Acta Biol. Univ. Daugavp. 21 (1) 2021
ISSN 1407 - 8953

PRELIMINARY STUDY OF MOSS *HOMALIA TRICHOMANOIDES* (HEDW.) BRID. GAMETOPHYTE DEVELOPMENT FROM SPORES *IN VITRO*

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Mežaka A., Plaksenkova I., Vanaga A., Petrova A., Svilāne I. 2021. Preliminary study of moss *Homalia trichomanoides* (Hedw.) Brid. gametophyte development from spores *in vitro*. *Acta Biol. Univ. Daugavp., 21 (1): 1 – 8.*

Bryophyte spore cultures are used in experimental research to understand not only fundamental problems in biology, but also to explore opportunities for practical application. The present study focused on the moss *Homalia trichomanoides* (Hedw.) Brid. gametophyte development from spores *in vitro*. The study allowed us to discover the optimal sterilization method of *H. trichomanoides* capsules. Study results show different protonemal developmental stages from spore germination to gametophyte development in controlled conditions. We suggest the future research to investigate rare species *in vitro* growth in different climatic settings. This was the first attempt in Latvia to cultivate moss *in vitro* under controlled conditions

Key words: Bryophyte, spore cultures, protonema, moss protonema.

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INTRODUCTION

Bryophyte cultures have been used to increase fundamental knowledge in the field of biology and also to explore possibilities of use for practical purposes. Culturing bryophytes *in vitro* may serve for the different aims, such as aiding our understanding of morphogenesis, clarifying the roles of protonema juvenile stages, juvenile characteristics and morphology (Alfayate et al. 2013) in phylogeny and systematics, maintaining rare taxa for conservation (Rowntree 2006, Rowntree et al. 2011) and possible reintroduction to nature and developing propagation methods for commercial exploitation. Moss culturing has advantages as long period of time that sporophytes are available in the natural environment and that the first structure from the spore, protonemata, spreads relatively quickly and it is easy to subculture (Duckett et al. 2004).

In maintaining bryophyte cultures *in vitro,* the main challenge is contamination (Duckett et al. 2004). In the *in vitro* spore capsule sterilization process, the concentration of the chlorine containing solution is crucial, and many researchers have tried and continue to try different concentrations of chlorite to eliminate any bacteria or other microorganisms which may contaminate the bryophyte culture and

damage bryophyte structures (Carey et al. 2015, Sabovljevic et al. 2003).

In addition to culture sterility, temperature and light conditions are important during the bryophyte growth *in vitro*. In study about moss *Dolichomitriopsis diversiformis* culture *in vitro* was found that temperature controls spore germination time. Continuous light within 20°C are suitable for protonemal elongation (Liu et al. 2016).

The evidence shows that most bryophytes grow *in vitro* in a 5-25°C temperature range (Duckett et al. 2004). A study of four mosses shows that gametophyte initiation *in situ* is related to light intensities of up to 27-40.5 μmol m^{-2} s^{-1} (Ahmed et al. 2010). However, various mosses respond differently to the effect of light on bud formation from protonema. It is important to understand the spore germination of various bryophyte species in different climatic conditions (Alfayate et al. 2013) to help us better understand moss adaptation abilities in a changing environment.

The spore size of the moss *Homalia trichomanoides* (Hedw.) Brid. is 14-16 μm and this moss species have been cultured *in vitro* before (Löbel & Rydin 2010), but we are still missing detailed information about gametophyte development. *Homalia trichomanoides* in Latvia is a Woodland key habitat indicator species (Ek et al. 2002), but it is rather common on tree basal parts in young and old-growth forests, and may be found in manor parks if suitable humidity conditions are met (Āboliņa 1968, Mežaka et al. 2020).

The aim of our study was to characterize *H. trichomanoides* gametophyte development in *in vitro*.

MATERIAL AND METHODS

Collected samples

Homalia trichomanoides turfs with capsules were collected from the basal part of *Acer platanoides* L. trunks in a nemoral forest near Aizkraukles purvs un meži Nature Reserve, Latvia, on 19th of October 2020. Collected turfs with almost mature capsules and with intact opercula were kept in a refrigerator for four days until spore inoculation.

Procedure of inoculation

On the fourth day after turf collection, we inoculated *H. trichomanoides* spores (Fig. 1) on the culture medium. Initially we removed five *H. trichomanoides* undehisced capsules. Each of these capsules was sterilized in the following order according to Ahmed et al. (2010) but slightly modified: 1) the capsules were immersed in 70% ethanol for a 10 seconds; 2) we applied different concentrations of sodium hypochlorite for different amounts of time (1% and 2% for 2 min and 4 min for both concentrations; 3% for 2 min) to the relevant capsule; 3) each capsule was rinsed in distilled water for a 10 seconds. After sterilization, we put the capsule on a glass slide and squeezed spores out of the capsule into a droplet of distilled water. Spore liquid was placed (around 30 μl) on a semi-solid agar medium (with added 1/8 MS solution after Ahmed et al., 2010) in petri dishes (size 9.20 cm in diameter). The MS basal medium was modified with one-eighth strength of macroelements and full-strength microelements of MS salts supplemented with 1.33% sucrose. The pH of this modified medium was adjusted to 5.7 with 0.1 N KOH and HCl before autoclaving for 20 min at 121°C. We used 1/8 MS solution (Murashige & Skoog 1962) because of the induction of relatively fast moss spore germination and protonema development in contrast to other solutions used *in vitro* (Ahmed et al. 2010).

Initially, cultures were grown in a growth chamber (Versatil Environmental Test Chamber MLR-352H, Panasonic) in +25°C under fluorescent light (31.98 μmol^{-2} s^{-1}) with a day/night regime of 16/8 h.

Because of the contamination and the prolific growth of protonema, protonema was subcultured in new petri dishes (size 9.20 cm in diameter) using the same medium at days 22, 42, 62, 83 and

98 after the initial inoculation and placed them under the same light and temperature conditions as initial petri dishes with spores. Due to the slow protonemal development, after 3.5 months of spore inoculation, the temperature in the growth chamber was decreased to 18°C (Sabovljević et al. 2012). All sterilization, inoculation and subculturing procedures were made under sterile air-flow.

In total, the cultures were observed for 224 days (from October 2020 until June 2021), twice a week. Culture observations were made using a Stereomicroscope SMZ 745T (Nikon) and Microscope Eclipse E200 (Nikon).

RESULTS

Four days after the initial inoculation of *H. trichomanoides* spores, healthy spores were found without contamination that were starting to germinate in the culture, where capsules were sterilized with 1% sodium hypoclorite for a duration of 4 min. In all other cultures, were found contamination or dead spores without indication of germination. Afterwards, there was observed and subcultured only *H. trichomanoides* protonema with the sterilization method of 1% sodium hypochlorite for 4 min. After 23 days of inoculation of this culture, greenish protonema were clearly visible. As protonema was rather dense, the first protonema subculturing were performed 22 days after the initial spore inoculation.

Protonemal developed as thread-like structures, mostly resembling chloronema (greenish threads), but some threads were also transparent, appearing as caulonema day 40 after inoculation (Fig. 2). Following two more subculturing events (days 42 and 62 after initial inoculation), more thread-like structures were observed in the next phases of protonemal development, but protonema did not show differentiation appearing as caulonema (Fig. 3,4). While some contamination was observed on day 71 after the initial inoculation, it did not progress until the final gametophyte observation. As protonema became dense, there were made two more subculturing events (82 and 98 days after the initial inoculation) until day 102 after the initial inoculation. Day 127 after the initial inoculation, protonema was brownish but still developing and healthy (Fig. 5). Only on day 148 after inoculation was the initial phase of gametophyte bud development visible with some initial stages of leaf-like structures on several buds (Fig. 6 A). Bud differentiation in leaves continued until day 158 after inoculation (Fig. 6 B). After the initial gametophyte development (Fig. 7 A, B), the gametophyte with stem and leaves was observed day 224 after spore inoculation (Fig. 1 J).

DISCUSSION

In the study, we found the most appropriate concentration of the main solution (1 % sodium hypochlorite for 4 min) in *H. trichomanoides* capsule sterilization process *in vitro*. In a study in Sweden, *H. trichomanoides* capsules were successfully sterilized with 1.5 % sodium hypochlorite for 1.5 min (Löbel & Rydin 2010). Duckett et al. 2004 suggested that sporophyte tissues could be sterilized better with 1% sodium dichloroisocyanurate for 3-6 min. In the present study also was observed culture contamination more than two months after the initial spore inoculation, but this contamination did not progress. It can be supposed that this contamination was induced by the developing protonema. Plants by themselves may induce changes in the medium during growth time as increasing medium pH and metabolite accumulation and may lead to the death of the cultures. To avoid this, regular subculturing is necessary (Duckett et al. 2004). Before inoculation there was observed that some capsules have dead spores (brownish color), probably the result of a cold autumn.

Spores in our study started to germinate day 4 after inoculation, similar to a study of three moss species cultures *in vitro* in South Korea, where spores started to germinate within 5-8 days after inoculation at 25°C (Ahmed et al. 2010). In a study of a *Dolichomitriopsis diversiformis*

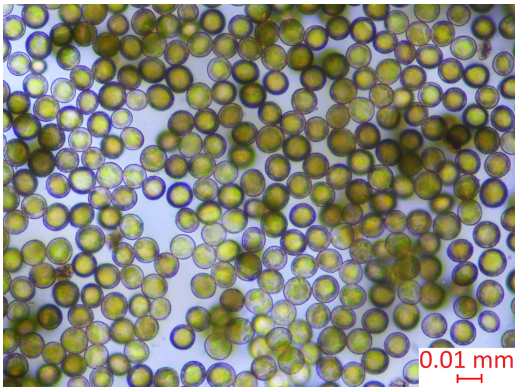

Fig. 1. *Homalia trichomanoides* spores before inoculation.

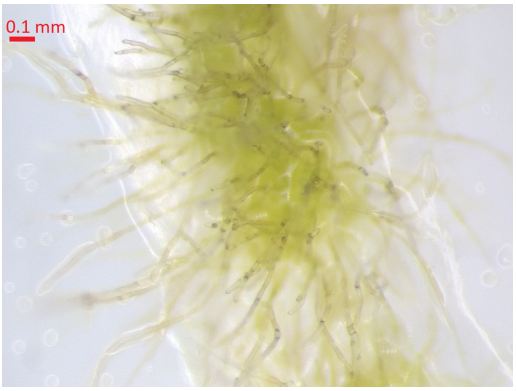

Fig. 2. Differentiating caulonema (transparent threads) and chloronema (greenish threads) of *Homalia trichomanoides* day 40 after incolulation.

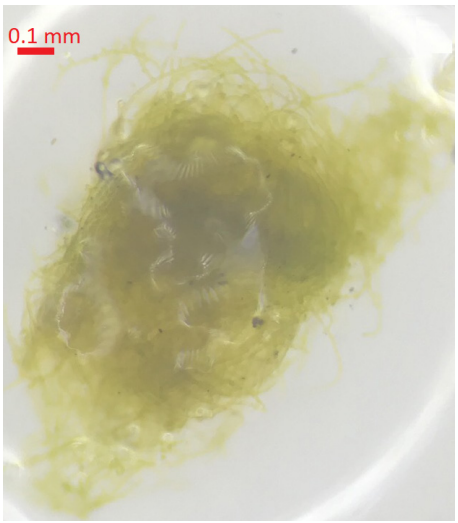

Fig. 3. *Homalia trichomanoides* protonema day 71 after inoculation.

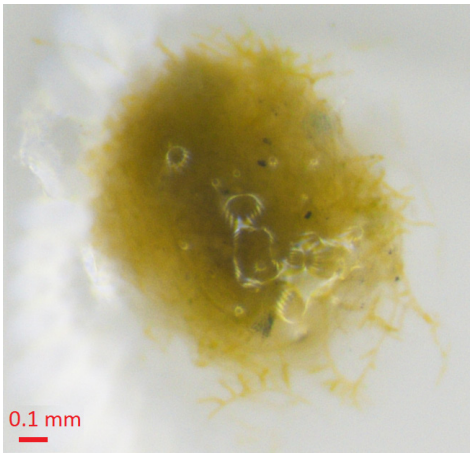

Fig. 4. *Homalia trichomanoides* protonema day 102 after inoculation.

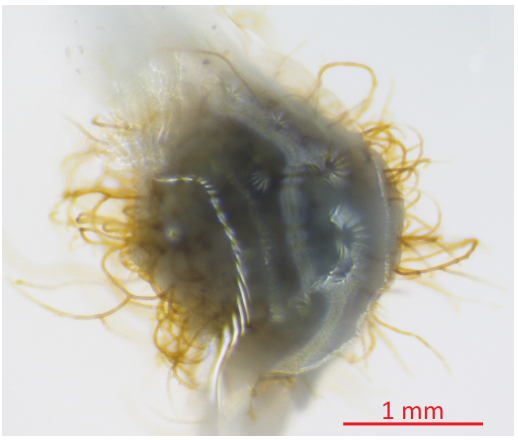

Fig. 5. *Homalia trichomanoides* protonema day 127 after inoculation.

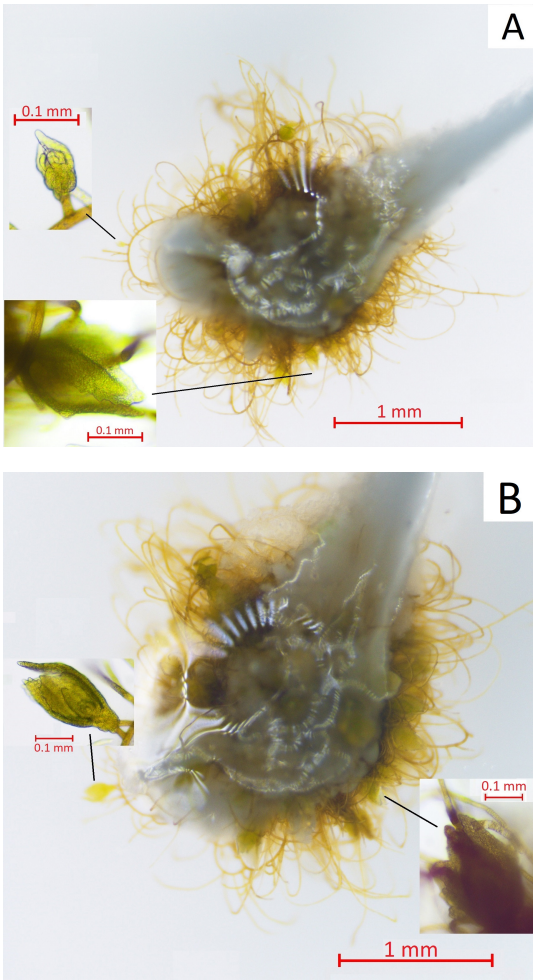

Fig. 6. *Homalia trichomanoides* gametophore bud development. Day 148 after inoculation (A) and day 158 after inoculation (B).

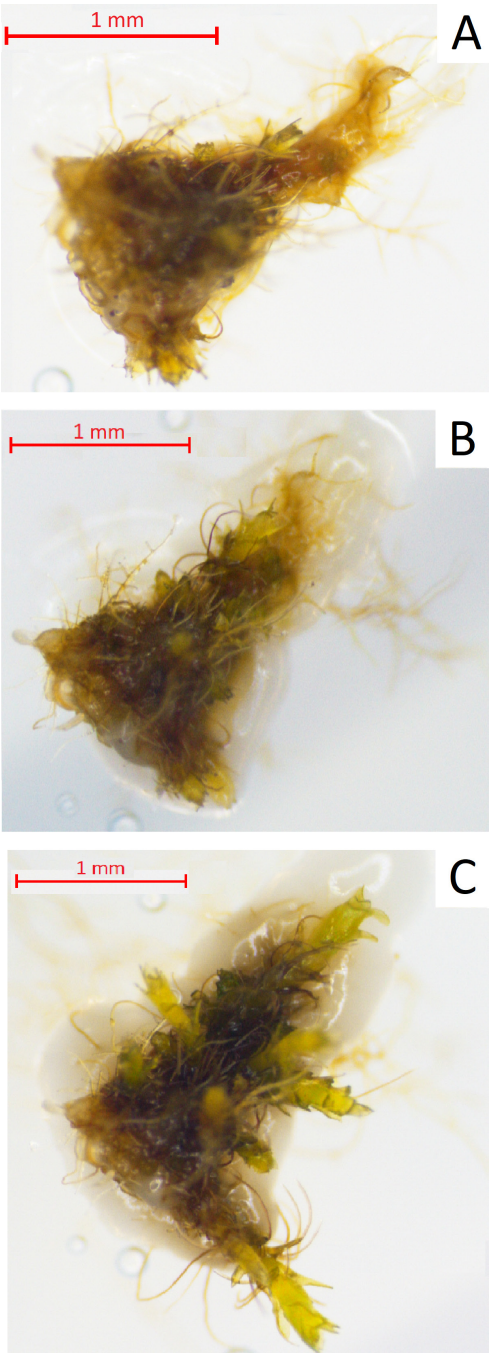

Fig. 7. Differentiation of *Homalia trichomanoides* gametophyte stem and leaves. Day 193 (A), day 200 (B) and day 224 (C) after inoculation.

(Mitt.) Nog. moss culturing experiment in China, it was found that temperature controls spore germination time, which is faster in higher temperatures (20-25°C) (Liu et al. 2016). Another study suggests that temperature above 20°C cause the moss gametophytes to slow down their growth in laboratory conditions (Glime 2017). It is likely that the decreased temperature in the growth chamber positively influenced also bud initiation.

In a study about culturing *Lindbergia brachyptera* (Mitt.) Kindb. moss from spores in China, it was revealed that if the temperature during the culture experiment is higher than 20°C, it may induce the growth of bacteria, but lower temperatures can prevent the development of protonema. The same study also revealed that temperature, culture, medium, pH and illumination have direct effects on the spore germination rate and protonemal development (Zhao et al. 2004). The greatest growth of four mosses (*Amblystegium serpens, Bartramia pomiformis, Hypnum plumaeforme, Polytrichum commune*) was found at 20°C in South Korea (Ahmed et al. 2010). Taxa from cooler climates grow better *in vitro* at 5-15°C, and for tropical taxa, 20-25°C would be the optimal temperature (Duckett et al. 2004). For other similar species, successful *Neckera pennata* Hedw. cultures *in vitro* were exposed to 15/7.5°C temperature in 16/8 h light/dark periods (Wiklund & Rydin 2004). In a study of European moss *Atrichum undulatum* (Hedw.) P. Beauv., cultures were kept successfully in 25°C in a fluorescent light of 47 µmol/m^{2}s (Sabovljević et al. 2006). It can be supposed that the influence of temperature in moss culture growth is species specific.

In the present study 1/8 MS medium was used, but changes in the composition of nutrients in media significantly influence the spore germination (Ahmed et al. 2010). A variety of organic media can be successful in bryophyte *in vitro* growth (Duckett et al. 2004). For bryophyte growth from *in vitro* spores, Murashige and Skoog (MS) medium (Sabovljević et al. 2006, Vujičić et al. 2011) as well as Knop's solution (Alfayate et al. 2013, Zhao et al. 2004, Silva-e-Costa et al. 2017) have been used with similar success.

In our study, similar to Zhao et al. (2004), it is possible to identify three stages in bryophyte gametophyte development: spore germination, protonemal development and gametophyte differentiation. In addition to these stages, was observed also further gametophyte growth with differentiation of stem and leaves. Spore germination and the speed of the following gametophyte developmental stages are probably species specific. In an *Atrichum undulatum* spore culture experiment *in vitro* in Serbia, it was found that spore germination appeared one month after inoculation; protonema developed 15 days after spore germination; and bud formation started two months after spore germination. Fully developed gametophytes occurred three months after spore germination (Sabovljević et al. 2006). In a study of European mosses, bud formation started 3.5 months after spore inoculation and shoots were found 4.5 months after the spore inoculation (Sabovljevic et al. 2003).

This was a first attempt in Latvia to grow *H. trichomanoides* spores *in vitro* in growth chambers in controlled conditions. In the future, we suggest *in vitro* spore growth of different rare species in various climatic settings.

CONCLUSIONS

In the present study was found that the best spore capsule sterilization results when using 1% sodium hypoclorite for 4 min, because all other concentrations led either to the contamination or death of the inoculated spores. We also found that subculturing is an important aspect in growing *H. trichomanoides* successfully *in vitro* and that protonema of *H. trichomanoides* pass through several stages before gametophyte development.

ACKNOWLEDGEMENTS

The study was financially supported by the project: "Epiphyte metapopulation dynamics in boreo-nemoral forest landscape" (Nr. 1.1.1.2/ VIAA/3/19/469). We are also grateful to Stock

Company "Latvia's State Forests" for co-operation. Thanks is given to Inese Kivleniece for technical assistance.

REFERENCES

Āboliņa A.A. 1968. Listostebeljnie mhi Latvijskoj SSR. (Mosses of the Latvian SSR). Riga, Zinatne, Pp. 329. (In Russian).

Ahmed M.G.U., Chang Y.D., Lee C.H. 2010. Factors affecting on in vitro gametophyte formation from spore culture of four moss species. *Korean Journal of Horticultural Science & Technology*, 28(1): 108–114.

Alfayate C., Ron E., Estébanez B., Á Pérez-Batista M. 2013. Mature spores of four pleurocarpous mosses in the Canary Islands: ultrastructure and early germination stages. *The Bryologist*, 116(2): 97–112.

Carey S.B., Payton A.C., Mcdaniel S.F. 2015. A method for eliminating bacterial contamination from in vitro moss cultures. *Applications in Plant Sciences*, 3(1): apps.1400086.

Duckett J.G., Burch J., Fletcher P.W., Matcham H.W., Read D.J., Russell A. J., Russel P.S. 2004. *In vitro* cultivation of bryophytes: a review of practicalities, problems, progress and promise. *Journal of Bryology*, 26(3): 3–20.

Suško U., Ek T., Auziņš R. 2002. Inventory of woodland key habitats. *Methodology*. 73.

Glime J.M. 2017. Photosynthesis: Limiting Factors. Chapt. 11-3. In: Glime J.M. Bryophyte Ecology. Vol. 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.

Liu S., Wei H., Peng X., Li J. 2016. Spore Germination and Protonemal Development of *Dolichomitriopsis diversiformis*. *Brazilian Archives of Biology and Technology*, 59: 1-8.

Löbel S., Rydin H. 2010. Trade-offs and habitat constraints in the establishment of epiphytic bryophytes. *Functional Ecology*, 24(4): 887–897.

Mežaka A., Stepanova D., Evarts-Bunders P. 2020. Epiphytic bryophytes in Latvian manor parks. *Arctoa*, 29(1): 195–200.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum*, 15: 473-497.

Rowntree J.K. 2006. Development of novel methods for the initiation of *in vitro* bryophyte cultures for conservation. *Plant Cell, Tissue and Organ Culture*, 87(2): 191–201.

Rowntree J.K., Pressel S., Ramsay M.M., Sabovljevic A., Sabovljevic M. 2011. In vitro conservation of European bryophytes. *In Vitro Cellular & Developmental Biology - Plant*, 47: 55–64.

Sabovljevic M., Bijelovic A., Dragicevic I. 2003. 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. *Turkish Journal of Botany*, 27: 441–446.

Sabovljević A., Cvetić T., Sabovljević M. 2006. Establishment and development of the Catherine's moss *Atrichum undulatum* (Hedw .) P . Beauv. (Polytrichaceae) in in vitro conditions. *Archives in Biological Sciences*, 58(2): 87–93.

Sabovljević M.S., Papp B., Sabovljević A., Vujičić M., Szurdoki E., & Segarra-Moragues J.G. 2012. *In vitro* micropropagation of rare and endangered moss *Entosthodon hungaricus*

(Funariaceae). *Bioscience Journal*, 28(4 SE-Biological Sciences).

Silva-E-Costa J. DA C., Luizi-Ponzo A.P., De Resende C.F., Peixoto P.H.P. 2017. Spore germination, early development and some notes on the effects of in vitro culture medium on *Frullania ericoides* (Nees) Mont. (Frullaniaceae, Marchantiophyta). *Acta Botanica Brasilica*, 31:19–28.

Vujičić M., Sabovljević A., Sabovljević M. 2011. Axenically culturing the bryophytes: establishment and propagation of the moss *Hypnum cupressiforme* Hedw. (Bryophyta, Hypnaceae) in in vitro conditions. *Botanica Serbica*, 35(1): 71–77.

Wiklund K., Rydin H. 2004. Ecophysiological constraints on spore establishment in bryophytes. *Functional Ecology*, 18(6): 907–913.

Zhao J.C., Huang S.L., Li M., Mamtimin S., He J., Zhang Y.M., Li X.Y. 2004. A study on the characteristics of spore germination and protonemal development in *Lindbergia brachyptera*. *Arctoa*, 13(1): 223–228.

Received: 14.05.2021.
Accepted: 15.07.2021.