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Research Article

In vitro Culture of Mosses: *Aloina aloides* (K.F.Schultz) Kindb., *Brachythecium velutinum* (Hedw.) B.S. & G., *Ceratodon purpureus* (Hedw.) Brid., *Eurhynchium praelongum* (Hedw.) B.S. & G. and *Grimmia pulvinata* (Hedw.) Sm.

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Abstract: In vitro cultures of five moss species were established on hormone-free MS medium, or on MS medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid and kinetin. In vitro culture of *Eurhynchium praelongum* (Hedw.) B., S. & G., has been initiated from the apical shoots of the gametophytes, and the cultures of *Aloina aloides* (K.F.Schultz) Kindb. *Brachythecium velutinum* (Hedw.) B., S. & G., *Ceratodon purpureus* (Hedw.) Brid. and *Grimmia pulvinata* (Hedw.) Sm. were initiated from the spores of immature sporophytes. In secondary protonema culture of *Eurhynchium praelongum,* spontaneous regeneration occurred successfully. Protonema cultures of *Aloina aloides, Brachythecium velutinum* and *Ceratodon purpureus* reversed to caulonema culture where bud formation occurred. *Grimmia pulvinata* cultures remained at the protonema stage.

Key Words: mosses, in vitro culture, *Aloina aloides, Brachythecium velutinum, Ceratodon purpureus, Eurhynchium praelongum, Grimmia pulvinata*

Aloina aloides (K.F.Schultz) Kindb., *Brachythecium velutinum* (Hedw.) B.S. & G., *Ceratodon purpureus* (Hedw.) Brid., *Eurhynchium praelongum* (Hedw.) B.S. & G. ve *Grimmia pulvinata* (Hedw.) Sm. Yosunlarının İn vitro Kültürü

Özet: Hormonsuz veya farklı konsantrasyonlarda oksin ve sitokinin içeren besi ortamlarında (MS) yaşamları tehlikede olmayan çok sayıdaki yosun türü için in vitro kültürleri oluşturulmuştur. *Eurhynchium praelongum* (Hedw.) B.S. & G.'nin *in vitro* kültürüne gametofitlerin uç sürgünlerinden, *Aloina aloides* (K.F.Schultz) Kindb., *Brachythecium velutinum* (Hedw.) B.S. & G., *Ceratodon purpureus* (Hedw.) Brid. ve *Grimmia pulvinata*(Hedw.) Sm.'nin in vitro kültürlerine ise olgunlaşmamış saprofitlerin sporlarından alınarak başlanmıştır. *Eurhynchium praelongum*'nin sekonder protonema kültürü do?al olarak başarılı bir şekilde oluşmaktadır. *Aloina aloides, Brachythecium velutinum* ve *Ceratodon purpureus*'nın protonema kültürleri tomurcuk formasyonunun gerçekleştiği kaulonema kültürüne dönüşmüş, *Grimmia pulvinata* ise protonema evresinde kalmıştır.

Anahtar Sözcükler: Yosunlar, in vitro kültürü, *Aloina aloides, Brachythecium velutinum, Ceratodon purpureus, Eurhynchium praelongum, Grimmia pulvinata*

Introduction

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 favoured research object; therefore most studies of plant morphogenesis are now being done on vascular plants. 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.

Bryophytes are the second largest group of higher plants after flowering plants, with approximately 15,000 species worldwide (Hallingbäck & Hodgetts, 2000). However, most bryophyte species have no commercial value and are therefore less attractive in a wide range of studies. Some 40% of this tiny species are endangered at present and in urgent need of active protection and conservation.

One of the latest ideas is to establish sterile in vitro cultures, then micropropagate plants and later consider methods for their reintroduction into potential native habitats. The first relevant steps in Yugoslavia have been taken and are presented in this study.

With the aim of improving the methodology dealing with bryophytes in vitro, we started our study with a few different but not endangered moss species as a model system for further studies. We have chosen these species because of their varying morphologies, life cycle, life form, ecology or biology. Some similar attempts have been made by Sokal et al. (1997), who also considered the problem in regeneration and callus induction in mosses.

Aloina aloides (K.F.Schultz) Kindb. is an annual xerophyte species which grows on very dry soils and in arid zones. It appears in loose groups or as single shoots. *Brachythecium velutinum* (Hedw.) B., S. & G. is cortico-terrestrial and *Eurhynchium praelongum* (Hedw.) B., S. & G. is terricolous, xeromesophytes and pleurocarpous and therefore different from all the other species. The former is often fructificated, while the latter spreads mostly vegetatively in Yugoslavia. *Ceratodon purpureus* (Hedw.) Brid. grows in dense cushions on nitrificated soils. It is a xerophyte, but is completely different in terms of its adaptation to dryness. *Grimmia pulvinata* grows in small cushions on rock faces, walls, and concrete etc. It is a pioneer species in such inhospitable places. *Grimmia pulvinata* is adapted to survive dry periods by growing in hemispherical dense cushions.

Materials and Methods

A. aloides and *E. praelongum* were collected from Kalemegdan Fort Park in Belgrade (C. Serbia), *C. purpureus* and *G. pulvinata* from Petnica, near Valjevo (W. Serbia), and *B. velutinum* from Vrsacki Breg, near Vrsac (N. Serbia). *A. aloides* and *E. praelongum* were collected in December 2000, while *B. velutinum*, *C. purpureus* and *G. pulvinata* were collected in February 2001. The material was kept in paper bags after drying, until the beginning of the experiment.

The in vitro culture of *E. praelongum* was initiated from the apical shoots of the gametophytes, while the culture of *A. aloides*, *B. velutinum*, *C. purpureus* and *G. pulvinata* was from the spores.

Apical shoots of *E. praelongum* were separated from the soil and washed under tap water for 30 min. In order to provide sterile plant material, small apical shoots were sterilised for 5 min in various concentrations (0.5, 1.0, 3.0, 7.0, 9.0, 10.0, 11.0, 12.0, 13.0, 15.0, 17.0 and 19.0%) of commercial bleach solution (8% active chlorine) supplemented with a few drops of liquid soap. The plant material was then rinsed three times in sterile water. Forty apical shoots were transferred to petri dishes with 20 ml basal media.

Immature sporophytes, containing spores of *A. aloides*, *B. velutinum*, *C. purpureus* and *G. pulvinata*, were surface-sterilised for 5 min in the same range of concentrations of bleach as those described for the apical shoots of *E. praelongum*. Capsules were then opened aseptically. Spores were transferred using a sterile needle to Petri dishes containing 20 ml of nutrient media. Medium (MS_1 medium) containing MS (Murashige & Skoog, 1962) mineral salts and vitamins, 100 mg/l myo-inositol, 30 g/l sucrose and 0.70% (w/v) agar (Torlak purified, Belgrade) was used for establishing in vitro cultures of *A. aloides*, *B. velutinum*, *C. purpureus*, *E. praelongum* and *G. pulvinata*. Additionally, we used other medium compositions (MS_1 medium supplemented with different concentrations of auxines and/or cytokinins) as well for bud induction and gametophyte regeneration:

MS_2: 1 mg/l of 2, 4-dichlorophenoxyacetic acid (2,4-D) and 2 mg/l of kinetin (KIN)

MS_3: 1 mg/l of 2,4-D

MS_4: 1 mg/l of 2,4-D and 1 mg/l of KIN

MS_5: 0.2 mg/l of indol-3-butyric acid (IBA) and 2 mg/l of benzyl-6-aminopurin (BAP)

MS_6: 0.2 mg/l of IBA and 2 mg/l of KIN

MS_7: 0.2 mg/l of indol-3-acetic acid (IAA) and 2 mg/l of BAP

The pH was adjusted to 5.8 before autoclaving at 114 °C for 25 min.

Cultures were kept at 25 °C, and light (16/8 hours of light to darkness) was supplied by cool-white fluorescent tubes at a photon fluence rate of 33.5-45 mmol/sm^2.

The plants were subcultured at 1-month intervals.

Results and Discussion

Surface sterilisation with various concentrations of commercial bleach solution was very effective for the sporophytes, but less effective for the gametophytes.

Considering the fact that filoides of mosses mostly contain one cell layer without cuticles, the concentration of commercial bleach used for surface sterilisation is critical. A low concentration (up to 9%) was not effective (Table 1). Fungal spores survived and proliferated as did algae and bacteria. Thus gametophytes sterilised in this way were not useful for establishing in vitro culture. Conversely, plant gametophyte material can be sterilised very well using higher concentrations of bleach solution but is lethally damaged. Seven and 9% concentrations of NaOCl proved efficient sterilisation for gametophytes of *E. praelongum.*

It was easier to sterilise sporophytes of the mosses studied, due to their morphology and anatomy. Immature capsules containing spores of *A. aloides*, *B. velutinum*, *C. purpureus* and *G. pulvinata* were sterilised best with a 12% commercial bleach solution. However, concentrations of bleach in the range 9-15%, (9.0, 10.0, 11.0, 12.0, 13.0 and 15.0%) were also suitable for sporophyte sterilisation. This is because capsules have better protection than filoids, and young capsules are usually smooth, and not as plicate as filoids. Twelve percent of commercial bleach solution showed the best results in capsule sterilisation. In non-damaged and non-mature capsules the spores are sterile, and this advantage was used to eliminate difficult procedures for the sterilisation of tiny and individual spores.

For establishing in vitro culture of *E. praelongum* (Fig. 1), MS_1 medium was more convenient than other types of media. Secondary protonema development occurred 3 months after establishing in vitro culture, while bud formation and shoot multiplication occurred 2 weeks and 6 weeks, respectively, after secondary protonema development on MS_1 medium (Table 2).

Spore germination in *A. aloides*, *B. velutinum*, *C. purpureus* and *G. pulvinata* in vitro cultures occurred 7 days after establishing axenic cultures of all these species. In *A. aloides* and *B. velutinum* cultures protonema and caulonema developed 8 and 12 days respectively after spore germination, while in *C. purpureus* and *G. pulvinata* axenic cultures they formed 8 days and 2 months after spore germination, respectively. Bud formation occurred 4 and 2 months after spore germination in *A. aloides* and *B. velutinum* cultures, respectively, while buds formed 5 months after spore germination in *C. purpureus* and *G. pulvinata* cultures. In *A. aloides* in vitro culture bud formation and gametophyte regeneration occurred on caulonema on the following media: MS_2, MS_6 and MS_7. In *B. velutinum* cultures gametophytes were regenerated on the following media: MS_1, MS_2, MS_4, MS_6 and MS_7, while in *C. purpureus* cultures gametophytes were formed on MS_1, MS_6 and MS_7 media (Fig. 1.).

According to Sargent (1988), axenic culture of the species from the genera *Eurhynchium* Schimp. and *Aloina* Kindb. have not been established previously. *In vitro* culture of *Brachythecium salebrosum* (Web. & Mohr.) B., S. & G. (Sargent, 1988) was conducted on Knop (1884) medium enriched with 0.5% glucose, which stimulated

Table 1. The concentrations of commercial bleach solution used for surface sterilisation of the moss plants for 5 min and the percent of explants that survived sterilisation.

Species	0.5	1.0	3.0	7.0	9.0	10.0	11.0	12.0	13.0	15.0	17.0	19.0	NaOCl concentration (%)
A. aloides (sporophytes)	0	0	0	7.5	12.5	30.0	32.5	77.5	37.5	20.0	0	0	
B. velutinum (sporophytes)	0	0	0	10.0	12.5	25.0	37.5	82.5	42.5	20.0	0	0	Survived
C. purpureus (sporophytes)	0	0	0	7.5	12.5	22.5	47.5	95.5	40.0	22.5	0	0	explants
E. praelongum (gametophytes)	0	0	0	27.5	67.5	25.0	17.5	0	0	0	0	0	(%)
G. pulvinata (sporophytes)	0	0	0	7.5	17.5	22.5	37.5	90.0	30.0	17.5	0	0	

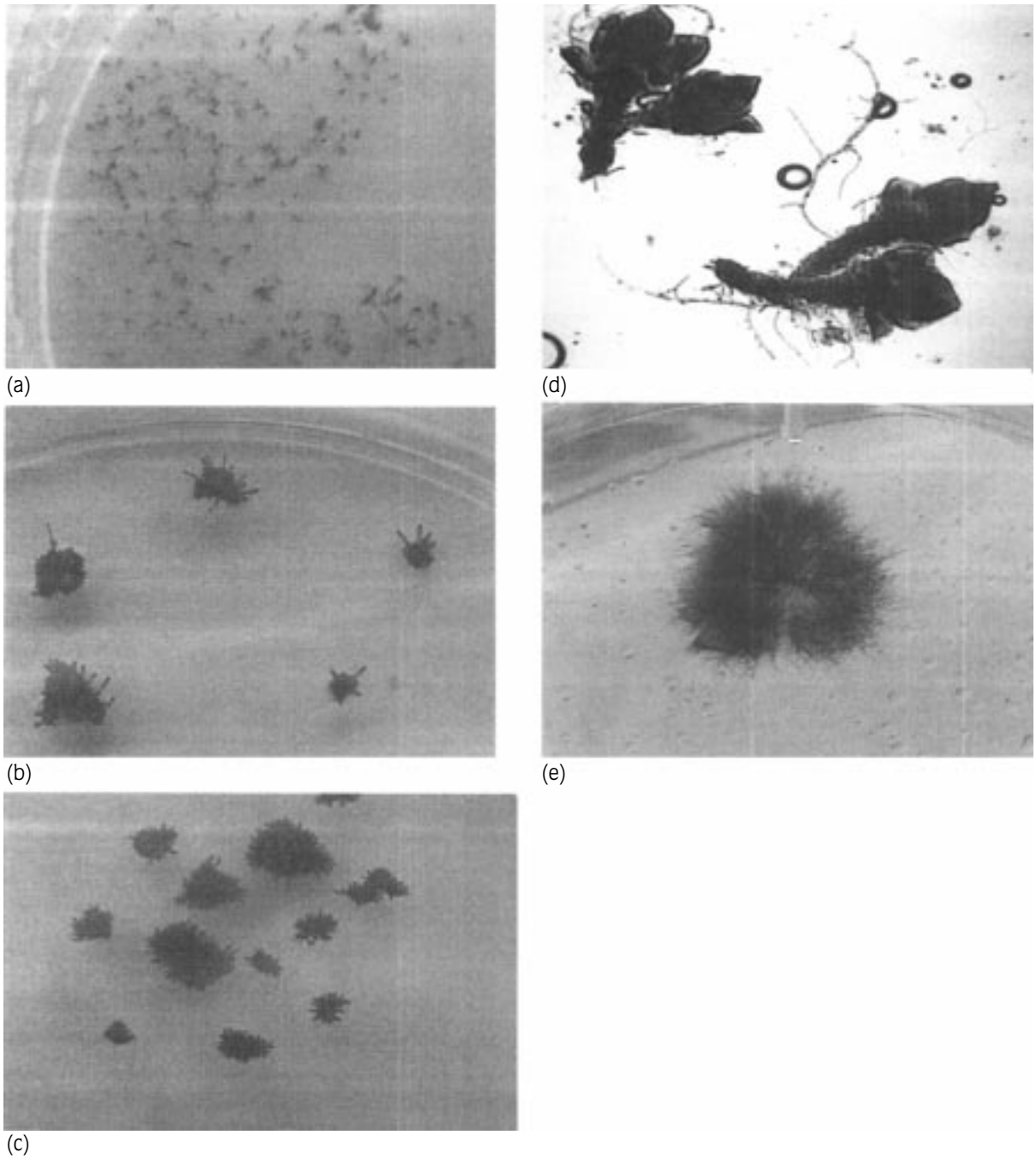

Fig. 1. Young shoots of *Ceratodon purpureus* (a); *Eurhynchium praelongum* (b); *Brachythecium velutinum* (c); *Aloina aloides* shoots developed in vitro 100x (d) and protonema of *Grimmia pulvinata* (e) in vitro.

initial growth of this species, but the growth form of *B. salebrosum* was markedly elongated and spindly. *B. velutinum* in vitro culture had normal morphology, but was smaller than the native form of this species. *G. pulvinata* has not been previously cultured in vitro. From the order *Grimmiales*, only *Schistidium apocarpum* Hedw. was growing in vitro. The propagation method for *E. praelongum* presented here allows fast and reliable clonal

Table 2. Moss development in different medium compositions.

Medium	Aloina aloides	Brachythecium velutinum	Ceratodon purpureus	Eurhynchium praelongum	Grimmia pulvinata
MS_1	P	S	G	S	P
MS_2	S	S	P	P	P
MS_3	P	P	P	/	/
MS_4	P	S	P	/	/
MS_5	P	P	P	/	/
MS_6	S	S	G	/	/
MS_7	S	S	G	/	/

P – protonemal development; G – normal gametophyte development; S – gametophyte development, but smaller than the normal one; / - not

propagation. However, each species has specific hormonal requirements for shoot regeneration and multiplication. It seems that Knudson (1946) medium is not necessary for successfully establishing in vitro moss cultures, at least for *E. praelongum*. Most of our efforts were directed towards establishing in vitro culture and regeneration of mosses, which we have succesfully performed by using hormone-free MS medium (MS_1), or MS medium supplemented with different concentrations of auxines and/or cytokinins (MS_2 – MS_7). Bopp (2000) and Reski (1998) confirmed that certain ratios and interactions of cytokinins and auxines are crucial for moss development, at least in *Physcomitrella patens* (Hedw.) B., S. & G. and *Funaria hygrometrica* Hedw. (both from *Funariales*). Bopp and Knoop (1984) and Bopp (1982; 1984) emphasised that the processes of moss development are not clear, not even in species like *P. patens* and *F. hygrometrica.* Hence, this study is also a contribution to the knowledge of the biology and physiology of mosses, bearing in mind that even *P. patens* and *F. hygrometrica* (species that are usually used as model systems) have insufficiently clear growth and development (Bopp, 1982; 1984).

In vitro cultures of these moss species may b useful as a model for establishing axenic cultures of rare, endangered and endemic mosses. The first phase would be multiplication and propagation, following by reintroduction of the cultures of specimens to native and potentially suitable habitats. The results of this study should be useful in further research as a base for investigations of insufficiently known phenomena in bryophytes such as the gametophyte-sporophyte junction, drought resistance in mosses, and heavy metal accumulations and bioindications in bryophytes. Since there is great morpho-anatomical, ecological and functional diversity in bryophytes, it is probably not possible to use one species as an axenic culture model system for all bryophytes.

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