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DEVELOPMENT OF THE MOSS *POGONATUM URNIGERUM* (HEDW.) P. BEAUV. UNDER *IN VITRO* CULTURE CONDITIONS

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Abstract – Pogonatum urnigerum (*Polytrichaceae*) *in vitro* culture was established from spores collected in nature. Both protonema and gametophore stages of gametophyte development were obtained. Also, a stable callus culture was established using hormone-free nutrient medium. The best nutrient medium for development was half-strength Murashige-Skoog medium supplemented with 1.5% sucrose. Auxin treatment enabled some gametophores to develop, but prolonged treatment induced early senescence. Tissues grown on cytokinin did not produce any gametophytes and did not survive prolonged treatment.

Key words: Moss development, Pogonatum urnigerum, *in vitro* culture

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INTRODUCTION

The gametophyte phase of mosses, which is dominant in their life cycle, is a favorable model system for genetic, biochemical, metabolic, and developmental studies (C o v e et al., 2006). It consists of a filamentous stage, the protonema, part of which bears buds that develop into leafy gametophores. However, the establishment of axenic culture is essential for obtaining relevant results, since material from nature is hard to separate from other moss species, microorganisms, invertebrates, and dust and soil particles.

Although it is sometimes stated that bryophytes are easily cultured *in vitro* (C o v e et al., 2006), most of this work has been done on *Physcomitrella patens*, and relatively few species have in fact been sucessfully induced for form stable axenic cultures.

As in higher plants, development under axenic conditions is species-specific. There are many descrepancies in the response of different moss species to the same treatment under *in vitro* culture (B i j e l o v i ć et al., 2004; S a b o v l j e v i ć et al., 2005). Mosses also contain numerous biologically active compounds (M u e s, 2000; S a b o v l j e v i ć et al., 2001, 2006a), but only a small percent of species have yet been thoroughly studied. Introduction of new species into axenic conditions and maintenance of stable cell and tissue cultures is therefore essential as a start for in-depth investigation of the physiology and potential uses of bryophytes.

Pogonatum urnigerum (*Polytrichaceae*) is a relatively robust moss with characteristic glaucous green leaves that grows on well-drained acidic soil. The aim of the present study was to establish stable *in vitro* culture of this species and examine its development under axenic conditions.

MATERIALS AND METHODS

Fully developed *Pogonatum urnigerum* plants were collected on Mt. Kopaonik (SE Serbia) in autumn of 2005. Fresh, unopened sporophytes were surface sterilized by dipping in 25% commercial bleach (8% active NaOCl) for 3 minutes, and thoroughly rinsed in sterile deionized water. The cap was then removed and spores released on nutrient medium.

Two nutrient media were tested: MS [containing mineral salts and vitamins according to Murashige and S k o o g (1962), 3% sucrose, and 0.7% agar] and MS/2 (containing half-strength Murashige-Skoog mineral salts and vitamins, 1.5% sucrose, and 0.7% agar). In some ex-

periments, 10^{-6} M indole-3-acetic acid (IAA) or benzyladenine (BA) was added to the MS/2 medium. Prior to sterilization, pH was adjusted to 5.8. Cultures were grown at 25±2ºC under long-day conditions (16 h light/8 h dark). Light was provided by fluorescent tubes, and irradiance on the plant-growth shelf was 45 $\mu mols^{-1}m^2$.

Fresh microscopic preparations were examined using a Leica DMLS microscope with a digital camera attached.

RESULTS

Spore germination began soon after introducing spores to nutrient media. Independent of the nutrient medium used (hormone-free MS or MS/2 medium), spore germination was evident after 8 days of *in vitro* cultivation. (Fig. 1). Primary protonemata that emerged from germinated spores developed rapidly, and after three weeks both chloronemata and caulonemata were visible (Fig. 2). Even at this early stage caulonemata began to create bud-like structures.

At this stage further development was inhibited on MS medium, causing disintegration of chlorophyll and cessation of growth. Only a few "islets" of green callus-like tissue remained and continued growth as a callus tissue when transferred to MS/2 medium. Protonemata obtained from spores germinated on MS/2 medium continued elongation and formed many buds. In all further experiments, only MS/2 medium was used. After 90 days of subcultivation, a small percent of buds growing on MS/2 medium developed into plantlets (Fig. 3), while at the same time some explants transformed into calli (Fig. 4).

To test factors that influence further bud development, protonemata with buds were transferred to MS/2 media containing either 1 µM IAA or 1 µM BA. In the control group grown on hormone-free MS/2 medium, some protonemata developed plants in the course of 30 days. In IAA treated protonemata, only a few plantlets appeared, while protonemata began to lose chlorophyll without callus formation. After 30 days of BA treatment only non-green senescent calli and protonemata were visible (Fig. 5)

Fig. 1. *Pogonatum urnigerum* germinated spores 8 days after introduction on nutrient medium; the bar represents 100 µm.

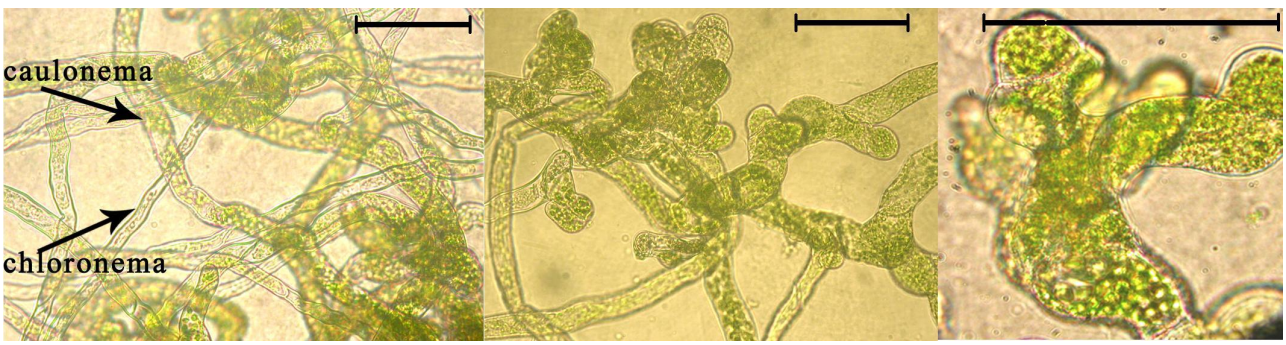

Fig. 2. Protonemata and early stages of bud formation 30 days after introduction of spores on MS/2 nutrient medium; the bar represents 100 µm.

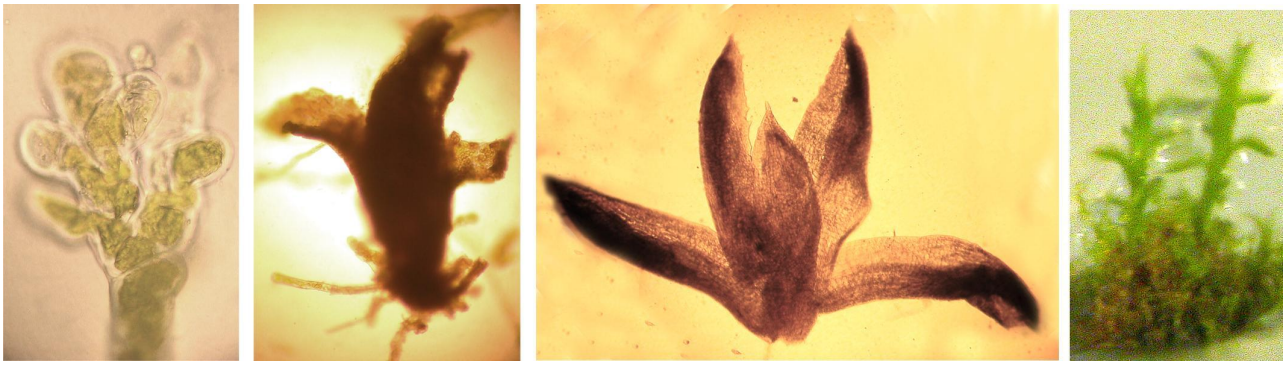

Fig. 3. Stages during development of a bud into a plantlet in axenic culture of *Pogonatum urnigerum*..

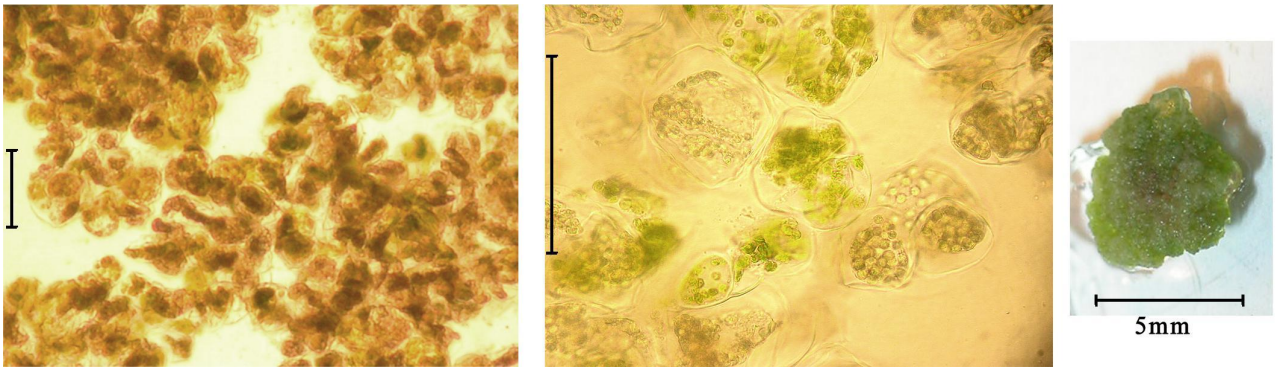

Fig. 4. Developed callus tissue after 120 days of subcultivation on MS/2 nutrient medium. The bar represents 50 µm.

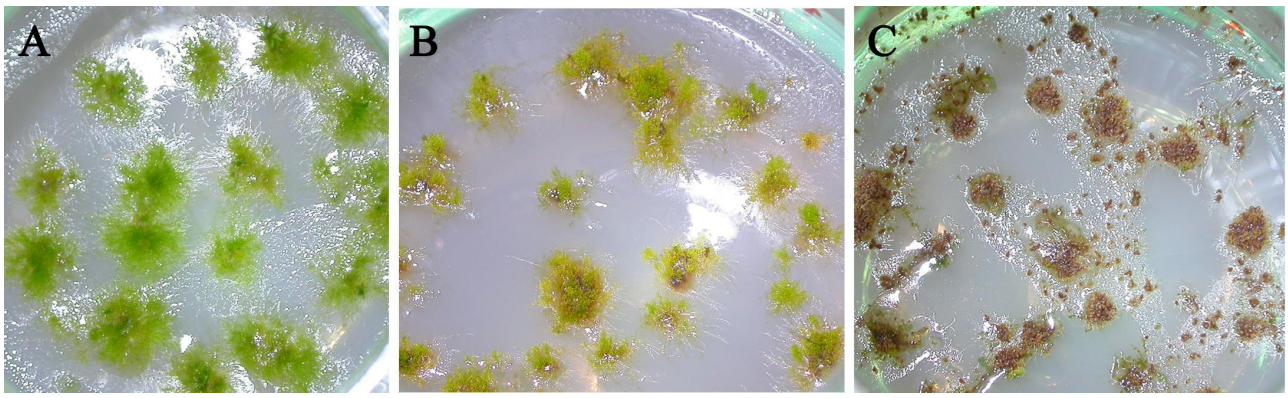

Fig. 5. Effect of hormone free MS/2 medium (**A**), MS/2 medium containing 1 µM IAA (**B**), and MS/2 medium containing 1 µM BA (**C**) on *in vitro* development of *Pogonatum urnigerum* protonemata.

DISCUSSION

The formerly accepted protocol for bryophyte introduction into axenic culture developed in our laboratory (S a b o v l j e v i ć et al., 2002, 2003) was also successful in the case of *Pogonatum urnigerum*. Hormone-free media with half-strength MS mineral salts and vitamins allowed full gametophyte development. Despite extensive bud formation, only a small fraction of buds developed into gametophores, indicating that other factors such as light regime, temperature, medium pH, and osmolarity should be varied in order to activate more exten-

sive gametophore development. Ours is the first report of callus formation in *Pogonatum urnigerum*, although this phenomenon has so far been reported for 18 other moss species (F e l i x, 1994).

Callus formation and relatively fast senescence of protonemata are probably due to the fact that the protonemata of this species are not persistent in nature. In keeping with the habitat of this species, growing in liquid medium resulted in somewhat faster growth and delayed senescence (data not presented).

Addition of 1.5% sucrose to the media positively affected gametophyte development in *P. urnigerum,* which was also observed in *Bryum argenteum* (S a b o v l j e v i ć et al., 2005) and another polytrichaceous moss, *Atrichum undulatum* (S a b o v l j e v i ć et al., 2006b). However, the level of sucrose influence on morphogenesis of different bryophyte species was not the same: in *B. argenteum in vitro* culture, a quite high index of multiplication was recorded with addition of 1.5% sucrose to the medium; in *A. undulatum*, on the other hand, normal gametophyte development was induced, as in the related polytrichaceous species *P. urnigerum*.

Of the five main groups of phytohormones, only auxins and cytokinins are documented as natural signal substances in mosses (C o v e and A s h t o n, 1984; B o p p and B h a t l a, 1985). Both of these hormone groups not only exist in mosses, but also have basic functions in the regulation of normal development. The known effects of auxins on moss development include inhibition of protonema growth, transformation of buds to filaments, torsion of young stems and complete suppression of leaves on gametophores (B o p p, 1953; S o k a l et al., 1997). Cytokinins have been shown to induce bud formation in protonemata cultures of some moss species (S p e i s s, 1975, 1976; T a k i o, 1989; C h r i s t i a n s o n and H o r n b u c k l e, 1999; B i j e l o v i ć and S a b o v l j e v i ć, 2003). In our study, sterile spores germinated and formed protonemata that were able to produce buds without exogenous cytokinin supply. However, as in the case of *Physcomitrella* (A s h t o n et al., 1979), cytokinin treated buds did not develop into gametophores. Rapid senescence of tissue grown on 1 μM BA might indicate high endogenous levels of cytokinins, senescence being the result of supraoptimal concentrations caused by exogenous cytokinin supply. When auxin was applied, some gametophores but no rhizoids were formed, opposite to what was observed in *Physcomitrella* (A s h t o n et al., 1979). In studies of cytokinin action on different moss species (S p e i s s, 1976), calli were obtained with most of the species, while the *Polytrichaceae* studied did not form calli. *Atrichum undulatum* formed calli when the growth medium contained 4% glucose and 0.2-2 mg/L benzyladenine (G a n g et al., 2003), and O n o et al. (1988) reported that high sugar levels and high medium osmolarity were essential for callus formation. In our study, callus was obtained at low sugar and osmolarity values, again indicating that endogenous factors determine the development pattern, these factors being species-specific and probably of adaptive rather than evolutionary origin.

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РАЗВИЋЕ МАХОВИНЕ *POGONATUM URNIGERUM* (HEDW.) P. BEAUV. ГАЈЕНЕ У УСЛОВИМА КУЛТУРЕ *IN VITRO*

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Стерилна култура маховине *Pogonatum urnigerum* (*Polytrichaceae*) успостављена је из спора сакупљених у природи. Гајењем на хранљивој подлози без биљних хормона из спора су се развиле протонеме и гаметофити. Успостављена је и стабилна култура калуса. Установљено је да је за гајење ове врсте најбоља хранљива подлога која садржи смањену концентрацију соли и витамина по Murashige-Skoog-у и 1.5% сахарозу. Третман ауксином није поспешио развиће гаметофита, док је продужени третман довео до постепеног одумирања протонеме. Ткива гајена на подлози која је садржала цитокинине уопште нису продуковала гаметофите и нису преживела продужени третман овим хормоном.

Овај рад представља прву студију развића врсте *Pogonatum urnigerum* у *in vitro* култури.