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Experimental Insights into *Ex Situ* Moss Conservation: A Case Study on *Anacamptodon splachnoides*

Bojana Z. Jadranin [1], Marija V. Vesović [1], Djordje P. Božović [1], Michal Goga [2,3], Milorad M. Vujičić [1,4], Pavel Širka [5], Beáta Papp [6], Aneta D. Sabovljević [1,4] and Marko S. Sabovljević [1,2,4,*]

1 Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia
2 Department of Plant Biology, Institute of Biology and Ecology, Faculty of Science, Pavol Jozef Šafárik University, Mánesova 23, 040 01 Košice, Slovakia
3 Center for Interdisciplinary Biosciences, Technology and Innovation Park, Pavol Jozef Šafárik University, Jesenná 5, 041 54 Košice, Slovakia
4 Center of Plant Biotechnology and Conservation (CPBC), Takovska 43, 11000 Belgrade, Serbia
5 Department of Phytology, Faculty of Forestry, Technical University in Zvolen, T. G. Masaryka 24, 960 01 Zvolen, Slovakia
6 Botanical Department, Hungarian Natural History Museum, H-1431 Budapest, Hungary
* Correspondence: marko@bio.bg.ac.rs

Abstract

A rare and threatened pleurocarpous amblystegiaceaen dendrothelmatic moss, *Anacamptodon splachnoides*, was the subject of *in vitro* establishment and propagation. Tests on growth and rapid propagation were applied with the aim of preparing plant material for outdoor *ex situ* conservation and reintroduction into nature. This integrative conservation approach provides novel methodologies applicable to conservation programmes and management strategies, while also providing fundamental insights into the species' biology. It further enables research on the species without harming its native populations, allowing for the exploration of questions raised during this investigation, such as spore biology and sex expression. *A. splachnoides* acts as both a shelter and signal species for a special microhabitat type, which also supports other rare organisms, and prolonged drought seems to affect reproduction, spore dispersal, and germinability more strongly than gametophore survival.

Keywords: integrative conservation; conservation physiology; knothole moss; propagation; acclimatisation

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1. Introduction

Rapid, unplanned and unpredictable environmental changes affect all biological entities. Many organisms have little or no capacity to adapt and survive, leading to population decline and, in some cases, silent extinction. Conservation initiatives often respond too late and tend to focus primarily on large plants and animals, while many other taxa remain neglected. Among these, bryophytes are frequently overlooked as being less significant than tracheophytes. However, they represent a fundamental component of all terrestrial ecosystems, playing a pivotal role in water retention and regulation, nutrient cycling and storage, and habitat formation. As organisms specialised for specific microhabitat types and environmental factors, bryophytes are particularly sensitive to habitat degradation, pollution, and climate change. Their specific morpho-anatomical features, such as a rootless thallus and the absence of a cuticle, further reduce their ability to withstand rapid environmental changes compared to vascular plants, resulting in heightened vulnerability and

extinction risk [1,2]. In this context, *ex situ* conservation emerges as a critical complementary strategy, providing controlled conditions for safeguarding threatened taxa and supporting future restoration efforts. Conversely, studies and experimental approaches, along with techniques and protocols for the conservation of bryophytes, remain insufficient.

Anacamptodon splachnoides (Froel. ex Brid.) Brid. (knothole moss) is a pleurocarpous, epiphytic moss belonging to the family Amblystegiaceae. It has an amphi-Atlantic distribution range, and outside Europe, it can be found in North America and the Caucasus [3].

It typically inhabits dendrotelmata, rainwater-retaining tree cavities, as well as moist, decomposing bark in shaded and humid forest environments. Unusually, in rather rare combinations of abiotic factors, it can also be found in composite substrates comprising rocks, soil and tree roots and wood [4]. Its threat status in Europe is currently regarded as Near-Threatened (NT) [5], but in a previous assessment, it was classified as Endangered (EN). This moss has a continental distribution, with a focus on Central Europe, and it is a naturally rare species, with a continuing decline in both the extent and quality of its habitat [6]. In many European regions, the species has not been rediscovered, and only a few recent records are available [4,7–9]. This scarcity of contemporary data suggests an NT conservation status, as the species' current population size and trends cannot be reliably assessed due to the predominance of old and incomplete records. The species requires very precise targeted searches and can easily be overlooked, resulting in substantial gaps in its known distribution. Nevertheless, it faces a high risk of extinction at the national level and was considered regionally extinct (RE) in Switzerland until its rediscovery in 2011. It is currently assessed as Endangered (EN) in Austria, Czechia, Hungary, Poland, and Slovakia, and as Vulnerable (VU) in Germany, Romania, and Ukraine [6,10]. In Slovenia, it is assessed as Endangered [11], while in Serbia it is classified as Data Deficient (DD) [12].

Modern forestry practices, including short rotation lengths, forest road construction, forest openings, and clear-cutting, together with the associated severe alterations of microclimatic conditions, as well as ongoing changes in global climate and precipitation patterns, represent major threats to this species. The fragmentation of suitable microhabitats further compromises its survival and self-sustainability within the areas it occurs. The species is evidently threatened with extinction, and continued monitoring of known populations, together with further research on its biology and ecology, is urgently needed [6].

With the aim of developing conservation protocols and proposing effective protection measures, we applied an integrative conservation and conservation physiology approach to improve understanding of the species' biology and ecological characteristics. This included *in vitro* propagation and mass production, as well as testing acclimatisation procedures and the establishment of new outdoor *ex situ* populations. Such an integrative conservation approach should be applied to halt overall population decline and to mitigate the impacts of natural microhabitat destruction.

2. Materials and Methods

2.1. Plant Material

The moss material used in this study originated from a less than one-year-old herbarium specimen from Hungary, Veszprém County, Balaton-felvidék, Szentbékkálla, Sátorma-tető, in *Quercetum cerris* prov., on the bark of *Quercus cerris* L. on a dendrotelma, 46°53′57,70″ N, 17°32′32,30″ E, 320 m a.s.l., 17 May 2016, leg. and det. Papp, B. (BP 192078). Some additional moss herbarium specimens from Slovakia and Hungary were also tested; however, the results suggested the presence of a persistent, possibly unbreakable spore dormancy. This phenomenon remains a subject for further study, as data on bryophyte spore biology are extremely limited [13].

2.2. In Vitro Culture Establishment

Axenic cultures were established from the spores of the above-mentioned Hungarian accession. Nearly mature, unopened capsules were selected from the herbarium material and surface-sterilised with NaDCC (5%) for 10 min. The capsules were then rinsed with distilled, sterilised water and opened under a laminar airflow chamber over Petri dishes containing KNOP medium, allowing the spores to be released directly onto the medium. Spores germinated after 19 days. The procedure was repeated with some other rather recently collected Hungarian and Slovakian herbarium material, but no spore germination was observed. Fragments of axenic primary protonema were transferred to fresh KNOP medium (for medium composition, please see [14]) and maintained at 18 ± 2 °C, relative humidity of ca. 70%, and a 12 h light/12 h dark photoperiod. Light was provided by fluorescent tubes (Tesla Pančevo) at an intensity of 60 µmol m^{-2} s^{-1}. After approximately six weeks, the first leafy gametophores appeared spontaneously. They were subsequently used for further propagation and secondary protonema development.

Once the axenic *in vitro* cultures were established, the plant material was maintained and multiplied on minimal KNOP medium until sufficient biomass was obtained for the experiments. The medium was adjusted to pH 5.8 prior to autoclaving at 121 °C for 45 min. Experiments were conducted using 1 cm long explants with the tips excised. The removal of the tips eliminated pre-existing meristematic activity, preventing its potential effect on regeneration and reproductive timing. Consequently, all the explants originated from comparable developmental conditions, allowing *de novo* meristem formation without the influence of prior growth (the equal possibility of differentiated cell dedifferentiation to meristematic ones and endogenous plant growth regulation). The experimental treatment consisted of five Petri dishes, each containing four explants, resulting in 20 biological replicates per treatment (n = 20). The experiments were conducted under sterile axenic conditions, following previously established procedures [15–17], to exclude the effects of associated microorganisms or uncontrolled environmental factors. The cultures were maintained in sterile Petri dishes under controlled conditions of 18 ± 2 °C, relative humidity of 60–70%, and a long-day photoperiod (16 h light/8 h dark). Light was provided by fluorescent tubes (Tesla Pančevo) at an intensity of 50 µmol m^{-2} s^{-1}.

2.3. Experimental Design

The effects of the exogenously applied plant growth regulators (PGRs) were evaluated in a PGR-type experiment aimed at promoting more rapid growth and increased biomass production. The explants were positioned either upright or prostrate (Figure 1) on the KNOP medium supplemented with varying concentrations (0.03, 0.3, or 3 µM) of indole-3-butyric acid (IBA) or 6-benzylaminopurine (BAP). Both upright and prostrate positions were tested to confirm whether the contact area between the explant and the medium significantly affects growth. Since bryophytes possess no cuticles, and uptake occurs across their entire surface, we hypothesised that a larger contact surface could provide more rapid growth. In addition, the lack of vascular tissue implies the slow transport of water, nutrients and signals from the organism's environment, and thus cell-to-cell transport can be replaced by rather shorter supply routes. The applied concentrations were selected based on previous studies on ambystegiacean species development under *in vitro* conditions [15–17]. After four weeks, the morphological parameters were measured, including the index of multiplication (IM), which reflects the number of new shoots developing from buds on the original explant, the diameter of the secondary protonemal patch, and the survival rate of the explants. For documentation and further analysis, images of the explants were captured using a Leica MZ stereomicroscope (Leica MZ 7.5, Bi-Optic Inc., Santa Clara, CA, USA).

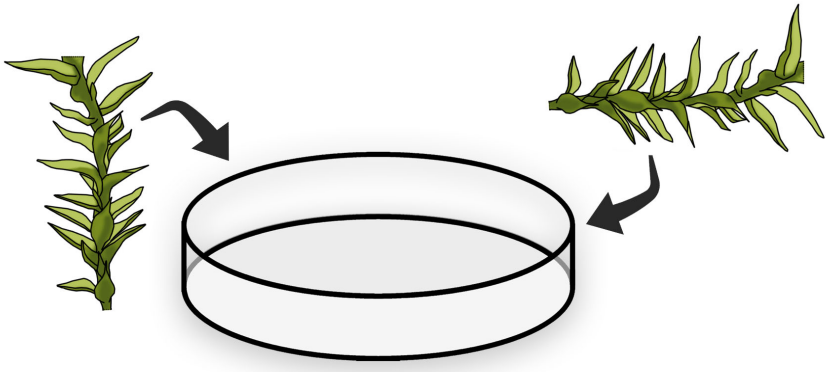


Figure 1. Schematic presentation of the position of the moss explants in the experimental setup—upright vs. prostrate. An explant is a decapitated gametophore (leafy part of gametophytes).

The acclimatisation experiments consisted of two setups. In the first setup, the plantlets were transferred from *in vitro* culture onto dead oak bark partly submerged in rainwater and maintained under controlled xenic conditions within Magenta vessels (18 ± 2 °C, relative humidity 80–90%, and a long-day photoperiod of 16 h light/8 h dark). In the second setup, the plants were moved from *in vitro* culture to a rainwater-retaining dendrotelmatic cavity in a beech trunk under outdoor, uncontrolled conditions in the Botanical Garden in Belgrade, Serbia. The holes were artificially created to a depth of 12 cm and a diameter of 8 cm, and the trunks were kept in the undercanopy shade of Acer trees, thus ensuring constant diffused light. The artificial cavities were maintained approximately two-thirds filled with water; if not naturally replenished by rain, water was added manually to maintain the microsite humid and moist and to prevent full desiccation. Despite this, summer desiccation occurred a few times during the hottest period, and the plants experienced a short-term absence of water (up to one week), but the knothole remained wet. The experiment was conducted at the Botanical Garden Jevremovac, University of Belgrade, Serbia, where the climate is mild-continental, with an average annual temperature of 12.8 °C, and 696 mm of precipitation unevenly distributed throughout the average year. Winter frosts and summer heat waves are common.

2.4. Statistical Analysis

All the statistical analyses were carried out in the R environment (v.4.3.2) [18]. Initial data exploration involved assessing distributional assumptions and variance homogeneity using the Shapiro–Wilk test for normality and Levene's test for homoscedasticity. The results indicated significant violations of normality and homoscedasticity; therefore, non-parametric statistics were applied. The measured parameters were analysed using a nonparametric factorial approach based on the Aligned Rank Transform (ART) methodology [19,20]. The ART procedure was applied to evaluate the main effects and interaction terms within factorial designs, followed by post hoc contrast testing to examine specific group differences employing the "ARTool" R package (v.0.11.0) [21]. Benjamini–Hochberg *p*-value adjustment method was used for multiple comparisons.

3. Results

The cultivation of bryophytes remains a major constraint in the development of bryophyte science, both in experimental and applied approaches. Here, we present the first successful axenic and xenic cultivation of the threatened and rare pleurocarpous moss *A. splachnoides*, a species of rather specific ecology (for a list of the species cultivated to date, please see [22]).

3.1. The Impact of Plant Growth Regulators and the Plant Orientation on the Morphogenesis of A. splachnoides

IBA had a significant positive effect on the multiplication index ($p < 0.05$) (Figure 2A). The lowest IBA concentration (0.03 µM) resulted in the highest multiplication index, and at lower concentrations, statistically significant differences between explant orientations were observed ($p < 0.05$), suggesting a hormesis-like effect on *A. splachnoides* development. In contrast, at the highest applied concentration (3 µM), these orientation-dependent differences were no longer evident, and the effect of IBA was similar regardless of explant orientation (Figure 2A). This pattern suggests that at lower IBA concentrations, prostrate explants may absorb more of the applied treatment due to their larger contact area with the medium. At higher concentrations, however, uptake may become saturated, resulting in comparable effects across explant orientations. Consistent with this interpretation, the factorial analysis revealed significant main effects of explant orientation (EO) and IBA concentration (C), as well as a significant EO × C interaction ($p < 0.001$), confirming that the effect of explant orientation depends on IBA concentration (Figure 2A).

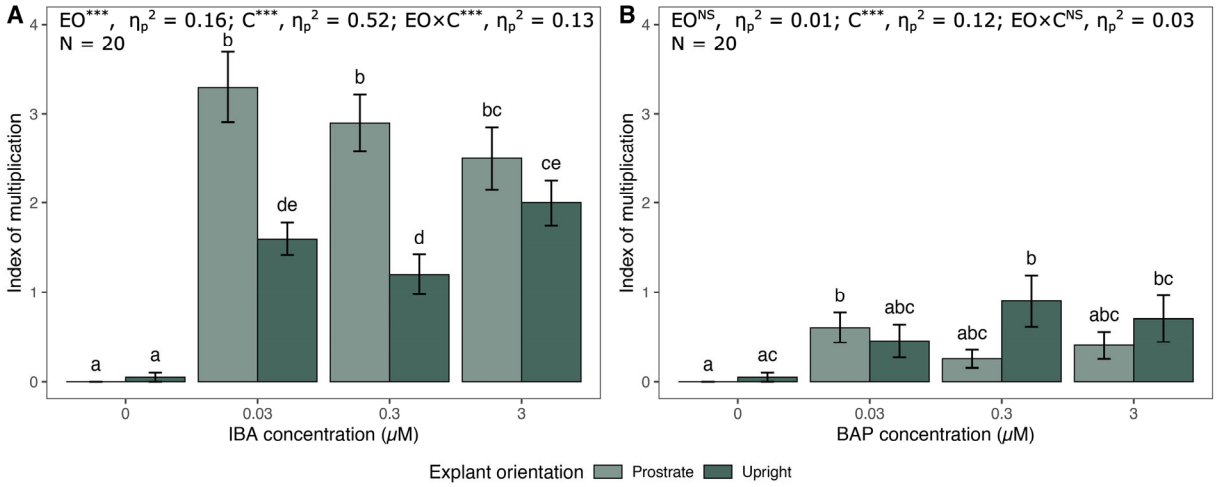


Figure 2. The influence of explant position and different concentrations of IBA (**A**) and BAP (**B**) on the multiplication index is shown. Values are expressed as means with corresponding standard errors. Distinct letters above the bars indicate significant pairwise differences among experimental groups ($p < 0.05$). The statistical significance and effect sizes, assessed using partial eta squared (η_p^2), of the main effects, i.e., explant orientation (EO), plant growth regulator concentration (C), and their interaction (EO × C) derived from factorial analysis are indicated by symbols (*** $p < 0.001$, ^{NS}—non-significant).

Similarly to IBA, lower applied concentrations of BAP also had a positive effect on the multiplication index (Figure 2B). Specifically, only the lowest tested BAP concentration significantly increased the multiplication index in the prostrate-oriented explants ($p < 0.05$), whereas in the upright-oriented explants this effect was observed at 0.3 µM BAP ($p < 0.05$). Consistent with the hormetic-like response observed for IBA, the prostrate explants required lower BAP concentrations to achieve a positive effect on multiplication compared to the upright-oriented explants. Furthermore, no significant differences between explant orientations were detected within individual BAP treatments, which is consistent with the factorial analysis showing that only the main effect of concentration (C) was significant ($p < 0.001$) (Figure 2B).

Plants grown on the KNOP minimal medium without the addition of plant growth regulators developed normally regardless of explant orientation, but produced relatively few new branches (Figure 3A,E,I). In contrast, the addition of IBA had a pronounced positive effect on plant phenotype, resulting in the formation of numerous new branches

(Figure 3B–D,F–H), especially in the prostrate-oriented plants. The application of BAP also promoted the development of larger plants with increased branching (Figure 3J–L), although this effect was less pronounced than that observed in the IBA-treated plants.

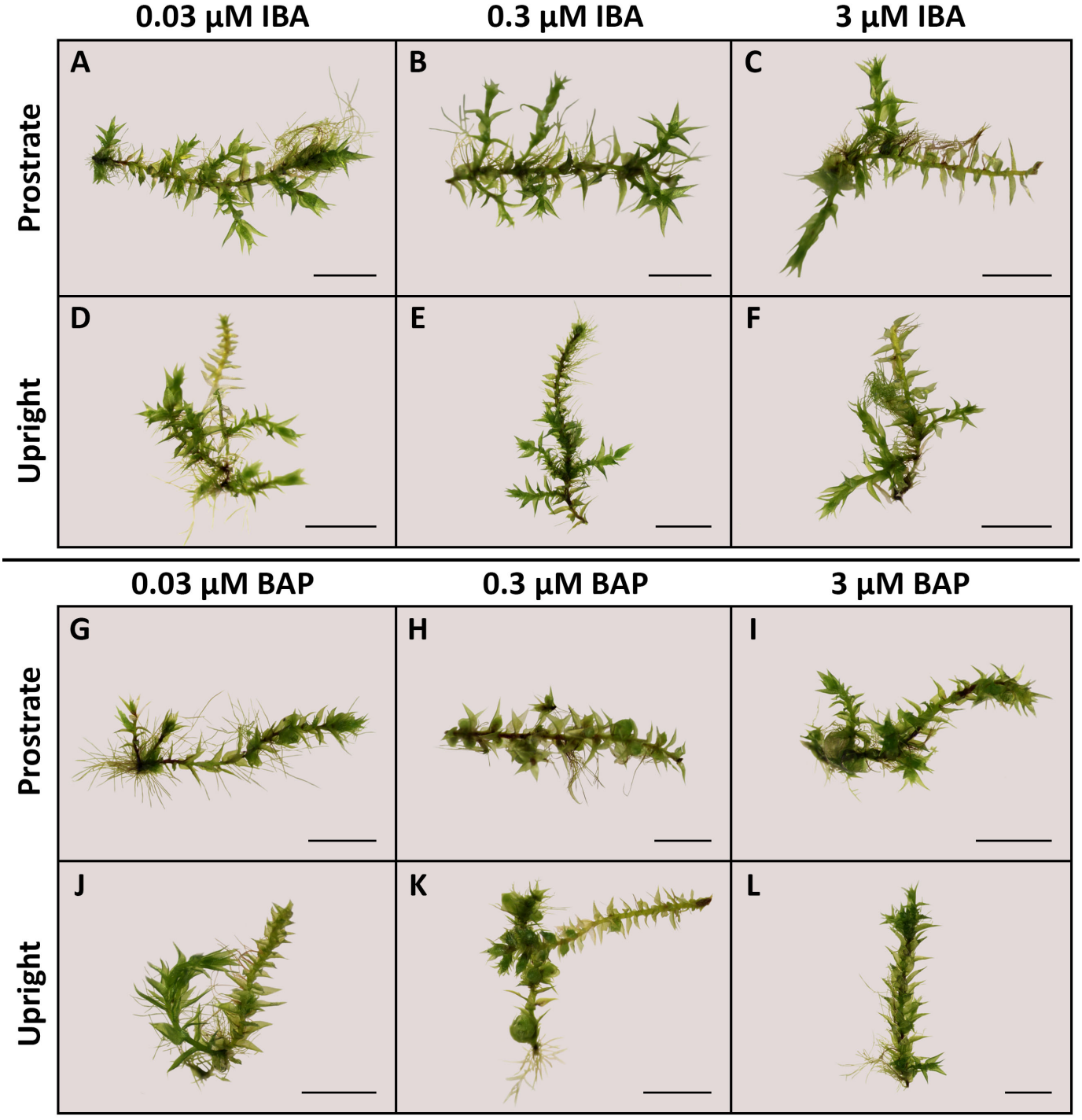


Figure 3. Phenotypic appearance of A. splachnoides explants grown on KNOP medium under different explant orientations (prostrate (**A**–**C**,**G**–**I**) and upright (**D**–**F**,**J**–**L**)) and supplemented with varying concentrations of IBA (In-dole-3-butyric acid; (**A**–**F**)) and BAP (6-benzylaminopurine; (**G**–**L**)). Under the treatments, decapitated and un-branched gametophores (lacking meristematic tetrads)developed secondary protonemal threads and new branches. The bars represent a size of 2 mm.

3.2. Acclimatisation of A. splachnoides on Oak Bark and Beech Trunk

Plantlets of *A. splachnoides* cleaned from medium remnants were transferred from *in vitro* conditions (Figure 4A) onto oak bark partly submerged in rainwater, attached to wet fissures of oak bark which were not directly submerged in water (Figure 4B). Within four weeks, the gametophytes showed a marked increase in size and intensive branching with numerous new shoots.

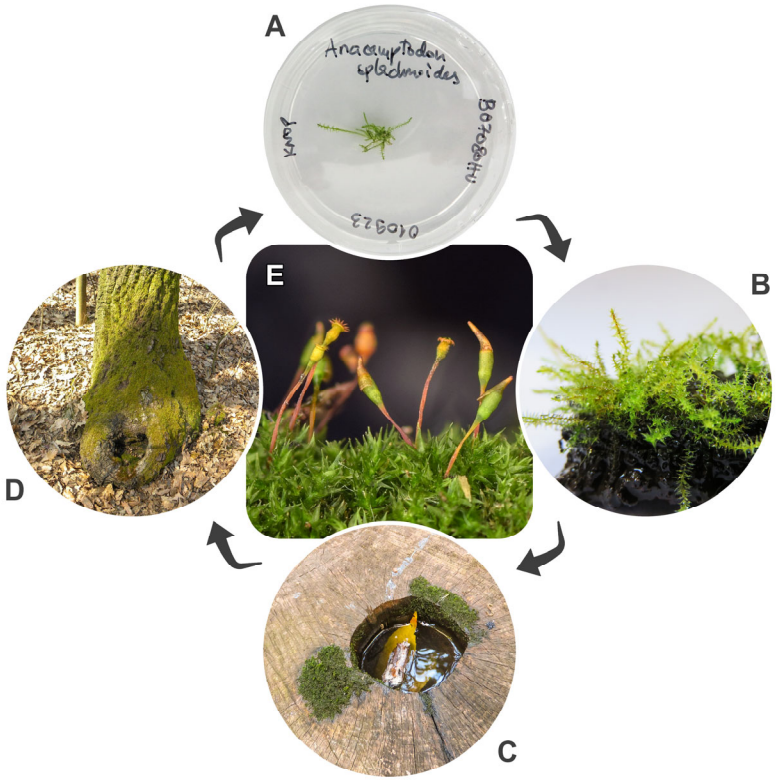


Figure 4. Plantlets of *A. splachnoides* in conservation continuum transferred from axenic *in vitro* culture (**A**) to oak bark partially submerged in rainwater with newly developed gametophores (**B**). Successfully acclimatised plants in rainwater-retaining man-made dendrotelmatic cavities of a beech trunk under outdoor conditions (green patches on the edge of the cavities) (**C**), imitating natural habitat conditions (**D**), with sporophytes developed (**E**), capable of spreading spores produced *ex situ*.

In the second setup, plantlets of *A. splachnoides* were transferred directly from *in vitro* culture to the edges of rainwater-retaining artificially made dendrotelmatic cavities in a beech trunk under outdoor conditions (Figure 4C), imitating the natural habitat type of the species (Figure 4D). These plantlets established successfully, remained viable, and continued to grow. After two years, the first sporophyte formation was observed (Figure 4E). The survival was 100%, and the spread around the edges was evident since only one cm^2 of plantlets were initially attached to the wood surface (Figure 4C).

4. Discussion

4.1. The Influence of Explant Position and Plant Growth Regulators on the Morphogenetic Response of A. splachnoides

Given the limited experimental data available for *A. splachnoides*, investigating the effects of plant growth regulators provides valuable insights into its *in vitro* growth and developmental biology. The selection of axenic conditions, PGR concentrations, and explant orientation was based on prior studies, allowing comparisons with other species, as summarised in [12].

Previous studies have demonstrated that PGRs influence bryophyte development and increase shoot formation [23,24]. Bryophytes not only synthesise endogenous hormones but also respond to exogenous PGRs [25]. Auxins and cytokinins play key, interconnected roles throughout gametophyte development [26]. Auxins regulate cell division, elongation, differentiation, tissue and organ development, and rhizoid formation, and they contribute to the chloronema to caulonema transition and bud initiation in combination with cytokinins [27–29]. Cytokinins promote the growth of chloronema and caulonema, influence protonemal differentiation, and induce caulonemal branching, even at low concentrations [30–32]. Bud formation is dependent on protonemal age, size, and endogenous hormone levels [33]. However, non-optimal concentrations or prolonged exposure to exogenous PGRs can disrupt hormonal balance and inhibit development, highlighting the need to optimise both concentration and treatment duration for effective bud induction [12,26,34].

The literature reports variable responses of bryophytes to PGRs. Some species, such as *Campyliadelphus elodes* (Lindb.) Kanda, *Podperaea krylovii* (Podp.) Z. Iwats. & Glime, and *Physcomitrella patens* (Hedw.) Bruch & Schimp. [16,17,23], can spontaneously form buds independently of exogenous PGRs, whereas in others, bud formation may be either stimulated or inhibited. In the present study, the IBA application resulted in a high index of multiplication, demonstrating the positive influence of PGRs on the *in vitro* development of *A. splachnoides*. The prostrate explants treated with IBA exhibited enhanced growth, likely due to increased contact with the medium surface, which may facilitate nutrient and PGR uptake. The upright explants also responded to IBA compared to the control, although the effect was less pronounced than in the prostrate orientation. The addition of BAP had a noticeable effect on the prostrate-oriented explants at the lowest concentration, whereas at 0.3 µM, an increase in the index of multiplication was observed in the upright explants. This pattern is likely due to the fact that explants in direct contact with the medium can access nutrients and PGRs more effectively, even at lower concentrations, while upright explants require higher BAP concentrations to achieve a similar effect.

In *Atrichum undulatum* (Hedw.) P. Beauv. [35], exposure to high cytokinin concentrations often results in developmental abnormalities, including gametophore deformation and the suppression of bud formation. Similar effects have been observed in the bryohalophytic species *Hennediella heimii* (Hedw.) R. H. Zander at 10 µM BAP and in *Bryum argenteum* Hedw. [24,35,36]. In *Hypnum cupressiforme* Hedw. elevated levels of IBA and BAP decreased the diameter of the secondary protonema patch, demonstrating the inhibitory influence of these growth regulators [37], whereas in *H. heimii*, lower BAP concentrations promoted secondary protonemal growth [36]. The application of IBA and BAP has also been reported to reduce bud numbers and suppress protonemal development in *Plagiomnium cuspidatum* (Hedw.) T. J. Kop. [38,39]. Inhibitory effects of exogenous BAP on protonemal growth have also been documented in *Garckea phascoides* (Hook.) C. Muell. [26]. Exogenous cytokinins have been shown to influence tissue senescence in *Pogonatum urnigerum* (Hedw.) P. Beauv., likely due to the disruption of endogenous cytokinin homeostasis [40]. Growth regulators, either alone or combined with sugars, have also been reported to induce callus formation in bryophytes [41,42]. They also affect gametangia development. In *Riccia frostii* Aust., specific concentrations of auxins and cytokinins stimulated archegonia formation [43]. Similar effects were observed in *Riccia crystallina* L. [44] and *Riccia gangetica* Ahmad [45]. In contrast to liverworts, auxin effects in mosses are more variable. In *B. argenteum*, auxins suppressed archegonia formation [24], whereas in *Leptobryum pyriforme* (Hedw.) Wilson, they had no significant effect on sex organ development [46]. Conversely, auxins stimulated antheridia formation in *Barbula gregaria* (Mitt.) A. Jaeger and *Bryum coronatum* Schwägr. [47]. In *Drepanocladus lycopodioides* (Brid.) Warnst., higher BAP concentrations triggered archegonia formation [15]. These effects were not observed in the present study during the *in vitro*

tests. Nevertheless, the finding that IBA and BAP influence the development of new buds is important for future research and applications for the selected species. During *in vitro* cultivation, gamete production was not observed. Explant orientation, particularly the prostrate position in certain pleurocarpous mosses, plays a critical role in enhancing the uptake of nutrients and growth regulators, thereby promoting more efficient multiplication and growth. These results are important for establishing pioneering *ex situ* conservation protocols for *A. splachnoides*.

The addition of both IBA (especially) and BAP positively impacted the multiplication index of *A. splachnoides.* For optimal *in vitro* propagation, we suggest applying small doses of IBA (0.03 µM) to plants grown in a prostrate orientation, as this yielded the highest multiplication index.

The results reported for various species do not seemingly indicate clear patterns, and further research—including that featuring combinations of various growth regulators and signalling molecules—is needed for a fundamental understanding of both species biology and practical conservation issues.

4.2. Acclimatisation of Knothole Moss A. splachnoides on Oak Bark and Dendrotelmatic Cavities of Beech Tree Trunk

The acclimatisation experiment consisted of two stages. In the first stage, propagated plantlets of *A. splachnoides* were transferred onto oak bark partially submerged in rainwater and maintained under controlled xenic conditions within Magenta vessels. Within four weeks, the plantlets showed a marked increase in size and produced numerous new branches. In the second stage, the moss was moved from *in vitro* culture to rainwater-retaining dendrotelmatic cavities in a beech trunk under outdoor, uncontrolled conditions at the Jevremovac Botanical Garden, University of Belgrade, Serbia. This placement ensured constant access to moisture and shade, thus preventing desiccation. After two years, the development of sporophytes confirmed the successful acclimatisation and sustained growth of *A. splachnoides* under outdoor conditions of the city botanical garden.

Once a stable axenic culture is established and sufficient plant material is available, gradual acclimatisation can begin, allowing plants to adjust to environmental factors such as temperature, light, humidity, and substrate. Previous studies have reported successful acclimatisation of several species outside their natural habitats. For example, *Ditrichum cornubicum* Paton, *Marchantia polymorpha* L., *Orthodontium gracile* Schwaegrichen ex B.S.G., and *Tetraphis pellucida* Hedw. were successfully transferred from nutrient media to natural substrates [48]. Similarly, *Entosthodon hungaricus* (Boros) Loeske [49] was efficiently acclimatised from sterile *in vitro* material to the saline substrate characteristic of its natural habitat, and a patch established at the reintroduction site produced sporophytes in the following growing season (one year later). *Physcomitrium eurystomum* Sendtn. has also been successfully transferred from sterile conditions to a substrate closely resembling its native environment [50]. Temperature acclimatisation experiments have been conducted for *Pleurozium schreberi* (Willd. ex Brid.) Mitt. [51]. *Drepanocladus lycopodioides* and *C. elodes* were initially grown under xenic, controlled conditions and then acclimatised on filter paper soaked in rainwater, enabling them to thrive in non-sterile conditions and resulting in a significant increase in biomass by the spring after establishment [15,16]. *Podperaea krylovii* was initially cultivated under axenic conditions and subsequently transferred to controlled xenic conditions. The plants were successfully placed in Magenta vessels containing distilled water and arranged on fine white gravel, where they grew vigorously, exhibiting rapid gametophyte development. Additionally, a transplantation test under controlled conditions onto a natural substrate was conducted. *P. krylovii* was successfully transferred to the new medium and maintained on the original substrate with regular watering to sustain moisture. The gametophytes developed well, whereas the sporophytes

were morphologically abnormal, likely apogamous due to low light intensity, and they failed to reach maturity [17]. Achieving successful acclimatisation from sterile to non-sterile conditions is crucial for endangered species and represents an essential step in *ex situ* conservation strategies. Proper acclimatisation not only facilitates the potential reintroduction of species into habitats from which they have disappeared, but may also contribute to the reinforcement of declining populations.

The rarity of *A. splachnoides* in nature appears to be closely linked to the limited availability and long-term instability of suitable microhabitats. The species does not seem to be particularly sensitive to air pollution, as it grows well in urban environments with relatively high levels of atmospheric contamination. Key ecological factors include the prolonged retention of rainwater in microsites where decaying wood and leaf litter increase nitrogen availability. Rainwater enriched with organic matter from slowly decomposing wood and fallen leaves accumulates in moist hollows, bark wounds, and trunk forks, as well as within moist bark fissures, providing suitable microsites for this species [3]. Beech and oak trees are the most common host substrates, and acclimatisation on oak bark and within beech knotholes proved to be successful in this study. Other tree species with acidic bark as hosts are also reported [3], although at much lower frequencies.

A. splachnoides is a dioecious moss species, with male and female plants occurring separately in natural populations. The inability to induce gametangia (i.e., sex organs) under axenic conditions suggests that specific environmental triggers may influence sex expression; however, this process, as well as spore biology, remains poorly understood in this species. The use of sex-linked molecular markers could be particularly valuable when dealing with sexually non-dimorphic gametophytes, especially for conservation purposes [52].

Although the species is documented to tolerate short periods of drought and even temporary desiccation, ongoing climate change, characterised by increasingly irregular precipitation patterns, together with forestry clear-cutting and rapid forest turnover, substantially increases the extinction risk of *A. splachnoides* by altering habitat suitability. Short forestry rotation cycles and the removal of older trees increase habitat uniformity and reduce the availability of suitable microsites. These changes may contribute to increased spore dormancy and high recalcitrance under newly established conditions. Furthermore, colonisation success and spore dispersal are likely reduced by the loss of cohabiting organisms within dendrotelmatic microsites, such as knotholes, which may otherwise facilitate spore transfer to suitable habitats.

The approach applied here can be useful for the propagation and conservation of other species. However, it should be taken into account that many species subjected to the same treatments do not respond in the same way. The substantial lack of data on the biological features for ecological counterparts and phylogenetically related taxa can cause further complications in the application of strict protocols, but may serve as guidelines for developing or adjusting new ones. The more tests conducted on the target taxon, the higher the likelihood of saving species from vanishing. This is especially important given the long time scales often needed for developing protocols.

The ethical and ecological issues of establishing cultivated material in the outdoor space of the botanical garden, the rarity of microhabitat types and dispersal vectors, and the generally low competence of bryophytes were all taken into consideration. Even if the material were to spread subspontaneously, this would not pose a problem since the origin of the material is from similar sites within fragmented locations in the Pannonian plane, in two neighbouring countries (namely Serbia and Hungary). Additionally, long-distance spore dispersal in bryophytes is well documented.

5. Conclusions

The integrative conservation and conservation physiology approach presented here offers improved prospects for the survival of threatened and rare species. The results can be applied to large-scale propagation under laboratory conditions and serve as a practical protocol for species reintroduction and monitoring programmes. Further studies are needed to address the species' survival constraints, such as drought stress tolerance, habitat and nutritional requirements, ecological interactions, and microhabitat longevity, as well as spore biology, sex barcoding, and genetic diversity maintenance. *A. splachnoides* also appears to function as an indicator species for a specialised microhabitat type which supports numerous other organisms. Therefore, its protection and recovery are likely to enhance associated biodiversity, as well as overall ecosystem functionality.

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Abbreviations

The following abbreviations are used in this manuscript:

NT	Near-Threatened
EN	Endangered
RE	Regionally Extinct
VU	Vulnerable
DD	Data Deficient
NaDCC	Sodium dichloroisocyanurate
PGR	Plant Growth Regulator
IBA	Indole-3-butyric acid
BAP	6-benzylaminopurine
ART	Aligned Rank Transform
IM	Index of multiplication

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