

A new and fast method to obtain in vitro cultures of *Huperzia selago* (Huperziaceae) sporophytes, a club moss which is a source of huperzine A

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Abstract

This study presents a protocol for a fast and effective in vitro axenic culture of *Huperzia selago* (Huperziaceae Rothm.) sporophytes, a club moss which is a source of huperzine A, an alkaloid of a considerable therapeutic potential extensively investigated for its uses as treatment for some neurodegenerative diseases. The proposed procedure allowed approximately tenfold shortening of the species developmental stages with the omission of the gametophyte stage while the sporophyte mass could be increased tenfold within a 6-month period. The cultures were established using vegetative propagules (bulbils) procured from sporophytes growing in the wild without degrading the habitats of this endangered plant species. Explants underwent surface and internal disinfection to eliminate the epiphytic and endophytic bacteria and fungi. In in vitro cultures, the optimum results were achieved using Moore (Mr) medium without growth regulators or supplemented with 0.015 mg/l IBA and 0.3 mg/l kinetin. These media ensured both viability of the propagules and their further development. The biomass growth index for *H. selago* sporophytes grown from propagules, determined at 3 months of culture (1 passage) on Mr medium with IBA and kinetin was 650%. At 6 months, the biomass growth index increased to 1114%. Vigorous growth of adventitious roots, especially on Mr medium with the addition of 0.25 mg/l NAA, and callus formation on shoot apices were observed. At 6 months of culture, some sporophytes obtained from the bulbils were used as the initiating material for shoot subcultures, which developed best on Mr medium with IBA and kinetin.

Keywords: *Huperzia selago*, *Lycopodium selago*, axenic culture, in vitro culture, bulbils, huperzine A

Introduction

Huperzia selago (L.) Bernh. ex Schrank et Mart. (= *Lycopodium selago* L.), fir club moss (Fig. 1) is the only European lycopod of the Huperziaceae Rothm. family distinguished by Rothmaler [1,2] following the taxonomic revision Lycopodiaceae sensu lato. The Huperziaceae family is now thought to include approximately 150 to 400 species with a worldwide distribution, though predominantly found in the tropical regions [3–5]. In Europe, *Huperzia selago* is commonly found in a widespread territory stretching from the Scandinavia to the northern Mediterranean countries [6]. It also grows in the boreal and temperate regions of Asia [5] and North America [7].

Up to now, a number of alkaloids have been isolated from *Huperzia selago* plant [8]. Huperzine A (selagine, HupA), one of these alkaloids, is an effective, reversible and selective acetylcholinesterase (AChE) inhibitor, now undergoing clinical

trials as a treatment for Alzheimer disease [8–10]. Nowadays, HupA for the pharmaceutical industry is mainly obtained from *H. serrata*, the Asian species of *Huperzia*. Its uncontrolled collection in recent years has considerably depleted the natural resources and according to Ma et al. [5,9] in China *H. serrata* is now an endangered species. Natural populations of the plant are small and the ecosystems in which it grows scarce. The species is characterized by very slow growth. Approximately 5–15 cm-long sporophytes are 15 to 20 years old and contain 0.007–0.01% of huperzine A in dry mass [9,11].

Huperzia selago is the only European species, which contains huperzine A [8,9,12–14]. Studies by Szypuła et al. [12,13] have shown that the plant is a rich source of HupA, much more abundant than the Chinese club moss *Huperzia serrata*. However, procurement of the raw material from naturally growing plants is impossible or difficult. In many countries, such as e.g. Poland [15], Luxembourg [16], Belgium [17] or some regions of the USA [7], *H. selago* has either a protected species or an endangered species status. Natural populations of the species are scant and found in unique habitats. In natural conditions, germination of the spores lasts from 3 to 5 years and this period may be only slightly shortened in in vitro culture [18–21]. The formation of young sporophytes takes another 5 years. According to Callaghan et al. [22], a fivefold increase in the biomass of *Lycopodium annotinum* sporophytes takes as long as 5 years. The growth rate of *H. selago* is likely to be similar. In view of the above, natural resources of *H. selago* should be not used to procure HupA, but at the same time the attraction of club

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Handling Editor: Beata Zagórska-Marek

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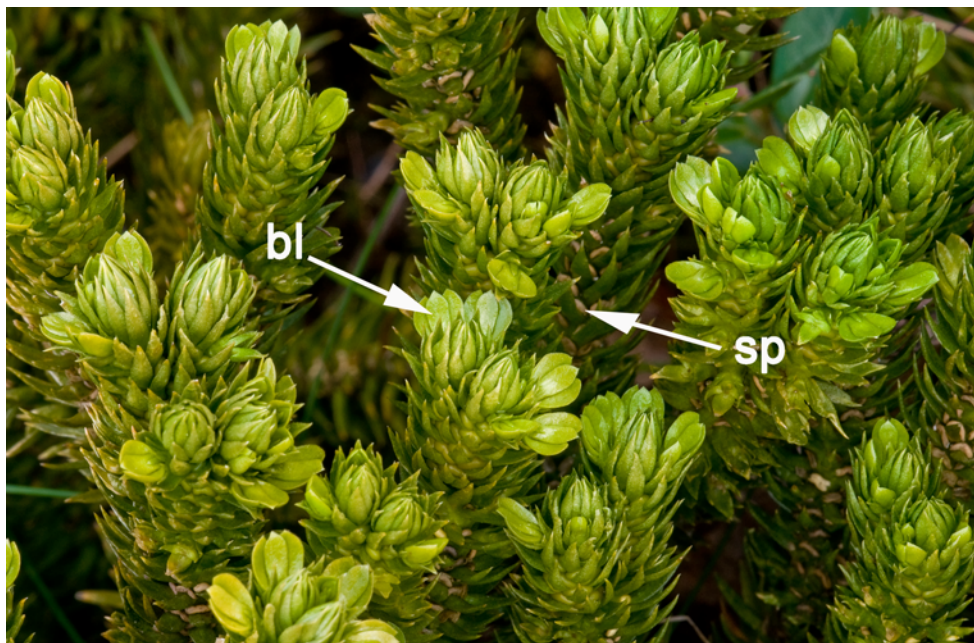


Fig. 1 *Huperzia selago* sporophyte in its natural habitat in the Babia Góra National Park, Poland. Side-view of the *H. selago* shoots presenting distribution of bulbils (bl) and sporangia (sp).

mosses with their rich content of acetylcholinesterase inhibiting alkaloids has increased with the rediscovery of therapeutic potential of huperzine A. This search for new sources has prompted international research into in vitro cultures of club mosses. Tissue cultures of *H. selago* and other club mosses are candidates for commercial procurement of huperzine A. In vitro micropropagation seems a feasible method to obtain sufficient amounts of the raw material to isolate alkaloids.

Up to date, studies on in vitro cultures have been conducted with a few club moss species only. Freeberg and Wetmore [23] and Freeberg [24,25] described gametophyte cultures and apogamous development of sporelings of *Lycopodium complanatum*, *L. cernuum* and *L. selago*. Atmane et al. [26] maintained in culture callus and sporophytes grown from somatic embryos of *Lycopodiella inundata*. Other successful cultures involved gametophytes of *Lycopodium obscurum* [27], *L. digitatum* [28] and *L. lucidulum* [18]. Recently, Whittier and Strochova [20] and Szypuła [21] reported in vitro cultures of *H. selago* gametophytes. However, there are only a few protocols for propagation of sporophytes of club mosses containing huperzine A in the literature. They describe shoot cultures and somatic embryogenesis of *H. selago* [12,29] and *Phlegmariurus squarrosus* sporophyte cultures [10]. Similarly tissue culture of various *Huperzia* species has been achieved and production of HupA has been confirmed in the callus of *H. pinifolia* [30]. Also, data on huperzine A content of sporophytes regenerated from vegetative propagules, or bulbils [13] has been recently reported.

These bulbils (Fig. 1, Fig. 2) which are young sporophytes initiated individually in the microphyll positions are important structures involved in vegetative reproduction and compensating a long cycle of generative development of *H. selago* and other *Huperzia* species such as *H. serrata*. This ability allows a relatively quick propagation and dispersal of a plant, which is a particularly important feature for a species with a long life cycle such as *Huperzia*. In this genus, the generative and vegetative reproduction occurs alternately during the vegetative season. Additionally, *H. selago* populations exhibit different reproduction strategies depending on the environmental conditions,

with the extensive bulbil production in the mountain locations contrasting with the lowland locations where the production of vegetative propagules is relatively limited [31]. This unique feature of the species may be utilized for the purposes of relatively simple and quick establishing of in vitro sporophyte cultures, omitting the gametophyte stage. A quick and efficient system to produce *H. selago* sporophytes using vegetative propagules (bulbils) as explants has been developed and the protocol is presented in this paper. It allows obtaining an axenic culture of *H. selago* sporophytes in 2 to 3 months, which may serve as a source of huperzine A or other alkaloids that are acetylcholinesterase inhibitors or provide material for further studies. So far, difficulties in establishing and maintaining cultures of *H. selago* and other club mosses have made complex studies of their biotechnology, embryology and morphogenesis virtually impossible. There are no protocols in the literature for quick and efficient culturing of *Huperzia* sporophytes with the exclusion of the spore stage.

Material and methods

Plant material

Huperzia selago sporophyte in vitro cultures were initiated using vegetative propagules (bulbils; Fig. 1, Fig. 2) procured from different local populations in the Babia Góra National Park (The Carpathians, Poland). They were harvested from the mother sporophytes and protected from drying during transport by placing on blotting paper moistened with water in tightly closed containers. The plants were collected in spring, from April to June, and in autumn, in September and October. In the laboratory, the plants were cleaned by manually removing litter, soil and leaves of other plants, and then rinsed in a stream of running tap water for 1 hour. Voucher specimens, including sporophytes with bulbils, were taken and deposited in the herbarium at Department of Biology and Pharmaceutical Botany, Medical University of Warsaw.

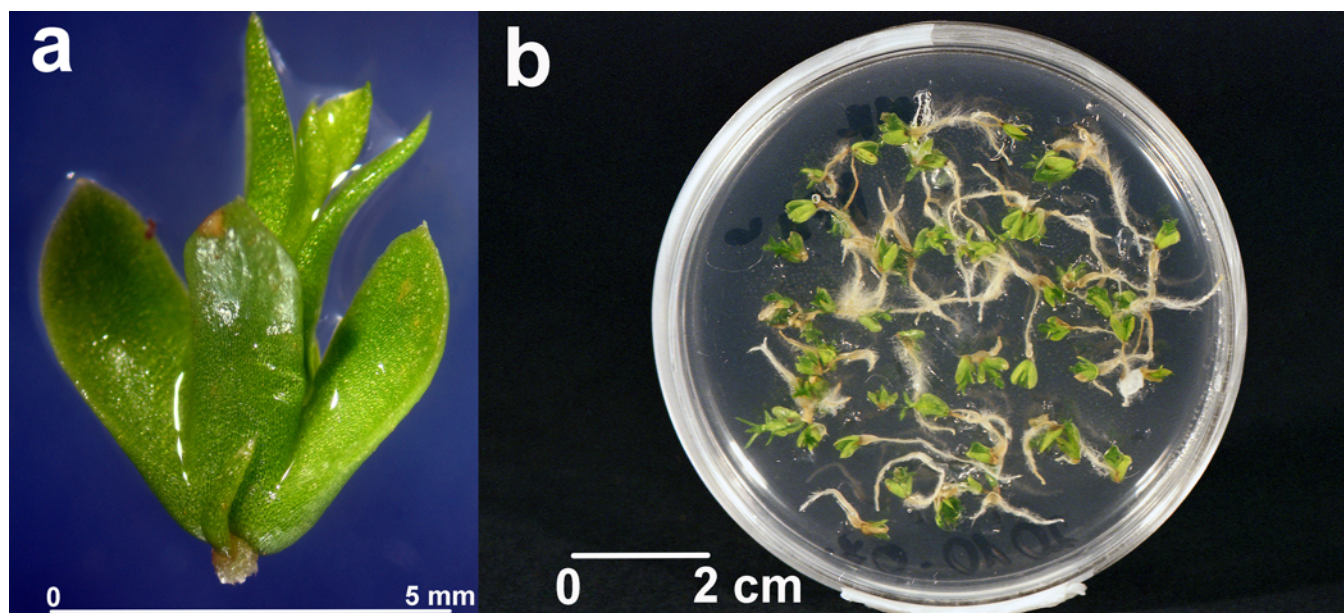


Fig. 2 a Bulbil of *H. selago* after surface disinfection and incubation with addition of PPM™. b Bulbils placed on the culture medium with addition of PPM™ 2 ml/l.

Explant disinfection and establishing of axenic cultures of *Huperzia selago*

Initial experiments in this study demonstrated that methods commonly used to disinfect plant material prior to establishing in vitro cultures of angiosperms were ineffective in trials to establish axenic cultures of *H. selago* [12,29]. According to the literature, *H. selago* is a species colonized by mycorrhizal and endophytic fungi and bacteria [25,32–35]. Exophytes (epiphytes) have been found on the surface of leaf epidermis while endophytes have been observed inside the organs, tissues and cells of gametophytes and sporophytes – in the mesophyll, intercellular spaces of the primary cortex and in the cells of conducting tissues [32–37]. That is why it was necessary to develop own methods of plant material disinfection. For that purpose various methods of surface and internal disinfection were tried using different chemicals, altering the order at which they were applied and the duration of explant exposure. The ultimately developed method, based on earlier experiments, employed in this study is highly effective and eliminates most epiphytic and endophytic organisms and at 4–8 weeks of culture up to 90% of explants did not show any signs of contamination [12,29]. The decontamination process was in two stages and consisted of surface and internal disinfection. In stage one, the explants were rinsed in a stream of tap water for 5 minutes, next soaked in water with the detergent Tween®20 (0.1% v/w) for another 5 minutes and then underwent surface disinfection by bathing in the following solutions: 70% ethyl alcohol for 1 minute, 5% ACE (Procter&Gamble) in H₂O (1:5 v/v) for 10 minutes, and 7% hydrogen peroxide (v/v) for 10 minutes. In stage 2 (internal disinfection), the plant material was rinsed several times in sterile distilled water and transferred onto sterile Petri dishes lined with filter paper moistened with tap water to which Plant Preservative Mixture™ (PPM™; Plant Cell Technology, Washington) 10 ml/l was added. After such pretreatment, the bulbils were incubated for 3–4 weeks in a phytotron, at 18 ±1°C (day) and 16 ±1°C (night), in light at 100 μM m⁻² s⁻¹ and 14 h photoperiod. After incubation young sporophytes growing from the bulbils were transferred on to Petri dishes (Fig. 2) or into 10-ml Erlenmeyer flasks which contained suitable culture media with the addition of PPM™ 2 ml/l.

Four basic media were used in in vitro cultures of *H. selago*: Murashige and Skoog (½ MS) [38] half strength mineral salt content and full strength of organic component, Moore (Mr) as modified by Freeberg and Wetmore [23], embryo production media (EP, ½ EP) and callus proliferation media (CP, ½ CP) with the mineral salt content modified according to Chée et al. [39] and full strength of organic component. The media were modified by altering the content of growth regulators (Tab. 1). Mr, Knudson [40] and Knop [41] liquid and solid media supplemented with 0.015 mg/l IBA and 0.3 mg/l Kin were used for experiments in in vitro culture of roots. Trials to initiate and maintain the isolated adventitious roots were performed on the Mr medium without or supplemented 0.15 mg/l IBA and 0.3 mg/l Kin or 0.25 mg/l NAA. Also WPM medium according to Lloyd and McCown [42] was tested.

The sporophytes were incubated in a phytotron, at 18 ±1°C (day) and 16 ±1°C (night), in light at 100 μM m⁻² s⁻¹ and 14 h photoperiod. The plants were transferred on to a fresh medium every 3 months. After the first 4 and 8 weeks of culture, the mean number of viable bulbils developing sporophytes was calculated (Tab. 1). At 3 and 6 months, the index of sporophyte biomass growth (*WP*) was calculated for 30 plants and the calculation was repeated in duplicate. The *WP* value was calculated according to Street and Henshaw [43] where $WP = \frac{\text{final plant mass (g)} - \text{initial plant mass (g)}}{\text{initial plant mass (g)}} \times 100$. The mean ($n = 10$) fresh mass of bulbils was 0.196 ±0.007 g. Statistical analysis was performed after 8 weeks with Graph Pad Prism version 6.0 (Graph Pad Software, San Diego, CA) using one-way analysis of variance (ANOVA) with Tukey–Kramer post-hoc test. The study was repeated thrice and data are presented as means ±SEM. $P < 0.05$ was considered significant.

Results

The literature data on in vitro cultures of club mosses are scant and that is why for the purposes of this study it was necessary to first determine which basic culture medium and types of growth regulators and their concentrations would be suitable for

the optimum growth of *H. selago* sporophytes. The selection of a culture medium was guided by published reports on in vitro cultures of gametophytes and sporophytes of different clubmoss species [18–20,23,26–28] and earlier own experiments [12,29]. The media had different effects on the growth and viability of *H. selago* explants in vitro. Moor medium without growth regulators or supplemented with 0.015 mg/l indole-3-butyric acid (IBA) and 0.3 mg/l kinetin (Kin) according to Atmane et al. [26] proved the most effective ensuring both bulbil viability and their further development (Tab. 1). Good results were also achieved using Mr medium supplemented with 0.25 mg/l 1-naphthaleacetic acid (NAA) as at 8 weeks of culture 30% of the explants began to develop into sporophytes (Tab. 1). Mr medium supplemented with other combinations and concentrations of growth regulators was found to be unsuitable since only a limited number of explants developed into sporophytes at 8 weeks of culture (Tab. 1). Embryo production media (EP, ½ EP) and callus proliferation media (CP, ½ CP) with mineral salt content according to Cheé et al. [39] (Tab. 1) also proved unsuitable as starting from week 4 of culture all *H. selago* explants died. That finding confirmed the literature data on high sensitivity of in vitro *Huperzia selago* or/and *Lycopodiella inundata* cultures to concentrations of growth regulators and mineral salts [12,26,29].

The modified Mr media allowed successful growth of sporophytes seen as early as 2 weeks of culture. The sporophytes were then approximately 1.5 cm in length, had several microphylls and beginnings of the root system (Fig. 3a). The sporophytes continued to grow and develop – at 3 months, numerous dichotomous branching of shoots and roots could be observed (Fig. 3b). At 6 months of culture, the sporophytes had 3–6 shoots and a well-developed root system (Fig. 3c). The sporophytes cultures were continued on the same media for another 12–24 months (Fig. 3d). The biomass growth index for *H. selago* sporophytes grown from explants, determined at 3 months of culture (1 passage) on Mr medium supplemented with 0.015 mg/l IBA and 0.3 mg/l Kin was 650% and at 6 months it increased to 1114%.

Sporophytes grown from bulbils incubated on Mr medium without growth regulators or supplemented with either 0.015 mg/l IBA and 0.3 mg/l Kin or 0.25 mg/l NAA developed adventitious roots (Fig. 4a). Medium with NAA (Tab. 1), the efficiency of root formation was $30 \pm 3\%$ and was significant different after 8 weeks ($P < 0.05$). Means received from Mr medium without growth regulators ($8 \pm 3.1\%$) or supplemented IBA and Kin ($10 \pm 4.3\%$) were not statistically significant. The roots grew out of the shoot above the medium level and gradually increasing

Tab. 1 Influence of media and growth regulators concentrations on bulbils survival and growth.

No.	Tested medium	Growth regulators (mg/l)						% of viable bulbil or/and young sporophyte after:	
		IAA	IBA	NAA	BAP	Kin	TDA	4 weeks ¹	8 weeks ^{1,2}
1.	½MS ³	-	0.015	-	-	0.3	-	70.0 ± 2.2	1.0 ± 0.3
2.	Mr	1.0	-	-	-	10	-	25.0 ± 6.1	0.7 ± 0.3
3.	Mr	1.5	-	-	-	15	-	15.0 ± 2.1	15.0 ± 2.1
4.	Mr	-	-	1.5	-	15.0	-	32.7 ± 3.9	10.0 ± 2.2
5.	Mr	-	-	2.0	-	25.0	-	5.3 ± 1.5	4.6 ± 0.3
6.	Mr	-	-	0.25	0.5	-	-	98.3 ± 1.7	20.0 ± 1.1
7.	Mr	-	-	1.5	10.0	-	-	0.0	0.0
8.	Mr	-	0.015	-	-	0.3	-	82.0 ± 2.1	65.0 ± 2.0
9.	Mr	-	0.015	-	-	-	0.015	97.7 ± 1.5	4.3 ± 0.7
10.	Mr	-	-	-	-	-	-	98.0 ± 1.5	60.0 ± 2.6
11.	Mr	-	-	0.25	-	-	-	98.0 ± 1.5	30.0 ± 4.5
12.	½CP ³	-	-	-	-	-	-	-	-
13.	½CP ³	-	0.015	-	-	0.15	-	-	-
14.	½CP ³	-	0.015	-	-	0.3	-	-	-
15.	½CP ³	-	-	0.125	-	-	-	-	-
16.	½CP ³	-	-	0.25	-	-	-	-	-
17.	CP	-	-	-	-	-	-	-	-
18.	CP	-	0.015	-	-	0.3	-	-	-
19.	CP	-	-	0.25	-	-	-	All explants turned brown and died within 4 weeks	
20.	½EP	-	-	-	-	-	-		
21.	½EP	-	0.015	-	-	0.15	-	-	-
22.	½EP	-	0.015	-	-	0.3	-	-	-
23.	½EP	-	-	0.125	-	-	-	-	-
24.	½EP	-	-	0.25	-	-	-	-	-
25.	EP	-	-	-	-	-	-	-	-
26.	EP	-	0.015	-	-	0.3	-	-	-
27.	EP	-	-	0.25	-	-	-	-	-

¹ The values represent the means ± standard error of 3 replicates with 12–55 explants per culture dish from 3 experiments. ² Variations among tested medium means are significantly greater than expected by chance (Tukey–Kramer multiple comparison test $P < 0.05$) with the exception of medium number 1 vs. 2,5,9, medium number 2 vs. 5,7,9 and medium 5 vs. 9 ($P > 0.05$). ³ ½ MS and ½ CP media containing half-strength of mineral salts and full strength of organic components.

in length grew into the medium developing numerous root hair (Fig. 4a). Trials to initiate and maintain the isolated adventitious roots from those plants failed. The isolated roots, transferred on to the same solid or liquid media and incubated in light 14 h (day) or dark died. Also unsuccessful were trials to culture adventitious roots on WPM medium according to Lloyd and McCown [42]. Callus formation on shoot apices was another finding in cultures of sporophytes initiated from bulbils (Fig. 4b). Small clusters of cells were found in the meristematic zone of shoots and on the first four visible leaves below. Callus cells were also seen on a few more well developed leaves. Loose, light callus, greenish-yellow in color, was composed of round parenchyma cells. Attempts to initiate and maintain callus culture and suspension culture of *H. selago* failed. Callus aggregates separated from the shoots and transferred on to solid and liquid media: Mr, Knudson [40] and Knop [41] supplemented with 0.015 mg/l IBA and 0.3 mg/l Kin died within 3 weeks in culture. $\frac{1}{2}$ MS medium though best for the purposes of culturing shoot fragments [12] proved unsuitable for use in bulbil cultures. Although the sporophytes remained viable for the first 4 weeks, they did not further develop.

At 6 months of culture, some sporophytes grown in vitro from bulbils were used to initiate subcultures of *H. selago* shoots on $\frac{1}{2}$ MS medium and Mr medium supplemented with 0.015 mg/l IBA and 0.3 mg/l Kin. On Mr medium, the explants grew with dichotomous branching and root formation. Their further development was the same as of sporophytes grown directly from bulbils. On $\frac{1}{2}$ MS medium, shoot fragments developed from bulbil-derived sporophytes formed neither nodular

structures described earlier in *H. selago* shoot cultures [12] nor adventitious roots although they grew roots in the next passage when transferred on to a fresh Mr medium supplemented with 0.015 mg/l IBA and 0.3 mg/l kinetin. Sporophytes grown from shoots cut from sporophytes grown from bulbils did not show any endophytic microbiological contaminants when examined using the methods employed by Budziszewska and Szypuła [34] and Budziszewska et al. [35].

Discussion

Our study is the first to report the conditions for successful establishing and maintenance of in vitro cultures of *H. selago* sporophytes obtained from bulbils (vegetative propagules). The protocol described in this paper allows a quick and efficient culture method. Bulbils proved a better material to initiate the in vitro culture than shoot fragments used earlier [12,29].

Previously we established *H. selago* sporophyte cultures using shoot fragments obtained from sporophytes growing in natural stations [12,29]. That required large amounts of plant material, which had to undergo time-consuming and complicated disinfection. On the other hand, when bulbils are procured from the wild, even small fragments of shoots of these rare and protected plants are not cut. Bulbils (Fig. 1, Fig. 2) are formed spontaneously in the apical shoot parts of *H. selago* sporophytes. They are best collected in the period of their complete development, preferably in spring and the collection does not cause any mechanical damage to the mother sporophytes. Both surface

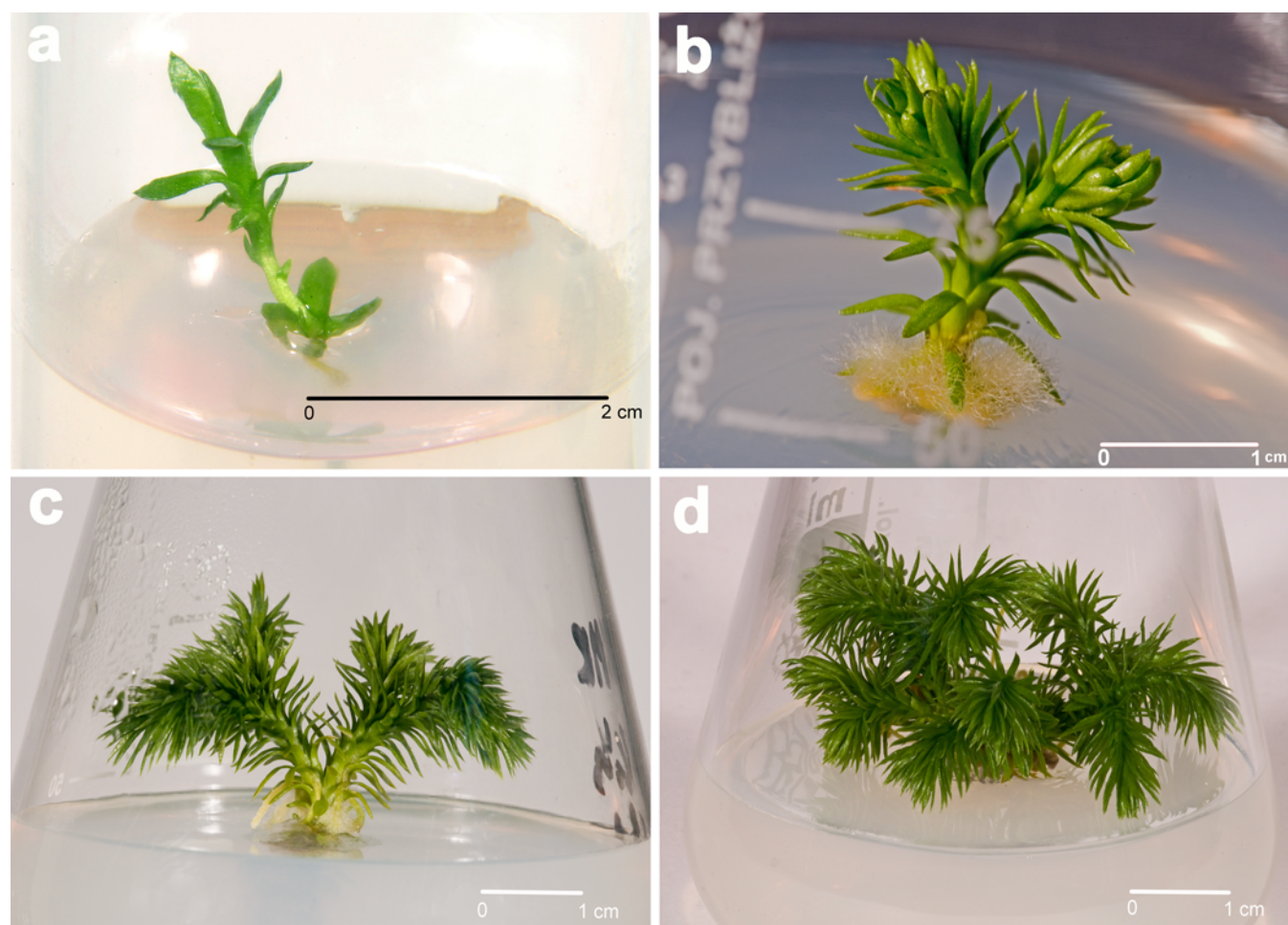


Fig. 3 Development of *H. selago* plants from bulbils. **a** Young sporophyte after 2nd week of incubation on Mr medium. **b** Young sporophyte after 3 months of culture. It has numerous lateral shoots and dichotomous branching shoots and roots. After 6 (c) and 12 months (d) of culture on Mr medium sporophytes had 3–6 lateral shoots and a well-developed root system.

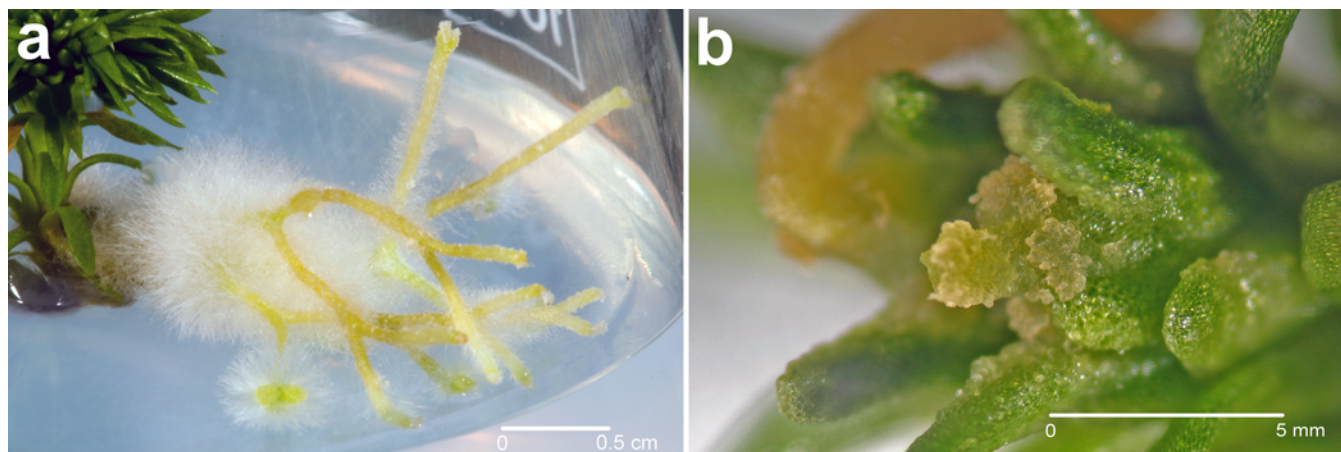


Fig. 4 a Adventitious roots formed from month 3 of Mr medium supplemented with either IBA (0.015 mg/l) and Kin (0.3 mg/l) or NAA (0.25 mg/l). b Callus developing in the meristematic zone of *H. selago* shoots growing on Mr medium with addition of IBA (0.015 mg/l) and Kin (0.3 mg/l).

and internal disinfection is easier with bulbils than with shoots. In the present study, with the disinfection methods described, up to 90% of explants were free from contaminants.

Sporophytes developed from bulbils and grew roots. Shoot apices procured from the sporophytes and incubated on culture media behaved likewise. Adventitious roots were formed from month 3 of culture on Mr medium supplemented with either 0.015 mg/l IBA and 0.3 mg/l Kin or 0.25 mg/l NAA (Tab. 1, Fig. 4a). Dichotomous branching roots grew mostly from the lower part of the stem. The first researcher to observe the development of adventitious roots and shoots in *H. selago* was Williams in 1933 [44]. His experiments, however, were carried out on a limited number of bulbils and shoots, incubated in non-sterile conditions on Petri dishes containing a medium according to Knop [41] or sand moistened with water. Williams [44] observed the development of adventitious roots and shoots on mechanically damaged shoots from which leaves were torn or the growing point removed. Shoots also developed from leaves separated from generative propagules. According to Williams [44], the adventitious organs of *H. selago* developed from epidermal cells in the stem and leaves under the influence of various factors, generally classified as stresogenic. In the present study, we observed the development of adventitious roots, but unlike Williams [44] we did not find any adventitious shoots. To date, no studies have been performed on the varying morphogenetic properties of club moss explants used to establish in vitro cultures. Also, there are no published reports on other factors, which may affect the morphogenetic response of explants used to establish in vitro cultures of *Huperzia* sp. These aspects should be investigated in the future.

Callus proliferation was observed in *H. selago* cultures (Fig. 4b). In the present study, sporophytes developed from bulbils formed callus exclusively on Mr medium with the addition of IBA and kinetin at doses according to Atmane et al. [26]. The callus was brittle and composed of loosely connected cells, which formed small aggregates (0.2–0.4 mm). The cells and their aggregates were found in the apical meristem and on 2–3 leaves below. Unlike the callus obtained in the culture of *H. selago* shoots [12], it did not demonstrate any embryogenic properties. The callus obtained from *H. selago* shoots was observed at approximately 12 weeks (3 months) and spontaneously produced somatic embryos [12]. It developed only when ½ MS medium was used without any growth regulators. The callus was

homogenous and nodular and for 4 weeks it continued to grow surrounding the apical meristem and 4–5 leaves below it. The process of callus formation observed in the present study and in shoot cultures described by Szypuła et al. [12] involved the apical meristems of the shoots. Still, possible factors affecting callus proliferation and its morphogenetic response remain unknown. To date, there have been only a few published reports of callus induction in *Huperzia* and *Lycopodium* cultures. DeMaggio [45] described *L. obscurum* gametophyte callus induction without the use of growth regulators while Atmane et al. [26] in cultures of *Lycopodiella inundata* shoots found that supplementation of ½ MS medium with IBA (0.05 μM) and kinetin (1.4 μM) stimulated callus proliferation.

The results of the present study confirm that in vitro culture of the club moss *H. selago* is a strategy effectively shortening its developmental stages, which in natural conditions lasts several years. Studies on the ecology and growth rate of *Lycopodium annotinum* [22] suggest that in natural conditions it takes 5 years for the sporophyte mass to increase approximately five-fold. The results of the present study show that with in vitro cultures the sporophyte biomass may increase over tenfold within 6 months. According to many authors [20,23,25,46], germination of *H. selago* spores in their natural environment takes 3–5 years while young sporophytes need another 5 years to develop. The in vitro culture allows approximately tenfold shortening of the developmental cycle. According to some authors, with such homophase cycle (sporophyte – sporophyte), the gametophyte phase, which is the most critical stage in the Pteridophyta life cycle, is omitted [47–51]. This may provide a good solution for plant biotechnology, which aims at producing the largest amount of biological material (e.g. for isolation of pharmacologically active alkaloids) in the shortest possible time. Although omission of the gametophyte stage and exclusion of syngamy may result in disorders of plant reproduction, findings from a number of studies demonstrate that incomplete cycles of alteration of generations are a sign of evolutionary progress and allow Pteridophyta to adjust to changing environmental conditions [47–51]. Considering recent increasing interest in alternative methods of huperzine A procurement for its uses in the pharmaceutical industry, in vitro micropropagation seems one of the methods of obtaining sufficient amounts of the raw material to isolate alkaloids. The protocol we propose is a step towards this goal.

Acknowledgments

The authors thank Babia Góra National Park authorities for permission to undertake the research in the park. This study was supported by the Polish Ministry of Science and Higher Education, grant No. NN 405 362237.

Authors' contributions

The following declarations about authors' contributions to the research have been made: concept of the study: WJS; laboratory research and data analyses: WJS, PM, OO; writing of the manuscript: WJS, PM, OO.

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