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AXENICALLY CULTURING THE BRYOPHYTES: A CASE STUDY OF THE LIVERWORT *Marchantia polymorpha* L. ssp. *ruderalis* Bischl. & Boisselier (MARCHANTIOPHYTA, MARCHANTIACEAE)

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ABSTRACT: Axenic culture of *Marchantia polymorpha* ssp. *ruderalis* were establish from the gemmae. The most appropriate condition of culturing were searched concerning mineral nutrition, light and temperature with other invariable conditions. The best material to start aseptic *in vitro* culture were to use gemmae and to use commercial bleach for surface sterilization (7%). The fully developed plants developed in long day condition at mild temperature 18-20°C and high humidity. The best biomass yields were at supstrate containing half strenght MS nutrient salts supplemented with 1.5% sucrose.

Key words: liverwort, *Marchantia polymorpha* ssp. *ruderalis*, *in vitro*, development.

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 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 and WOOD, 1997; SHUMAKER and DIETRICH, 1998; RESKI, 1998; WOOD *et al*., 2000; CVETIĆ *et al*., 2005).

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*., 2006; 2007; ROWNTREE, 2006; CVETIĆ *et al*., 2007; BREZEANU *et al*, 2008; VUJIČIĆ *et al*., 2009). 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 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 bryophyta, the liverworts are diverse haploid-dominant plants. Some have the thalli that are morphologically simple and usually composed of only a few cell layers, while the others have multicellular complex thallus. Liverworts, the most basal lineage of extant land plants, have been used as model systems in the reconstruction of adaptations to life on land (KUTSCHERA and KOOPMANN, 2005). Recently, liverworts 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 to propagate it for the wanted purpose.

In this study, we have focused to liverwort *Marchantia polymorpha* L. ssp. *ruderalis* Bischl. & Boisselier (Marchantiaceae), a large thalloid liverwort, distributed worldwide that shows interesting biological properties. The aim of the present study was to establish stable *in vitro* culture of this species and examine its development under axenic conditions. The true challenge was to establish the axenic culture of this liverwort, having in mind that endobiotic fungi was reported from *Marchantia* previously (eg. LIGRONE *et al*., 2005).

MATERIALS AND METHODS

M. polymorpha ssp. *ruderalis* has a complex thalli, and is known to contain different axenical organisms within and on thallus. Besides, it has separate sexes and its sporophytes are not easy to find in proper stage. Therefore, an attempt was made to start axenic culture from the meristemal part of thallus and from the gemmae collected on the thalli grown spontaneously in greenhouse in Belgrade. The voucher specimen was deposited in the Belgrade University Herbarium (BEOU s/n).

After collection, the chosen thalli were separated carefully from the mechanical impurity placed in glasses, covered with cheese cloth, and rinsed with tap water for 30 minutes. Gemmae and thallus parts 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 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 developed the *in vitro* developed plantlets 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/m2s. Cultures were subcultured for a period of 4-6 weeks. For analysis of condition set influence to development 10mm long apical segments (gametophyte) or spores were transferred to nutrient media. For each medium composition combined with light conditions, approximately 40 transplants of *M. polymorpha* ssp. *ruderalis* were cultivated.

The influence of tested environmental condition was quantified by measuring seep of plant gametophyte development, biomass production in time (dry weight) after 30 days and the morpho-anatomical similarity with nature plants estimated by the range 1-10, where 1 is the axenically developed plant looks like nature developed plant.

RESULTS AND DISCUSSION

Today *M. polymorpha* is considered as a complex species that contains of at least three entities/species well separated ecologically and bio-chemically, but rather not morpho-anatomically (BOISSELIER-DUBAYLE *et al*. 1995). The study gives the first concrete insight into biology of the *M. polymorpha* ssp. *ruderalis*.

The best bleach concentration for surface sterilizing of *M. polymorpha* ssp. *ruderalis* gemmae was 7%. The percentage of both tested propagules survive decrease with concentration increase. However, the bleach could not offer proper sterilization for thallus part since either thallus parts suffer from contamination afterwards with fungi, algae or

bacteria, or the high concentration is lethal (above 10%). For gemmae the bleach concentration under 7% are functional since the propagules survived in high percentage (Fig. 1, 2) but not 100% of used material was axenical. Bleach concentrations above 7% for five minutes exposure kill most of the propagules and is not appropriate for this liverwort *in vitro* culture establishment.

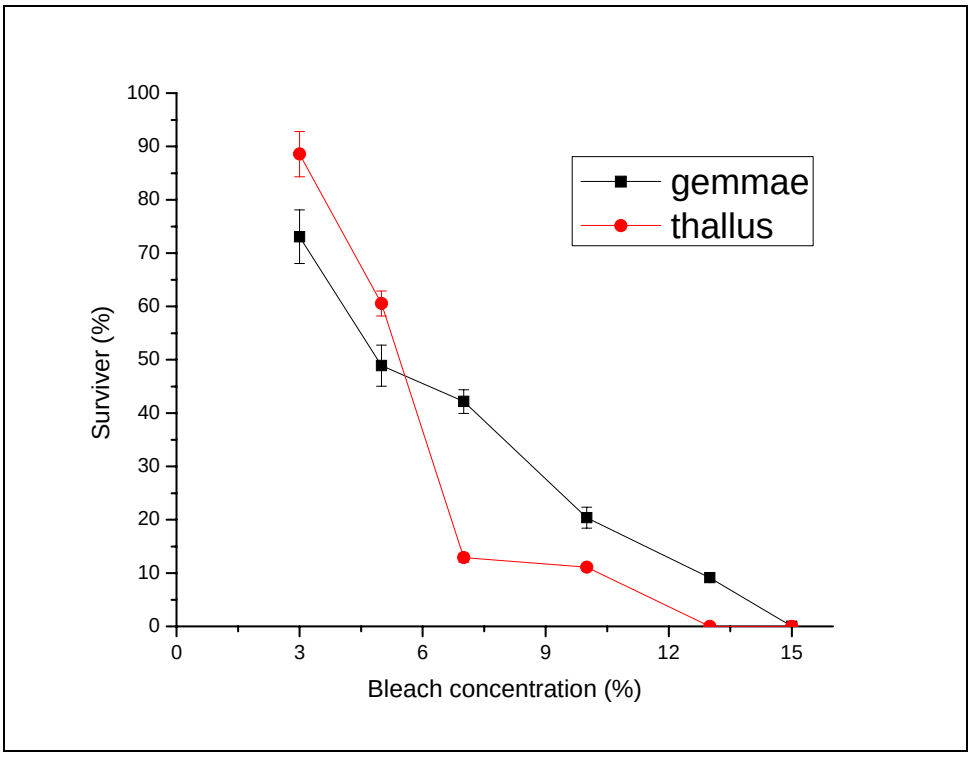

Fig. 1. Percentage of surviving of gemmae and thallus parts after bleach treatment.

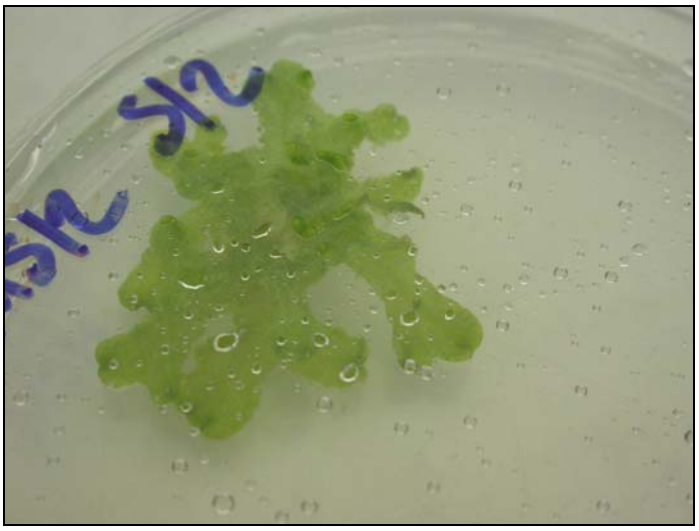

Fig. 2. Tender plantlet developed from germinated gemma of *M. polymorpha* ssp. *ruderalis*.

Once the material were axenically obtained it was propagate for the experimentations on selected condition of nutrition and light in the estimated humidity of 80±5%.

The axenically cultivated growth of *M. polymorpha* ssp. *ruderalis*, is the best on the half strength MS substrate enriched with 1.5% of sucrose, kept in long day condition (16/8 light) at mild temperatures (18 or 20±2°C). The plants in this condition are similar to those grown in nature and the biomass is the greatest comparing to other tested environmental condition combination (Fig. 3, 4).

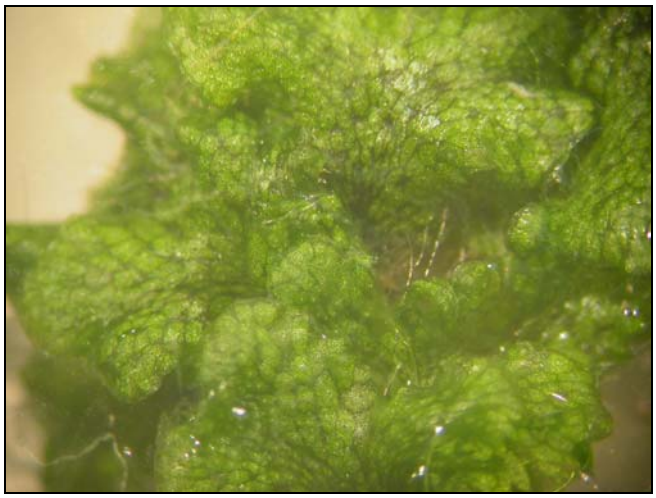

Fig. 3. Well developed thallus (gametophyte) of *M. polymorpha* ssp. *ruderalis*.

Fig. 4. The liverwort *M. polymorpha* ssp. *ruderalis* in *in vitro* condition.

Previous reports of aseptic culture are rare. ADAM and BECKER (1993) cultivated *M. polymorpha* on modified B5 liquid medium with 2% sucrose without phytohormones and vitamins. They used constant light and achieved some increase in biomass with double the light intensity. ADAM (1996) reported two way of aseptic culture establishment (by thallus parts and spores), this paper report newly methods of axenical culture of this liverwort starting from gemmae.

The long day preferential was rather expected since WAN (1925) stated that *M. polymorpha*, is apparently a "long-day" plant and might respond to artificially lengthened

days in the winter and thus be brought into proper condition for class study of the sex organs and sporophytes at any desired time.

However, the temperate regimes are not mentioned and it is not clear which of the subspecies were studied. *M. polymorpha* gametophytes respond to the photoperiod in a manner similar to that characterizing "long-day" plants among the flowering plants. When subjected to artificially lengthened days in the winter, mature antheridiophores are produced in 3 to 4 weeks, mature archegoniophores in 6 to 8 weeks, and mature sporophytes in 10 to 12 weeks according to WAN (1925). However, in any of environmental combination tested our species have not express sexuality neither production of gemmae cups. Even the high humidity was present in all our experimental designs, and a relatively high humidity tends to hasten the sexual response according to WAN (1925) the sex organs were not appeared. WAN (1925) assumed that a relatively low humidity tends to retard or may inhibit the production of sexual branches, especially archegoniophores. However, his experiments were not aseptic and so strictly controlled and no attempt was made in his experiments to control the temperature.

VOTH and HAMMER (1940) reported interesting comparison of plants grown on long photoperiod and on short photoperiod. The differences between the lots of plants grown on the two photoperiods were both quantitative and qualitative. Irrespective of the range of nutrient supply, plants on long photoperiod were larger and had greater dry weight than similar plants on comparable solutions on short photoperiod. This is in accordance with the results obtained in this study. Irrespective of the range of nutrient supply, plants on short photoperiod produced a greater total number of gemmae cups but on long photoperiod a greater number of gametangiophores than comparable plants on short photoperiod (VOTH and HAMMER, 1940). In the axenically strictly controlled experiments conducted by us (Tab. 1), any of gametes and gemmae cups were not appeared.

Tab. 1. The effects of selected substrata and light-temperature conditions on the development of *Marchantia polymorpha* ssp. *ruderalis*. For the abbreviations 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	BCD1	BCD2	BCD3
C1	-+	+-	-+	-+	+-	-+	-+	-+	-+
C2	-+	+-	-+	-+	+-	-+	-+	-+	-+
C3	+-	++	+-	+-	+-	-+	-+	-+	-+
C4	+-	++	+-	+-	+-	-+	+-	+-	+-

The choices of the medium tested were made according to the data presented in VOTH (1941).

VOTH (1941) stated that in experiments with *M. polymorpha,* the omission of K^+, Ca^{2+}, NO_3^- or PO_4^{3-} ions results in characteristic differences in the gross appearances of the plantlets. The lack of K^+ produces plants with tan-colored bases and slightly narrower tips. Absence of Ca^{2+} results in almost immediate death of the growing tips. Deficiency of NO_3^+ and PO_4^{3+} is indicated by reddening of scales, of rhizoids and of lower epidermis (VOTH, 1941). Plants lacking the former ion become light green, possess few gemmae cups and fork infrequently. He also find that plants growing on solution lacking PO_4^{3+} soon become dark green, have abundant gemmae cups and because of frequent dichotomy are rosettes. Deficiencies of Mg^{2+} and SO_4^{2+} are not indicated by any characteristic symptoms. When potassium is over-sufficient in media death and degeneration of plant apices occurred.

VOTH (1941) also stated the different reaction in gemmae cups production in male or female plants according to nitrates available.

VOTH (1941) find out that medium should contain the following 0.5M salts: KNO_3, $Ca(NO_3)_2$, $Mg(NO_3)_2$, $KHPO_4$, $MgSO_4$.

One of the problems of culturing *M. polymorpha* is the algal contamination which is hard to dispose of. All known algicidal compounds (e.g. copper acetate, tartarate and sulphate) letally damaged liverwort as well. Potassium permanganate in saturated solution was shown to kill algae but damaged the older plants equally, so not to be useful in algal contamination control. Propamidine, pentamidine and to some extent phenamidine and stilbamidine are useful in suppression of algal contamination in *M. polymorpha* culture. However, VOTH (1945) stated that not all the clones react equally to algicidal treatment, and that some geographical clones can not be cleared from algae with the same treatment. This is rather the consequence of different algal contamination in connection with various geographical origins.

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