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 (Cove 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 (Cove et al., 2006), most of this work has been done on Physcomitrella patens, and relatively few species have in fact been successfully induced for form stable axenic cultures.

As in higher plants, development under axenic conditions is species-specific. There are many discrepancies in the response of different moss species to the same treatment under in vitro culture (Bijelović et al., 2004; Sabovljević et al., 2005). Mosses also contain numerous biologically active compounds (Mues, 2000; Sabovljević 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 Skoog (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 benzyl-
adenine (BA) was added to the MS/2 medium. Prior to
sterilization, pH was adjusted to 5.8. Cultures were
grown at 25±2°C under long-day conditions (16 h light/8
h dark). Light was provided by fluorescent tubes, and ir-
radiance on the plant-growth shelf was 45 µmols·m$^{-2}$.

Fresh microscopic preparations were examined us-
ing a Leica DMLS microscope with a digital camera at-
tached.

RESULTS

Spore germination began soon after introducing
spores to nutrient media. Independent of the nutrient me-
dium used (hormone-free MS or MS/2 medium), spore
germination was evident after 8 days of in vitro cultiva-
tion. (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 tis-
sue when transferred to MS/2 medium. Protonemata ob-
tained from spores germinated on MS/2 medium contin-
ued elongation and formed many buds. In all further ex-
periments, 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 develop-
ment, 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 vis-
ible (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.
The formerly accepted protocol for bryophyte introduction into axenic culture developed in our laboratory (Sabovljević 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-

**DISCUSSION**

**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.
sive gametophyte 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 (Felix, 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 ić et al., 2005) and another polytrichaceous moss, *Atrichum undulatum* (S a b o v l j e v ić 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 ić and S a b o v l j e v ić, 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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REFERENCES


DEVELOPMENT OF THE MOSS *POGONATUM URNIGERUM*  


**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. Гајење на хранљивој подлози без биљних хормона из спора су се развијеле протонеме и гаметофити. Успостављена је и стабилна култура ка- луса. Установљено је да је за гајење ове врсте најбоља хранљива подлога која садржжи смештени концен- трацију соли и витамину по Murashige-Skoog-у и 1.5% сахарозу. Третман ауксином није поседио 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 продуже- ни третман овим хормоном.

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