Wulfenia **25** (2018): 117–130

Wulfenia
Mitteilungen des
Kärntner Botanikzentrums
Klagenfurt

Ecology, distribution, *in vitro* propagation, *ex situ* conservation and native population strenghtening of the rare and threatened halophyte moss *Entosthodon hungaricus* in Serbia

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Summary: Conservation biology of *Entosthodon hungaricus*, a rare bryophyte in Serbia, has been studied. Active protection measures, i.e. study of distribution, ecological and biological features of the target species and *in vitro* propagation, have been taken into account to overcome threat problems affecting native populations. The final goal was to execute reintroduction/introduction to known and potential sites in Serbia. A conservation continuum in protection of one endangered moss species in Serbia is presented. This is the first time that laboratory produced mosses have been released into the wild with the aim to establish or strengthen existing declining populations.

Keywords: bryophytes, active protection, *in vitro*, *ex situ*, *Entosthodon hungaricus*

Conservation biology is an arising discipline of biology that is framed by ecology and biodiversity science on the one side and by biotechnological achievements, management and environmental politics on the other. Conservation is a process that starts with the identification of threatened habitats/species/genotypes, continues with analyses of threats and finally results in actions to ensure the long-term survival of the species (Söderström 2006). Bryophytes appeared in conservation literature about 30 years ago. Since then, more than 100 papers dealing with some aspects of conservation biology have been published and most of them have a 'passive' aspect of conservation like the identification of threatened entities (e.g. Fife et al. 2010; Lazarević et al. 2016), threats (e.g. Hodgetts 1996; Hallingbäck et al. 1996, 1998) or even giving a fundamental basis for management (e.g. Wyatt 1992; Longton 1992; Orban 1992; Sabovljević & Frahm 2008; Sabovljević et al. 2011). Very few papers reported on the active protection of bryophytes (Rowntree et al. 2011; Ros et al. 2013; Sabovljević et al. 2014a, 2016), mainly some kind of *ex situ* conservation, although *ex situ* is one of the goals of the Convention of Biodiversisity, and also stated by Hallingbäck & Tan (2010) as one of the future bryophyte conservation strategies. Extremly rarely, case studies have reported on the reintroduction of threatened bryophytes like Kooijman et al. (1994) who documented the translocation of *Scorpidium scorpioides* (Hedw.) Limpr. from different sites in the Netherlands. However, no reports on lab establishing and managing propagation of threatened bryophytes and on release into nature have been recorded to date.

The moss *Entosthodon hungaricus* (Boros) Loeske (syn. *Funaria hungarica* Boros) was described from Hungary (Boros 1924). It was long considered to be an European endemic species with scattered distribution. However, it was found out of Europe in Israel, Kazakhstan, Kirgizia and because of its synonymization with *E. maroccanum* (Meyl.) Hebr. & Lo Giudice also in Morocco (Pisarenko et al. 2001 and the references therein). Prior to its synonymization with

E. maroccanum, it was considered to be a typical species of the central European and Aral-Caspian steppes. In Europe it has quite a strange range extending from the Mediterranean to Central and Eastern Europe, similarly to some other species often considered as xerothermic (Pócs et al. 2004). It has been reported from Austria, Canary Islands, Greece, Germany, Hungary, Malta, Moldova, Montenegro, Portugal, Romania, Russia, Serbia, Sicily, Slovakia (Hodgetts 2015; Ellis et al. 2016). It is considered to have Continental-Mediterranean distribution and to be a moss halophyte inhabiting saline areas (Papp 2002).

Nonetheless and despite its widespread European distribution, the populations are scattered and the range is highly discontinuous and fragmented. In addition to its distribution range characteristics, its ephemeral habit and specialized ecology and habitat type on gypsum and saline substrates have contributed to its consideration under different categories of threat. Thus, it has been considered as rare and threatened in Europe (ECCB 1995) and it has been included in the red lists from several European countries (Hodgetts 2015).

In this study, the conservation biology of *Entosthodon hungaricus*, an endangered species in Serbia (Sabovljević et al. 2004) has been studied and the results are presented.

The ecology, biology, recent distribution in Serbia, lab population establishment, *in vitro* propagation, release into wild and wild population establishment are presented as well.

Materials and methods

Field Investigation. In Serbia, *E. hungaricus* grows in habitats with cold winters and very hot and dry summers, in salty areas and it exhibits an ephemeral life strategy to avoid extremely harsh conditions. All known localities from the literature and potential new suitable habitats were investigated during early spring, a wet and cool period during the past decade. This species copes with extreme periods by a rapid development during suitable rainy spring seasons (Pisarenko et al. 2001). Continental inland salt steppes and salt meadows are a typical type of habitat, in which it is possible to find *E. hungaricus*. These are rare and endangered types of habitat of outstanding ecological and conservation value in Serbia. This habitat type is found in Pannonia, mainly in the eastern part of Austria, Hungary, Slovakia and Romania (Zechmeister 2005) but also in Serbia. When researching bryophytes in salt meadows in Austria, Zechmeister (2005) found out that only *E. hungaricus* and *Pottia heimii* (Hedw.) Hampe were restricted to the salt meadows, while other collected species were also found in non-saline habitats and thus considered them as moss-halophytes.

Conservation assessment. The new regional IUCN (2012a, b) threat category (for Serbia) is assessed according to guidelines for bryophytes by Hallingbäck et al. (1998) and due to the results of its recent distribution and population knowledge.

Lab population establishment and propagation. For all laboratory and experimental treatments presented here, *E. hungaricus* was derived from genotypes out of herbarium bryophyte collections from Hungary deposited in BEOU, Bryophyte collection of Belgrade University Herbarium (Bács-Kiskun County, Kisrét at Szabadszállás, saline-alkali area, 46°51'46,5" N, 19°12'14,6" E, 100 m, 01. April 2010 and from Bács-Kiskun County, Szappanszék at Fülöpháza, saline-alkali area, 46°53'11,1" N, 19°25'35,3" E, 115 m, 01. April 2010). This species was established in axenic *in vitro* culture according to Sabovljević et al. (2012) and Ros et al. (2013), using spores as starting plant material. Once the axenic cultures were obtained, the optimization of moss growth has been studied by application of exogenously added plant growth regulators to the basic medium type in different concentrations and combinations with aim to achieve fully developed gametophores, bud formation to a great extent and secondary protonema development (see Sabovljević et al. (2014b) for details). The plants were cultured in Petri dishes, containing the basic type of medium (BCD), during six weeks for axenic optimization of large-scale propagation. Cultures were grown at 18±2°C under a 16 h photoperiod at 47 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (provided by cool-white fluorescent tubes). The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 25 min. The media were supplemented with different auxins and cytokinins, separately or together applied in concentrations of (0.03–10 µM) of indole-3-butyric acid (IBA) and N_6-benzyladenine (BA). BCD hormone free media (Sabovljević et al. 2009) were used as a basic medium for the control group. The gametophores multiplication rate (index of multiplication) and protonemal patch diameter were recorded twice during the cultivation, four and six weeks of treatment. Index of multiplication was considered as number of newly developed shoots, while the secondary protonema development was measured by the diameter increase within a certain time frame. For each treatment, shoot explants were cultured in 90 mm Petri dishes. The effects of tested growth regulators were evaluated by measuring the multiplication index and secondary protonema development. The microscope Leica DMLS and the stereomicroscope Leica MZ75 were used for observation.

Statistical analysis. For each treatment, we made four replications, each representing 10 gametophyte shoots of the same height (10 mm). The experimental procedure was repeated twice. Data were analyzed by factorial analyses of variance followed by the separation of mean values by Fisher's least significant difference test. The term significant was used to indicate differences for which $P \leq 0.05$.

Acclimation and acclimatization. Acclimatization is the process by which the axenic moss material (taken from *in vitro*) is transferred to a natural substrate and kept under controlled lab conditions while acclimation is the transfer of material already established on a natural substrate to natural conditions as elaborated in Sabovljević et al. (2014a).

Release into wild. Ten well grown, healthy, fully hydrated and well established on natural substrate (salty soil, namely Solonetz) taken from the release site acclimated patches (ca. 30 cm^2 each) of *E. hungaricus* born and originated from axenic lab culture were released into the previously chosen locality. The locality was a salty grassland with *Festuca pseudovina* L. (i.e. *Festucetum pseudovinae*), used as pasture for cows, but not overloaded by large machines and agro-measures. The site for release was chosen to be in no-man land in border region of Serbia and Romania, in the area of Banat. This was done with aim to avoid habitat changes or destruction and strong human influence (in N. Serbia 90% of lands are arable fields) as well as complicated bureaucracy. Ten sites within the same salty grassland field were chosen by chance, but trying to get small naked soil surfaces or small earth hills. The release was done in February 2014, marked by red sticks and georeferenced. The results of population establishment were recorded in March 2015 and 2016 respectively, collecting the empirically noticed threats and ecological remarks.

Results and discussion

Historically, there are only two references which cite *E. hungaricus* in Serbia (all in northern province, Vojvodina) (Guelmino 1970, 1972). There have been no records of this species till the collection made in 2010 (Papp et al. 2016). Although, it has not been found for almost 40 years, Papp et al. (2016) could confirm three previously cited localities.

Here we report the historical presence of *E. hungaricus* in Serbia (Vojvodina):

Bačka, Senta, Roman Catholic cemetery, salt meadow, May 1969. N 45°55'4.0", E 20°5'41.2", altitude 80 m (Guelmino 1970), also reconfirmed in Papp et al. (2016).

Bačka, Senta, Veliki rit, N 45°53'52.9", E 20°10'20.5", altitude 77 m (Guelmino 1972).

Bačka, Bačka Palanka, Miškov salaš, salt meadow, May 1970. N 45°15'26.1", E 19°25'40.9", altitude 80 m (Guelmino 1970).

Bačka, Žabalj, N 45°22'41.1", E 20°2'27.7", altitude 82 m (Guelmino 1972).

Bačka, Novi Sad, Veternik, on salt meadow, in scattered association with *Festuca pseudovina,* May 1970. N 45°14'50.4", E 19°45'20.3", altitude 80 m (Guelmino 1970).

Banat, Padej, on a salt meadow, near the road, in scattered association *Artemisio-Festucetum pseudovinae*, May 1969. N 45°49'54.3", E 20°11'2.9", altitude 79 m (Guelmino 1970, also reconfirmed in Papp et al. (2016).

Banat, Sanad, on a slope, near the road, in scattered association with *Festuca pseudovina,* May 1969. N 45°58'30.2", E 20°7'23.3", altitude 80 m (Guelmino 1970).

Banat, Novi Bečej, Novo Miloševo, N 45°40'27.3", E 20°15'25.9", altitude 79 m (Guelmino 1972).

In spring 2010, new data of *E. hungaricus* occurrence in Serbia were recorded (Papp et al. 2016). Additional new localities of *E. hungaricus* in Serbia were found in spring of 2014 and 2015 during field work in Vojvodina:

Bačka, Velebit near Senta, saline grassland, N 46°0'38.78", E 19°57'9.34", altitude 90 m. Date: 06.04.2010. leg. Papp B. (Papp et al. 2016).

Bačka, Tresnjevac (Oromhegyes) near Senta, saline grassland, N 45°59'18.90", E 20°0'32.20", altitude 85 m. Date: 06.04.2010. leg. Papp B. (Papp et al. 2016).

Banat, Padej near Senta, saline grassland, N 45°50'17.7", E 20°9'58.1" and N 45°50'28.80", E 20°9'53.40", altitude 80 m. Date: 06.04.2010. leg. Papp B. (Papp et al. 2016).

Banat, Crne Bare, N 45°58'42,8", E 20°15'43,1", altitude 78 m. Date: 11.03.2014, salt grassland, leg. Janković I., Nikolić N., Pantović J., Sabovljević M. [BEOU].

Banat, Margita N 45°12'44.22", E 21°11'26.36", altitude 75 m. Date: 20.03.2015, salt grassland, leg. Nikolić N., Sabovljević M. [BEOU].

Banat, Banja Rusanda, near Melenci, N 45°31'41.87", E 20°17'54.42", altitude 79 m. Date: 22.04.2015, salt grassland, leg. Nikolić N., Pantović J., Sabovljević M. [BEOU].

Banat, Slano Kopovo N 45°36'40.44", E 20°12'56.92", altitude 74 m. Date: 22.04.2015, salt grassland, leg. Nikolić N., Pantović J., Sabovljević M. [BEOU].

After more than 40 years, seven new localities of *E. hungaricus* are documented in Serbia (the total distribution in Serbia is given in Fig. 1). During previous research of Bačka region's bryoflora, *E. hungaricus* was not recorded (Sabovljević & Stevanović 2006). This species is protected by Serbian Nature Protection Law ('Official Gazette of the Republic of Serbia' No. 36/09 and 88/2010) and it is categorized as EN (endangered) species according to the Bryophyte Red List

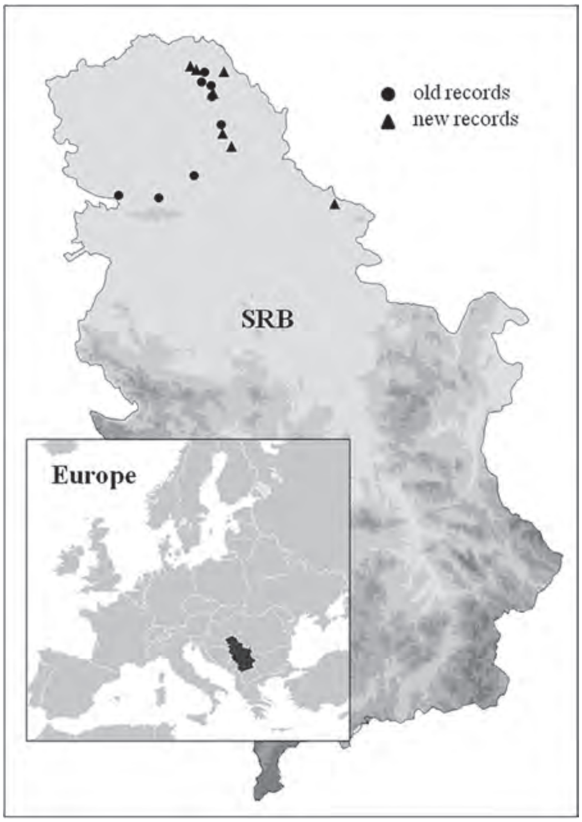

Figure 1. Distribution of *E. hungaricus* in Serbia (old (circles) and new (triangles) records).

of Serbia and Montenegro (Sabovljevic et al. 2004). Due to new data, a revision of threat status has been carried out, and the species stays in category Endangered. Nevertheless, some historical localities were ruined by construction or significant ecological changes in the sites, but nearby localities need to be checked in more detail. We assume that the disruption and fragmentation of generally rare habitat type (salt grasslands) for *E. hungaricus* are the reason for the rarity of this species. Another threat can be assumed because the suitable habitats are often considered as lands of low economic importance and thus are used for construction or raw material deposition. Sometimes, it is easily permitted to change the legal purpose of land due to unknown biological and/or low economic and agricultural values. The ephemeral character of this species and low bryophyte diversity in salt habitats are potential reasons for possible overlooking of *E. hungaricus*. From our experience, it turned out that the optimal time for rapid grow of *E. hungaricus* in Serbia is during February, March and April, but it also depends on the yearly climate condition variation. Unfavourable weather conditions during that period are often not pleasant for bryological search, due to coldness, strong winds and partially flooded terrain.

This species is established in axenic *in vitro* culture (Fig. 2) as suggested by Sabovljevic et al. (2012) and Ros et al. (2013). With the aim to achieve a rapid multiplication and propagation, the best developmental patterns were examined by adjusting growth conditions such as light and temperature, but also by exogenously added auxin and cytokinin both separately and jointly. Significant differences in protonemal patch diameter among plants after four and six weeks of treatment were noticed (Figs 3 and 4). Secondary protonema diameter was largest on media

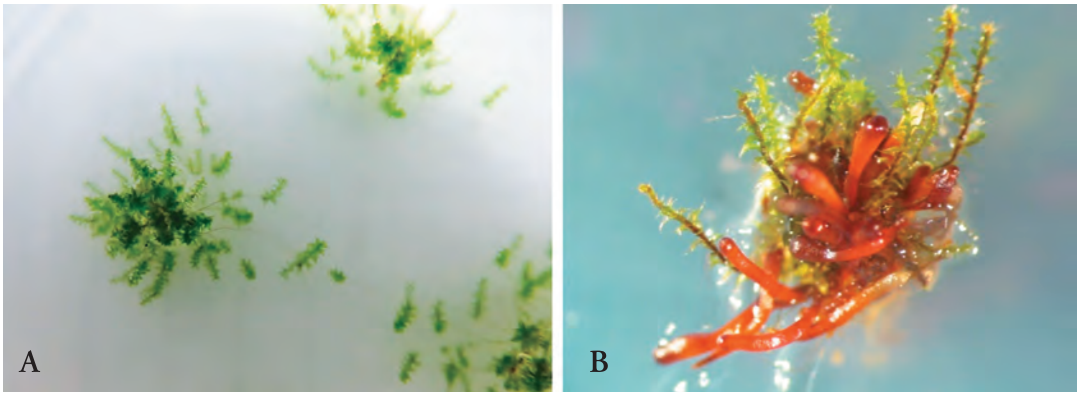

Figure 2. A – *in vitro* culture of the moss *E. hungaricus* with fully developed gametophores; B – apogamous sporophytes developed in *E. hungaricus* in *in vitro* conditions with insufficient light regime.

enriched with IBA and free of BA, compared to all other combined treatments, especially when results were recorded six weeks after the cultivation compared to four weeks development (Fig. 2). If plants were grown for four weeks, protonemal patch diameter was high on medium free of BA or with low BA content (0.03 µM) combined with low IBA concentrations (0–1 µM) (Fig. 3). Secondary protonema was up to two times larger if plants were grown for six weeks compared to plants grown for four weeks. In six weeks treatment, secondary protonema diameter was the highest when IBA was applied in low concentrations (0–1 µM) with no BA addition (Fig. 4). With increasing BA content in the medium, protonema patch diameter decreased. This proved to be quite significant since it was shown that protonema is a good material for propagation, where each cell could establish a new colony both in wild and in controlled conditions (Fig. 5).

On secondary protonema, buds appeared on BCD hormone free medium and also in media containing IBA, BA or combination of these two, but to different extent. The differences in IBA and BA effects on multiplication index in four- and six- weeks treatments were noticed (Figs 6 and 7). Multiplication index recorded after four weeks treatment showed the highest values on BCD enriched with 0.03 µM BA and 0.1 µM IBA (Fig. 6).When *E. hungaricus* was grown for six weeks, the multiplication index was the highest on the medium with 0.03 µM BA and

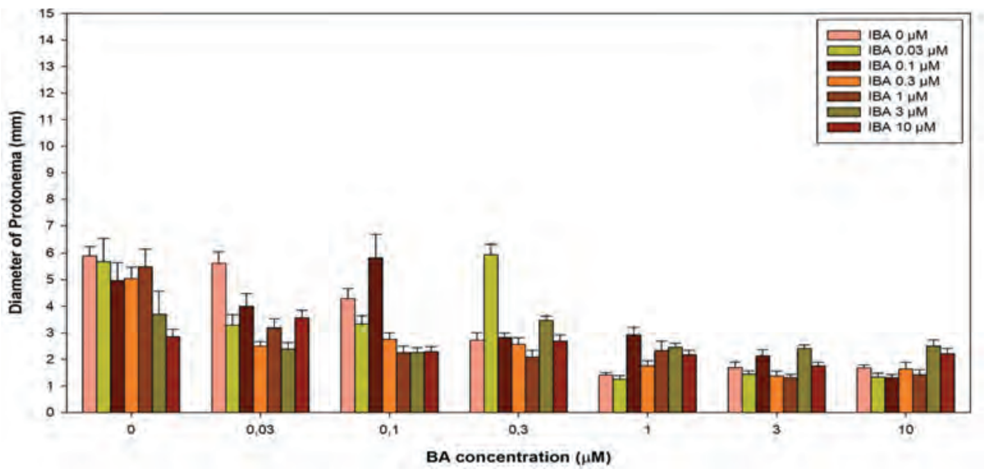

Figure 3. Protonemal patch diameter of *E. hungaricus* after 4 weeks.

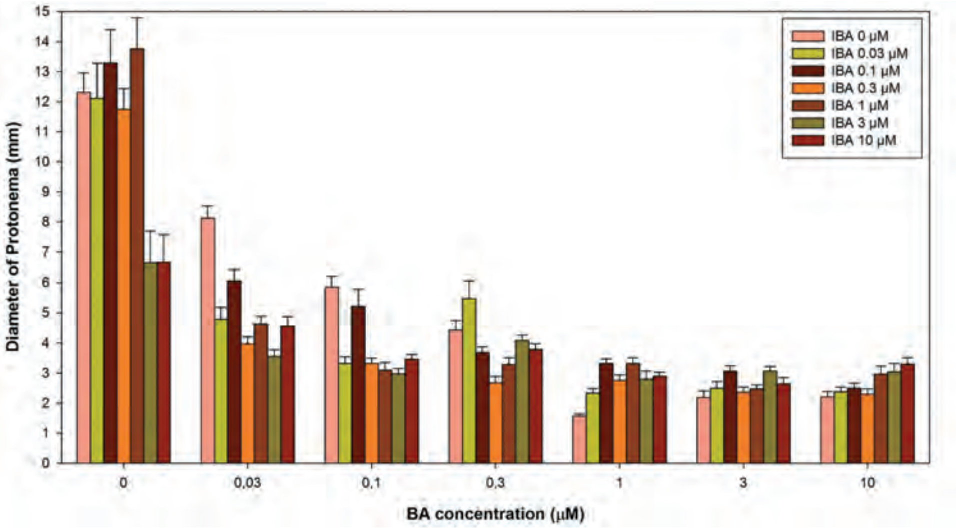

Figure 4. Protonemal patch diameter of *E. hungaricus* after 6 weeks.

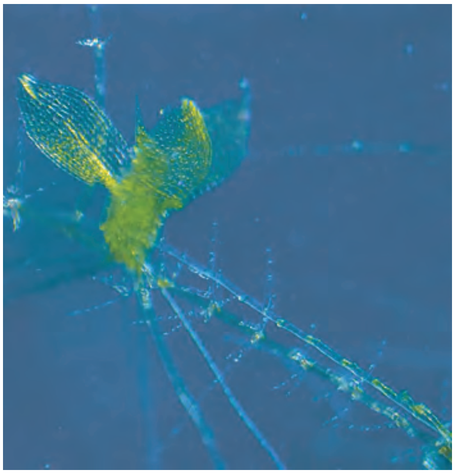

Figure 5. Protonema of *E. hungaricus* with a small developing gametophore.

0.1 µM IBA, as well as on BCD medium with no BAP and with 0.1–0.3 µM IBA (Fig. 7). Also, the growth regulator IBA applied alone had a positive effect on the multiplication of shoots after six weeks. These facts once achieved would serve in the procedure of propagation if gametophytes with gametophores (green plantlets) and not just protonema should be obtained (Fig. 8). This is significant since protonema cannot be observed and identified in the field easily.

There are many evidences that the effects of various growth regulators on various bryophytes seem to be rather specific and in some cases contradictory. Auxins and cytokinins have basic functions in the regulation of normal bryophyte development.

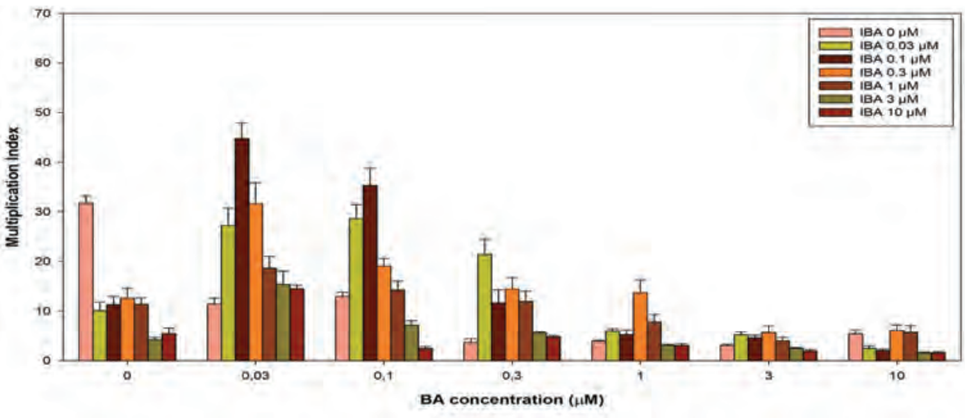

Figure 6. Multiplication index (number of newly established gametophores) after 4 weeks.

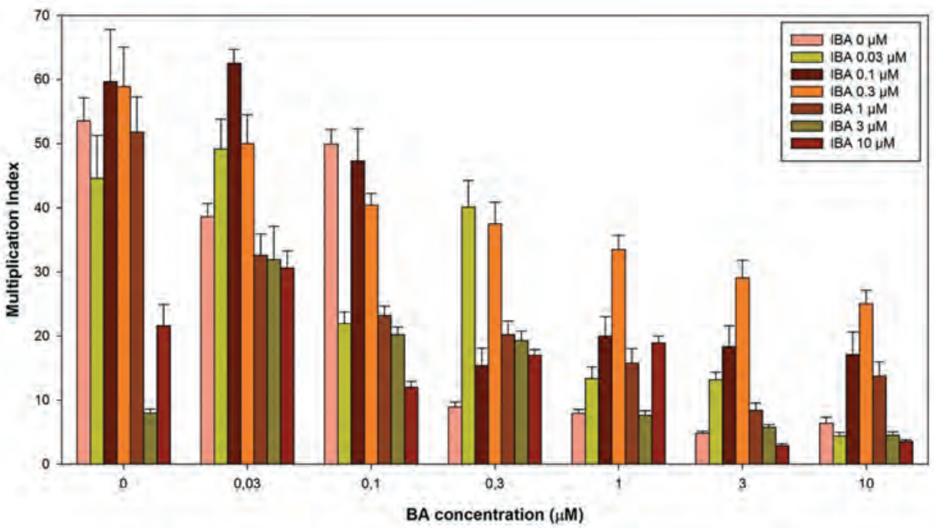

Figure 7. Multiplication index (number of newly established gametophores) after 6 weeks.

Although the effects on exogenously applied auxins and cytokinins on moss development were discovered many years ago, results are very scattered. Only few bryophyte species have been studied so far, in particular not the rare ones and the general patterns have many exceptions (Sabovljević et al. 2014a, b). Growth regulators such as IBA and BA play an important role in forming of buds and growth of secondary protonema in mosses (Figs 5 and 8). In general, ratio between these two hormones effect inductions of buds, new gametophore appearance and growth of protonemal patch in related moss *Physcomitrella patens* (Hedw.) Bruch & Schimp. (von Schwartzenberg 2009). Our results also imply that time plays a role in the autonomous plant regulator production and in reaching the effective level of plant regulators. This was already stated by Bopp (1990) and Johri & D'Souza (1990), who assumed that the size of protonema patch is among prior parameters which are important in gametophore development. This is in accordance with the results we achieved for *E. hungaricus* in the six week treatment. Auxins promote the development of caulonema in *P. patens*, but the mechanisms, by which these regulators interact during development, remain rather unknown (Jang & Dolan 2011).

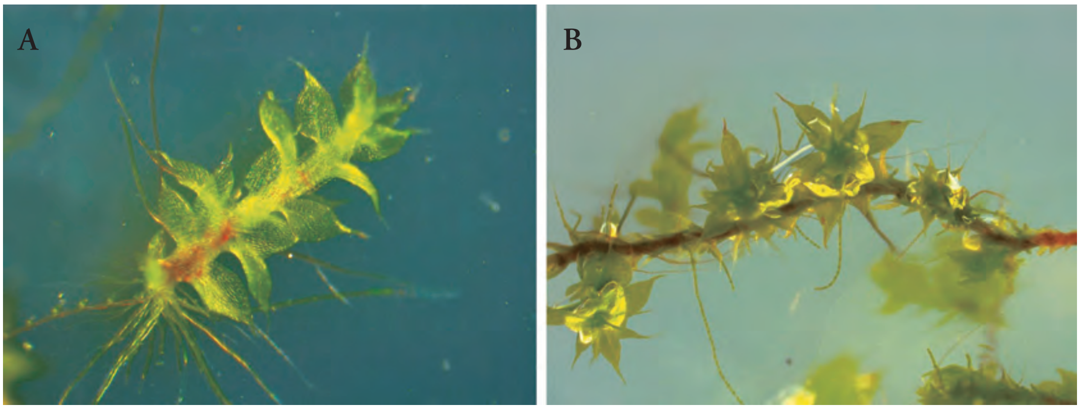

Figure 8. *E. hungaricus.* A – well developed gametophore; B – numerous new gametophores developing on medium enriched with plant growth regulators.

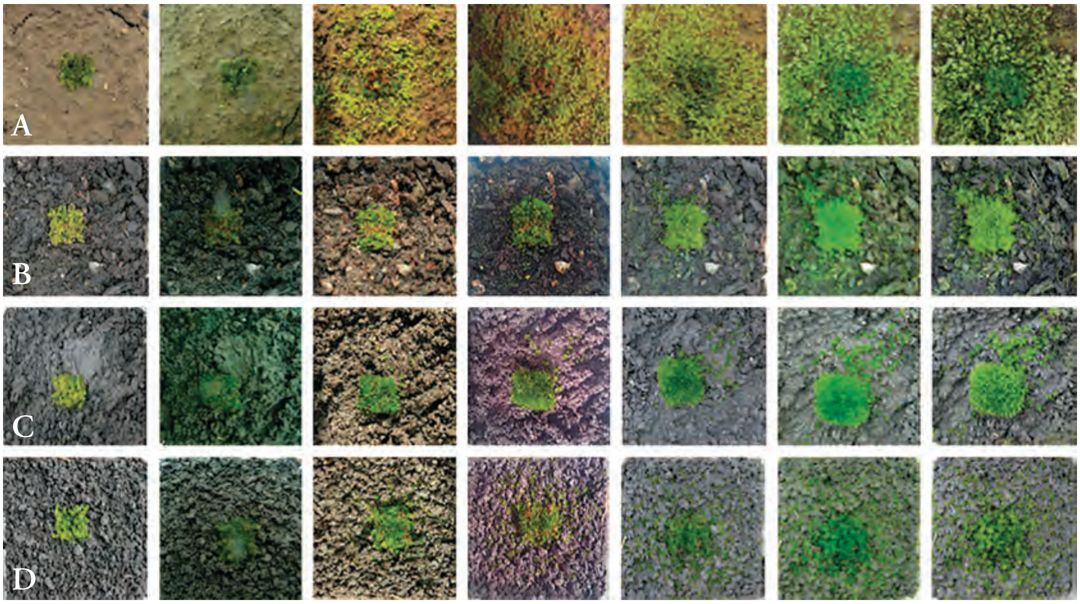

Figure 9. Acclimatization of *E. hungaricus* propagated under *in vitro* condition for *ex situ* purposes on different soil types for 6 weeks (each column represents a week more of growth). A – plants transferred from medium to non-salt loaded soils are overgrown by other mosses completely; B – plants transferred from medium to humus; C – plants transferred from medium to prior-heat sterilized salt soils; D – plants transferred from medium to natural salt soils.

The results presented here seem to fit the ecological requirements of this species, too. Bud production and reproductive effort happen simultaneously in accordance to harsh environmental conditions. Thus, as an adaptation to relatively dry springs, the effort in bud production should start exclusively if humidity is high and long enough (in reality rather short). So, the achievement of the effective protonemal patch size does not suppress the gametophore production, sex organs and sporophyte formation. In contrast, the suppressed protonemal patch development leads to tmema cell production by just short positive environmental stimuli with the aim to survive the drought period.

It is assumed that the low light intensity induces apogamy in *E. hungaricus* and the spores directly produce sporophytes (Fig. 2B) (Vujičić et al. 2013), but the ploidy level of spores and sporophytes remain unclear. The ecological sense of these can be observed in providing survival by rapid spore production in case of overgrowth by vascular plants and resources overtake during the short suitable period for *E. hungaricus* growth. Also, the rapidness saves the energy of this autoecious plants in developing reproductive organs.

Once, the propagation and optimization achieved, it leads to mass production of *E. hungaricus* under laboratory conditions. With such lab produced moss materials, we started the experimentations of transferring the moss material on various soil types (Fig. 9). Although *E. hungaricus* is thought to be an obligate halophyte (Papp 2002; Zechmeister 2005), which is true in ecological sense, it could fully develop on basic media (e.g. BCD, Knop, Murashige and Skoog) with and without additional salt. Thus, *E. hungaricus* should be considered as facultative moss halophyte *sensu* Sabovljević & Sabovljević (2007).

The experimentation for adjustment of moss patches developed axenically to soil conditions including its abiotic and biotic properties showed that *E. hungaricus* can develop on and tolerate

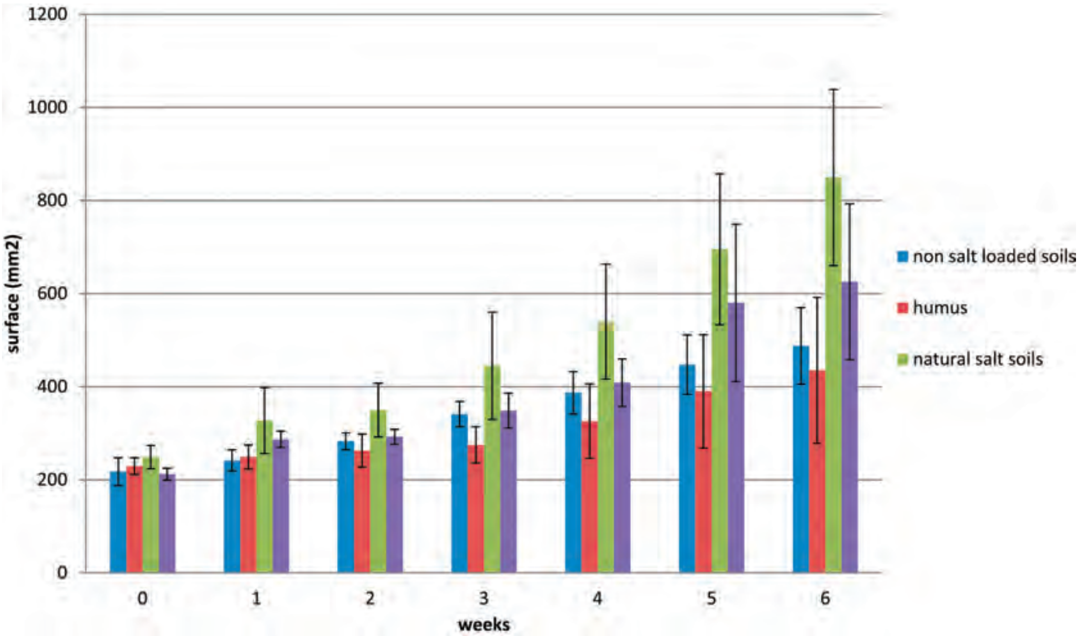

Figure 10. Acclimatization of *E. hungaricus*. Development (yield) measured by surface occupied by the lab propagated plant; start material ca 200 mm^2.

various soil types (Figs 9 and 10), but if the soil is not overloaded with salt and/or not sterilized priorily, it will be easily overgrown by other mosses such as *Barbula unguiculata* Hedw. However, on salty and unsterile soils, *E. hungaricus* is highly competitive with other mosses, and this is probably the reason of being such a specialist in salt habitat. It can survive and develop on non-salty habitats, but it is no competitor to the other moss species. Nevertheless, this investigation also confirms *E. hungaricus* to be a facultative halophyte moss species.

The moss patches with developed gametophores on salty soils in controlled lab conditions were used for establishment in a chosen polygon for reintroduction and native population strengthening in the area of the southern region of the Great Pannonian Plain. The area is situated in Banat in Northern Serbia and the mosses were transferred by the end of February 2014

Figure 11. Reintroduction of *in vitro* propagated and acclimatized material of *E. hungaricus*.

Figure 12. Patch of *E. hungaricus* developed in reintroduction site in the next vegetation season (one year later) with sporophytes.

(Fig. 11). Ten populations were transplanted and left for one year, marked with red sticks and georeferenced with the aim to look for them in spring 2015 and 2016. Three of ten populations were reproducing the next year on the same places (30% achievement, Fig. 12), which seems to be a rather successful reintroduction (bearing in mind that April 2014 in Serbia was extremely dry compared to average April weather). In 2016, the authors found 2 patches in this field as well (the third one was physically destroyed by cows).

Additional factor affecting reintroduced population were cows, which moved rather more than usually during dry months to look for good grass and thus ruined some of populations.

Our experience showed that cows in a balanced number of individuals and time spent in the salt pastures with *Festuca pseudovina*, keep some soil patches open both by grazing and moving and favour micro-sites for *E. hungaricus* development, while in cow-overpopulated salt pastures, cows can be regarded as threat factor. Another threat factors of this species in Serbia are habitat degradation, changes and devastation which have probably the highest impact to this species. According to the present distribution and population size of *E. hungaricus* in Serbia and the application of the IUCN criteria slightly changed for bryophytes, this species is regionally (for Serbia) assessed in endangered category (EN) under criterion B. Spores are often produced in Serbia and neighbouring countries and this species is assumed to have a rather good dispersal capacity by both wind and animals (e.g. birds). The overtaken measures (legislative protection and *ex situ* conservation) and a good dispersal capacity lead to downgrade the threat category to Vulnerable in Serbia (VU) at present. However, due to the absence of stepping stone habitats, present habitat degradation and changes, continuous monitoring on this species status is needed and thus, at present, it remains in endangered threat category (EN) in Serbia.

Nevertheless, further investigation and proper conservation management can downgrade or even get this species off from the red-listed bryophytes of Serbia.

Acknowledgements

The study was supported by the Ministry of Education, Science and Technological development of the Republic of Serbia (grants no. 173024 and 173030).

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