



# Axenically culturing the bryophytes: a case study of the moss *Dicranum scoparium* Hedw. (Dicranaceae, Bryophyta)

Milorad VUJIČIĆ, Aneta SABOVLJEVIĆ and Marko SABOVLJEVIĆ\*

Institute of Botany and Garden Jevremovac, Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia

**ABSTRACT:** The aim of this study was to establish axenic culture of the moss *Dicranum scoparium* a counterpart of rare and widely endangered *D. viride*. The media contents, as well as light and temperature were varied to find the optimal conditions for spore germination, protonema growing, bud formation and gametophyte development. The best conditions for micropropagation or axenically bryo-farming is to grow *D. scoparium* on the MS medium enriched with sucrose (1.5%), at 18-20°C independent of light length condition.

**KEY WORDS:** moss, *Dicranum scoparium*, *in vitro*, axenical culture

Received 30 September 2009

Revision accepted 30 November 2009

UDK 582.32.085.2

## INTRODUCTION

Bryophytes, although the second largest group of terrestrial plants, received much less attention in conservation and protection in comparison to vascular plants and higher animals. However, bryophytes are documented to decrease in nature affected by habitat devastation directly or indirectly.

Thus, for some species there is urgently need of the active protection and conservation with aim to save some species from extinction.

For many of the species in threat of extinction, there are very few data on their biology which so diminish the attempts to work on their conservation.

By definition, material for the initiation of *ex situ* collection (i.e. *in vitro* culture, *in vitro* farming) of threatened species is rare (ROWNTREE & RAMSAY 2009). Consequently, it is important that technique used have a high success rate and to be applicable to related species at least. The importance of the development of the methods of *in vitro* and/or *ex situ* maintaining of bryophytes was already elaborated elsewhere (e.g. ROWNTREE & RAMSAY 2005; ROWNTREE 2006).

One of the species in high risk of extinction is *Dicranum viride* (Sull. & Lesq. in Sull.) Lindb. (species of community interest listed in the Habitat Directive 92/43 EEC under annex II, Bern Convention Species, appendix 1). It is ranged in northern hemisphere but rare all over. In Europe, this species is threatened all over (ECCB 1995). In Serbia, *D. viride* is estimated by available data as vulnerable (VU) (SABOVLJEVIĆ *et al.*, 2004).

Since this species is very rare, hardly accessible in enough amounts, a more spread counterpart of it was used to study axenically establishing, growing and propagation of *Dicranum* species with aim to apply the knowledge acquired to the *D. viride*, when starting project of its active conservation and protection and material propagation for spreading in nature.

The problem of establishing *in vitro* culture of bryophytes, even they were the first plants grown in *in vitro* condition (SERVETTAZ 1913) remain up to date. Besides the problems with bryophyte establishment in axenic culture, it is often problem of material availability, genetic variability of material, disposal of axenic organisms living on bryophytes and low level of species biology knowledge.

\*correspondence: marko@bio.bg.ac.rs

The problems, perspective and potential usefulness of *in vitro* bryophyte farming overpass the issue of bryophyte *ex situ* conservation and can be found only in few studies (BIJELOVIĆ & SABOVLJEVIĆ 2003; SABOVLJEVIĆ *et al.* 2003, 2006, 2009; BIJELOVIĆ *et al.* 2004; DUCKETT *et al.* 2004; CVETIĆ *et al.* 2005; GONZÁLEZ *et al.* 2006; MALLÓN *et al.* 2006; ROWNTREE 2006; SABOVLJEVIĆ & SABOVLJEVIĆ 2008).

## MATERIALS AND METHODS

The complete developed plants (both sporophyte and gametophyte) of *D. scoparium* from the Tara Mt. (Western Serbia) were collected in the May 2007. Collected materials were stored in plastic bag till laboratory and then in paper bag at room temperature until manipulation and establishing axenical culture. The collected material of *D. scoparium* were chosen so that the sporophytes contain also ripen unopened capsules with intact opercula. The voucher specimen was deposited in the Belgrade University Herbarium BEOU2158.

Cultures initiation was tried from almost mature spores, from unopened capsules that were taken for sterilization and young gametophyte vegetative shoots (i.e. not bearing sex organs or sporophytes). After collection, the chosen sporophytes or gametophytes were separated carefully from the impurity and each other, placed in glasses, covered with cheese cloth, and rinsed with tap water for 30 minutes. Sporophytes and gametophyte shoots were then disinfected for 5 minutes with a 3, 5, 7, 10, 13% or 15% solution of sodium hypochlorite. Finally, the sporophytes and gametophyte shoots were rinsed three times in sterile deionized 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). In order to observe the influence of sucrose and/or mineral salts on the morphogenesis of this species, the following medium compositions were used:

- 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 1: 16/8 hours of light to darkness, at  $25 \pm 2^\circ\text{C}$ .

Combination 2: 8/16 hours of light to darkness, at  $20 \pm 2^\circ\text{C}$ .

Combination 3: 16/8 hours of light to darkness, at  $20 \pm 2^\circ\text{C}$ .

Combination 4: 16/8 hours of light to darkness, at  $18 \pm 2^\circ\text{C}$ .

Light was supplied by cool-white fluorescent tubes at a photon fluency rate of  $47 \mu\text{mol}/\text{m}^2\text{s}$ . Cultures were subcultured for a period of 4-6 weeks. For analysis of condition set influence to development 10 mm long apical segments (gametophyte) or spores were transferred to nutrient media. For each medium composition, approximately 40 transplants of *D. scoparium* or 0.5 ml of water containing spores from one unopened capsules were cultivated in four Petri-dishes. The influence of tested environmental condition was quantified by measuring elongation of initial gametophyte explants and the index of multiplication, protonemal development (both secondary and primary) and new bud formation.

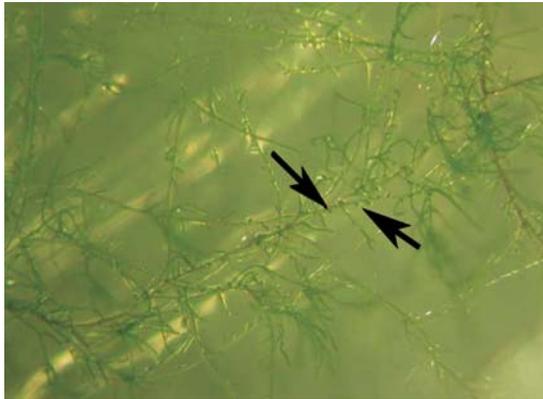
## RESULTS AND DISCUSSION

Surface sterilization of sporophytes was effective at concentration of 7, 10, 13% and 15% solution of sodium hypochlorite for five minutes. Concentrations of 10-15% of sodium hypochlorite solution for five minutes period were lethal for gametophyte shoots. Even, if the period of sterilization were shortened, the green part of plants could not survive applied concentrations. In lower sodium hypochlorite concentration gametophyte shoots survived but also contaminants, xenical organisms living on mosses.

Thus, contaminated starting material produced secondary protonema and after ca. one week period it was overgrown with fungi, algae or bacteria and could not be used for further developmental researches. So, the further developmental studies were focused on plants development deriving from spores on different media.

The highest percentage (95%) of spores germinated on basal MS medium (supplemented with 3% sucrose). Spore germination of *D. scoparium* occurred 22 days after establishing *in vitro* culture. Spores germinated ineffectively of light duration but prefer slightly lower temperature. At temperature of  $25 \pm 2^\circ\text{C}$ , the germination rate is estimated up to 60%.

Visible protonema can be noticed already 30 days after spore germination, and it grows rapidly at  $18^\circ\text{C}$  and  $20 \pm 2^\circ\text{C}$ . Media containing sugar slightly increased the germination speed, but after germination sugar available in substrate seems to play role in bud induction on protonema since protonema developed on media without sugar remain in that stage for a long period.



**Fig. 1.** Protonema of *Dicranum scoparium* with buds indicated by arrows.



**Fig. 2.** A detail of *Dicranum scoparium* gametophyte developed in *in vitro* condition



**Fig. 3.** *Dicranum scoparium* gametophyte developed in *in vitro* condition

Four weeks after bud development, buds started to grow to fully developed gametophytes (Fig. 1). The number of developed buds was not high, and they remained for a long time in the bud phase without growing to fully developed gametophytes.

The best medium for the plantlets regeneration both from primary and secondary protonema was MS5. The plants developed at this media were most likely to the ones developed in nature, both morphologically and in size (Figs. 2, 3). The optimal temperature for growth was 20°C, as well as 18°C. At 25°C, bud formation decreases or even lack and the caulonema stage remain for the long period. The day length was irrelevant for the plant development at the media tested.

The majority of *ex situ* collections consists of angiosperm seeds (e.g. Millennium Seed Bank), ignoring the vast diversity of non-vascular plants (ROWNTREE & RAMSAY 2009).

The spore germination of *D. scoparium* are increased on media supplemented with 3% sucrose. However, to reach bud induction and fully developed gametophytes

one should use less sugar in growing substrate, and such material is applicable for micro-propagation on the same media where secondary protonema, followed by bud formation and gametophyte plantlets appear in highest percentage.

## CONCLUSION

Surface sterilization is easier to achieve on sporophyte than to gametophyte. The best conditions for micro-propagation or axenically bryo-farming is to grow *D. scoparium* on the MS medium enriched with sucrose (1.5%), at 18-20°C independent of light length condition. The results obtained can be used for axenically culturing *D. viride*, a rare and endangered counterpart of tested species.

**Acknowledgement** – This work was supported by Serbian Ministry of Science and Technological Development – grants No. 143015 and 143031.

## REFERENCES

- BIJELOVIĆ A & SABOVLJEVIĆ M. 2003. Callus induction and plant regeneration in the moss *Aloina aloides* (Schultz.) Kindb. (*Pottiaceae*, *Bryopsida*). *Arch. Biol. Sci., Belgrade* **55**: 77-80.
- BIJELOVIĆ A, SABOVLJEVIĆ M, GRUBIŠIĆ D & KONJEVIĆ R. 2004. Phytohormone influence on the morphogenesis of two mosses (*Bryum argenteum* Hedw. and *Atrichum undulatum* (Hedw.) P. Beauv.). *Israel J. Plant Sci.* **52**: 31-36.
- CVETIĆ T, SABOVLJEVIĆ M, SABOVLJEVIĆ A & GRUBIŠIĆ D. 2005. *In vitro* culture and apogamy – alternative pathway in life cycle of the moss *Amblystegium serpens* (Amblystegiaceae). *Arch. Biol. Sci., Belgrade* **57**: 267-272.
- DUCKETT JG, BURCH J, FLETCHER PW, MATCHAM HW, READ DJ, RUSSEL AJ & PRESSEL S. 2004. In vitro cultivation of bryophytes: a review of practicalities, problems, progress and promise. *J. Bryol.* **26**:3-20

- ECCB. 1995. Red Data Book of European Bryophytes. European Committee for Conservation of Bryophytes (ECCB), Trondheim.
- GONZÁLEZ ML, MALLÓN R, REINOSO J & RODRÍGUEZ-OUBIÑA J. 2006. *In Vitro* micropropagation and long-term conservation of the endangered moss *Splachnum ampullaceum* Hedw. *Biol. Plant.* **50**:339–345
- MALLÓN R, REINOSO J, RODRÍGUEZ-OUBIÑA J & GONZÁLEZ ML. 2006. *In vitro* development of vegetative propagules in *Splachnum ampullaceum*: brood cells and chloronematal bulbils. *Bryologist* **109**:215–223
- MURASHIGE T & SKOOG F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plantarum* **15**: 473-497
- ROWNTREE JK. 2006. Development of novel methods for the initiation of *in vitro* bryophytes culture for conservation. *Plant Cell Tissue Organ Cult.* **87**:191-201.
- ROWNTREE JK & RAMSAY MM. 2005. *Ex situ* conservation of bryophytes: progress and potential of a pilot project. *Bol. Soc. Esp. Briol.* **26-27**:17-22.
- ROWNTREE JK & RAMSAY MM. 2009. How bryophytes came out of the cold: successful cryopreservation of threatened species. *Biodivers. Conserv.* **18**: 1413-1420.
- SABOVLJEVIĆ M, BIJELOVIĆ A & DRAGIĆEVIĆ I. 2003. *In vitro* Culture of Mosses: *Aloina aloides* (K.F.Schultz) Kindb., *Brachythecium velutinum* (Hedw.) B.S., G. and *Grimmia pulvinata* (Hedw.) Sm. *Turk. J. Bot.* **27**:441–446
- SABOVLJEVIĆ A & SABOVLJEVIĆ M. 2008. Bryophytes, a source of bioactive and new compounds. In: GOVIL JN & SINGH VK (eds.). *Phytopharmacology and Therapeutic Values IV, the Series "Recent Progress in Medicinal Plants"*. Studium Press, Houston, Texas, USA. pp. 9-25.
- SABOVLJEVIĆ A, CVETIĆ T & SABOVLJEVIĆ M. 2006. The establishment and development of the Catherine's moss *Atrichum undulatum* (Hedw.) P.Beauv. (Polytrichaceae) in *in vitro* conditions. *Arch. Biol. Sci., Belgrade* **58**: 87-93.
- SABOVLJEVIĆ A, SABOVLJEVIĆ M & JOCKOVIĆ N. 2009. *In vitro* culture and secondary metabolite isolation in bryophytes. In: JAIN SM & SAXENA PK (eds.) *Protocols for in vitro cultures and secondary metabolite analysis of aromatic and medicinal plants. Methods in Molecular Biology* **547**. Humana Press, pp. 117-128.
- SABOVLJEVIĆ A, SABOVLJEVIĆ M, GRUBIŠIĆ D & KONJEVIĆ R. 2005. The effect of sugars on development of two moss species (*Bryum argenteum* and *Atrichum undulatum*) during *in vitro* culture. *Belg. J. Bot.* **138**: 79-84.
- SABOVLJEVIĆ M, CVETIĆ T. & STEVANOVIĆ V. 2004. Bryophyte Red List of Serbia and Montenegro. *Biodivers. Conserv.* **13**: 1781-1790.
- SERVETTAZ C. 1913. Recherches expérimentales sur le développement et la nutrition des mousses en milieux stérilise. *Ann. Nat. Bot. Biol. Veg.* **17**: 111-223.

## REZIME

## Aksenična kultura briofita: mahovina *Dicranum scoparium* Hedw. (Dicranaceae)

Milorad VUJIČIĆ, Aneta SABOVLJEVIĆ, Marko SABOVLJEVIĆ

Cilj rada bio je da se uspostavi aksenična kultura mahovine *Dicranum scoparium*, srodnik retke i ugrožene vrste *D. viride*. Sadržaj podloge, svetlost i temperature su varirani da bi pronašli optimalni uslovi za klijanje spora, rast protoneme, formiranje pupoljaka i razvoj gametofita. Najbolji uslovi za mikropropagaciju ili aksenični uzgoj ispitivane vrste *D. scoparium* su MS mineralna podloga sa dodatkom 1.5% saharoze na temperaturi od 18-20°C nezavisno od dužine dana.

**Ključne reči:** mahovina, *Dicranum scoparium*, *in vitro*, aksenična kultura