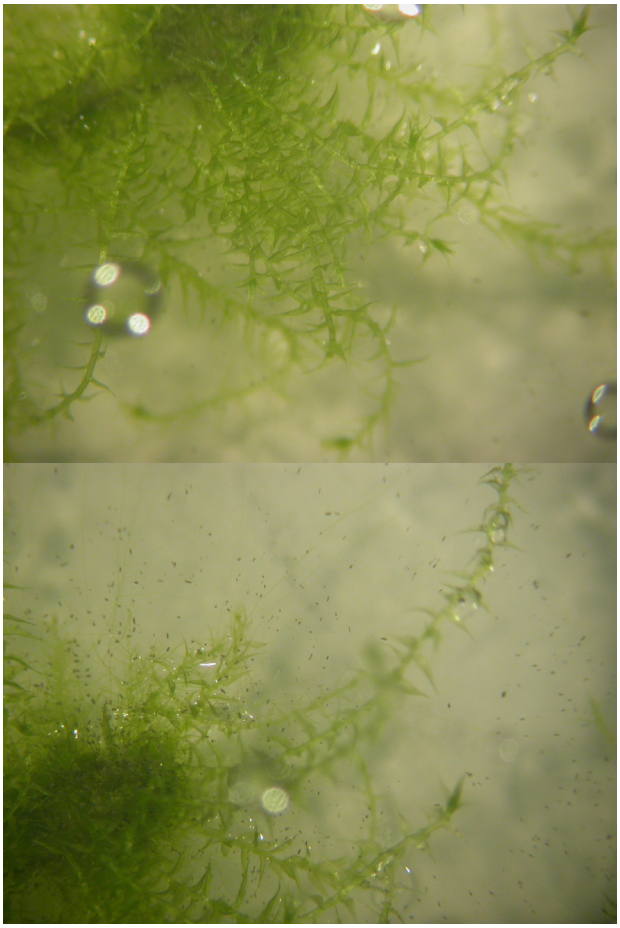
Figs. 2. and 3. A detail of *Herzogiella seligeri* gemetophyte developed in *in vitro* condition

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 with sucrose was not a signal for plantlets to pass to the next developmental stage. The protonema is not spreading far from spore in any media tested. The variation of light-length and temperature condition in *H. seligeri* cultures did not show significantly different behavior (**Figs. 2 and 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 (MS2, 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 (7-10 days) than the spore germination on sucrose free media. Interestingly, difference in gametophyte development was achieved when sucrose was put by (MS1, MS4).

On BCD sugar free (BCD3), the bud formation was noticed after a month. 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.

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. 4 and 5**). A rather very humid air condition of the growth-dishes favors elongation and growth.



Figs. 4. and 5. *Herzogiella seligeri* gemetophyte developed in *in vitro* condition

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 MS1 and BCD3 at temperature of 18°C or 20±2°C, at both day length. In the temperature of 25±2°C the plants produced slightly smaller, shorter, tinny, fragile and unbranched shoots, often developing contamination of blue-green algae. Spore germination was not effected by the day length and it was similar in all temperatures.

When the plantlets tips transferred to new media, they produced the secondary protonema, not far from the plantlets, developing shot on them which spread further and branching depending on conditions of growth (**Fig. 5**).

Axenically culturing *H. seligeri* 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 *Herzogiella* counterpart species conservation and propagation. Secondarily, the problems of contamination with blue-green algae can appear but they rather do not harm the plantlets like green algae, bacteria and fungi do.

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