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ARTICLE TITLE	THE ROLE OF ACETYLCHOLINE IN THE BIOSYNTHESIS OF ALKALOIDS IN IN VITRO CULTURES OF <i>HUPERZIA SELAGO</i> (L.) GAMETOPHYTES
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THE ROLE OF ACETYLCHOLINE IN THE BIOSYNTHESIS OF ALKALOIDS IN IN VITRO CULTURES OF *HUPERZIA SELAGO* (L.) GAMETOPHYTES

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ABSTRACT

Introduction: *Huperzia selago* is a natural source of huperzine A (HupA), a potent and reversible acetylcholinesterase (AChE) inhibitor with therapeutic potential in Alzheimer's disease. Due to limited natural resources, alternative methods for alkaloid production—such as in vitro cultures—are being explored. Acetylcholine (ACh) functions as a signaling molecule in plants, but its influence on alkaloid biosynthesis in clubmosses has not yet been investigated [1-5].

Aim: To evaluate the effect of exogenous ACh on the biosynthesis of HupA, huperzine B (HupB), and lycopodine in *H. selago* gametophyte cultures.

Methods: Gametophytes were grown in vitro on Whittier and Storchova media supplemented with acetylcholine chloride at concentrations of 0 (control), 1, 10, 100, and 1000 μ M. Biomass was collected after 2, 4, 6, 8, and 10 weeks, lyophilized, and extracted with methanol. Quantitative analysis of HupA, HupB, and lycopodine was performed using HPLC-DAD [2, 4-12].

Results: Control cultures showed high HupA content (mean 3.34 mg/g DW), ranging from 3.01 to 4.22 mg/g DW, with a maximum at week 8. HupB was detected only at week 8 (0.44 mg/g DW). In contrast, none of the ACh-treated cultures contained detectable HupA or HupB. A significant increase ($p < 0.05$) in lycopodine content was observed in cultures treated with 10 μ M ACh, reaching up to 16.72 mg/g DW at week 8. Other ACh concentrations did not significantly affect lycopodine levels compared to the control.

Conclusions:

- *H. selago* gametophytes in vitro are an efficient source of HupA and HupB.
- Acetylcholine inhibits the biosynthesis of HupA and HupB.
- Acetylcholine at 10 μ M significantly enhances lycopodine biosynthesis.
- Culture duration influences alkaloid content. The results indicate a significant and selective role of ACh in regulating alkaloid biosynthetic pathways in *H. selago*.

KEYWORDS

Huperzia Selago, Acetylcholine, Huperzine A, Huperzine B, Lycopodine, Alkaloid Biosynthesis, In Vitro Cultures

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AD – Alzheimer's Disease

HupA – huperzine A

HupB – huperzine B

ACh – acetylcholine

ARIA – Amyloid-Related Imaging Abnormalities

AChE – acetylcholinesterase

MCI – Mild Cognitive Impairment

PET – Positron Emission Tomography

CDR-SOB – Clinical Dementia Rating Scale Sum of Boxes

iADRS – Integrated Alzheimer's Disease Rating Scale

ADCOMS – Alzheimer's Disease Composite Score

Introduction

Alzheimer's disease (AD) represents a major public health challenge in aging societies. Current symptomatic treatment relies mainly on acetylcholinesterase (AChE) inhibitors such as donepezil, rivastigmine, and galantamine. Huperzine A (HupA), an alkaloid isolated from clubmosses of the genus *Huperzia*, is a strong, reversible, and selective AChE inhibitor characterized by good blood–brain barrier penetration and a favorable safety profile [1–3]. The main source of HupA is the sporophytes of *Huperzia serrata*, but their exploitation has led to the species becoming endangered. Consequently, alternative methods for obtaining this alkaloid, including in vitro tissue cultures, are being explored [3–5].

Gametophytes of *H. selago* grown in vitro have proven to be an exceptionally efficient source of HupA, reaching levels up to 4.8 mg/g dry weight, which significantly exceeds the typical content found in *H. serrata* sporophytes. To increase alkaloid biosynthesis efficiency in cultures, approaches such as supplementation of media with precursors or elicitors are commonly used [1–2, 5, 7–8].

In addition to its key role as a neurotransmitter in animals, acetylcholine (ACh) is also present in plants, where it may function as a local messenger or regulatory molecule influencing growth, development, and stress responses. Literature reports suggest a potential link between endogenous ACh and the biosynthesis of AChE inhibitors in plants [5–7]. However, data on the effect of exogenous ACh on alkaloid biosynthesis in clubmosses are lacking [8–12].

The aim of this study was to evaluate the influence of different concentrations of exogenous acetylcholine on the biosynthesis of huperzine A, huperzine B, and lycopodine in in vitro cultures of *Huperzia selago* gametophytes.

Materials and methods

Plant material and culture conditions: Aseptic cultures of *H. selago* gametophytes were used in the study. The cultures were maintained on solid media according to Whittier and Storchova, modified by the addition of a sterile solution of acetylcholine chloride (ACh·HCl) at final concentrations of 1, 10, 100, and 1000 μ M. The control consisted of cultures grown on medium without ACh supplementation. The cultures were kept in a phytotron under a 12 h light / 12 h dark cycle at 20°C/18°C (day/night) and 70% humidity.

Sample collection and alkaloid extraction: Gametophyte biomass was collected at weeks 2, 4, 6, 8, and 10 of culture. The material was lyophilized, weighed, and subjected to triple ultrasonic extraction in methanol. After solvent evaporation, the resulting dry extracts were dissolved in methanol to a concentration of 5 mg/mL.

HPLC-DAD analysis: Quantitative determination of HupA, HupB, and lycopodine was performed using HPLC-DAD according to the procedure developed by Szypuła et al. [11]. A Shimadzu chromatograph equipped with a Hypersil GOLD RP18 column (250 $\times$ 4.6 mm) and UV detection was used. Identification and quantification of alkaloids were based on retention times, UV spectra, and calibration curves for HupA and HupB. Lycopodine content was estimated using the calibration curve for HupA due to limited availability of lycopodine standard.

Statistical analysis: Differences in lycopodine content between groups were analyzed using Student's t-tests for independent samples with Statistica software. The significance level was set at $p < 0.05$.

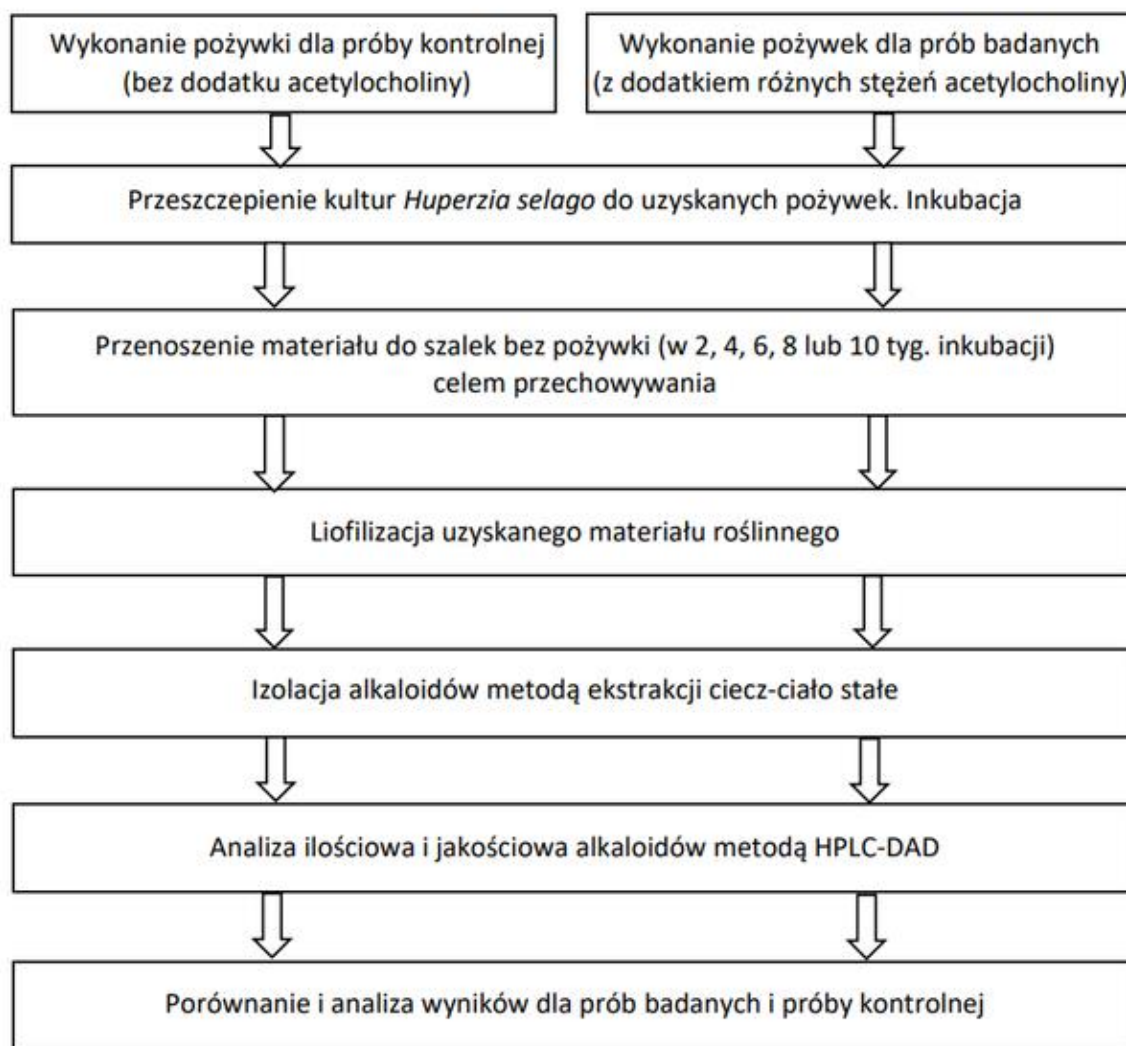


Fig. 1. Scheme of the work organization (own work).

Results

For the determination of huperzine A (HupA), huperzine B (HupB), and lycopodine content, the HPLC-DAD method was used [11]. Methanolic solutions of alkaloid extracts were analyzed. The quantities of HupA and HupB were calculated based on calibration curves developed by Turulska [13] and Waliszewski [14], with results expressed both per 1 g of dry gametophyte mass and per 1 g of dry methanolic extract. Due to the limited amount of lycopodine standard, its preliminary quantification was performed using the HupA calibration curve. Identification of lycopodine peaks was confirmed by comparing UV-VIS spectra with the reference spectrum obtained in earlier studies [15]. As indicated by the results of Szypuła [8], differences between lycopodine content determined using the HupA curve and its actual content are minimal. A summary of the results is presented in the table and in Figures 19 and 23–25, while UV-VIS spectra of HupA, HupB, and lycopodine are shown in Figures 20–22. Data on alkaloid content in gametophytes grown on media with different ACh concentrations and collected at subsequent time points are presented in Table 3 and Figures 26–34. Mean lycopodine values are shown in Figure 35.

In the chromatograms of standards, clear separation between HupA and HupB peaks was observed. The retention time of standard HupA was 12.712 ± 0.5 min, and that of HupB was 11.455 ± 0.5 min. In the extracts, these values were slightly higher: 13.325 ± 0.5 min for HupA, 11.849 ± 0.5 min for HupB, and 34.503 ± 0.5 min for lycopodine. The highest number of peaks (60) was recorded in the extract from gametophytes grown on medium supplemented with $10 \mu\text{mol}$ ACh and collected at week 2. The lowest number of peaks (6) was found in the 10-week control sample (Fig. 25).

In the control sample, HupA content remained high—on average 3.34 ± 0.37 mg/g DW of gametophyte, with values ranging from 3.01 ± 0.76 to 4.22 ± 0.33 mg/g DW. The highest content was recorded at week 8, and the lowest at week 6 (Fig. 26). HupB was detected only in the material collected at week 8, at 0.44 ± 0.18 mg/g DW (Fig. 27). Lycopodine in the control sample occurred at an average level of 3.34 ± 0.37 mg/g DW, ranging from 3.03 ± 0.03 to 3.97 ± 1.26 mg/g DW. The highest content was found at week 2, and the lowest at week 8 (Fig. 28). A comparison of HupA and lycopodine content in the control sample is shown in Figure 29.

In the experimental samples—i.e., cultures treated with acetylcholine—neither HupA nor HupB was detected. At the same time, significantly higher lycopodine content was observed compared with the control (Figs. 34 and 35). Its levels ranged from 2.19 ± 0.14 to 16.7 ± 0.68 mg/g DW. The highest value was obtained at week 8 in the culture supplemented with $10 \mu\text{mol}$ ACh (mean 11.92 ± 3.6 mg/g DW). The lowest value—also at week 8—was recorded in the culture with $1 \mu\text{mol}$ ACh (mean 3.20 ± 0.68 mg/g DW) (Figs. 30, 31, and 34).

In the next part of the study, alkaloid content was recalculated per 1 g of dry plant extract (Table 3). The highest HupA content was found in the control extract from week 8 (49.91 ± 3.95 mg/g DW), and the lowest in the material from week 2 (27.63 ± 1.75 mg/g DW). The highest lycopodine content was detected in the extract from gametophytes treated with $10 \mu\text{mol}$ ACh and collected at week 8 (54.25 ± 7.98 mg/g DW). The lowest content was found in the extract from the $1 \mu\text{mol}$ ACh culture at week 6 (16.80 ± 0.28 mg/g DW).

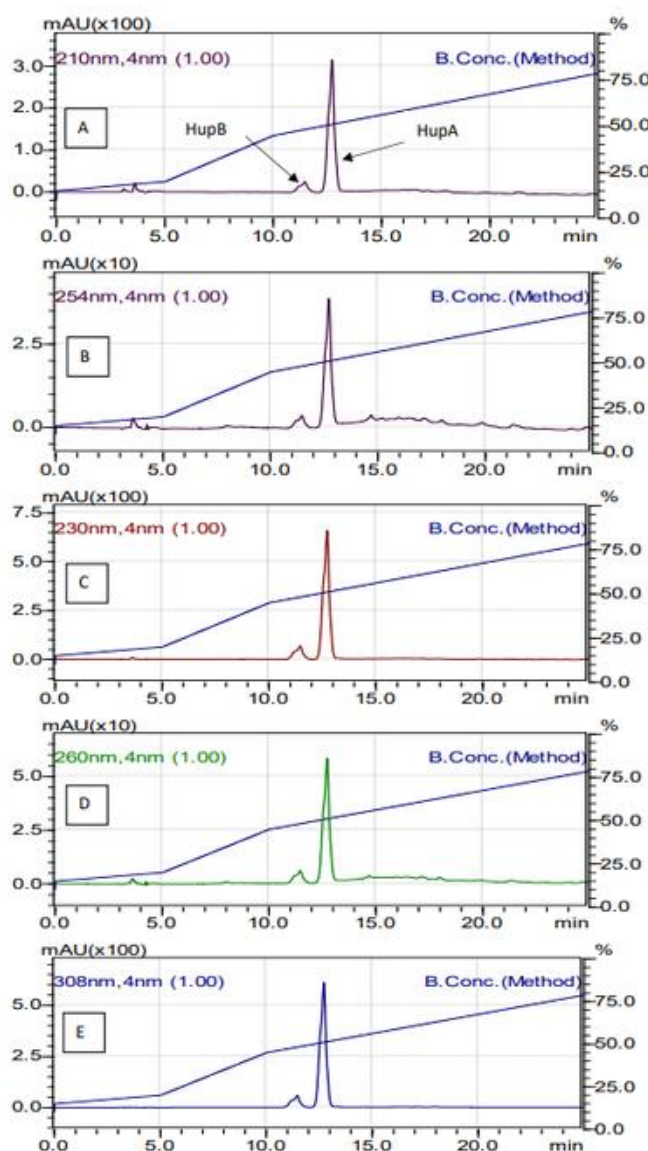


Fig. 2. Chromatograms of the HupA and HupB standard solution. For huperzine A $R_f = 12.712$ min, and for huperzine B $R_f = 11.455$ min; A – $\lambda = 210$ nm, B – $\lambda = 254$ nm, C – $\lambda = 230$ nm, D – $\lambda = 260$ nm, E – $\lambda = 308$ nm.

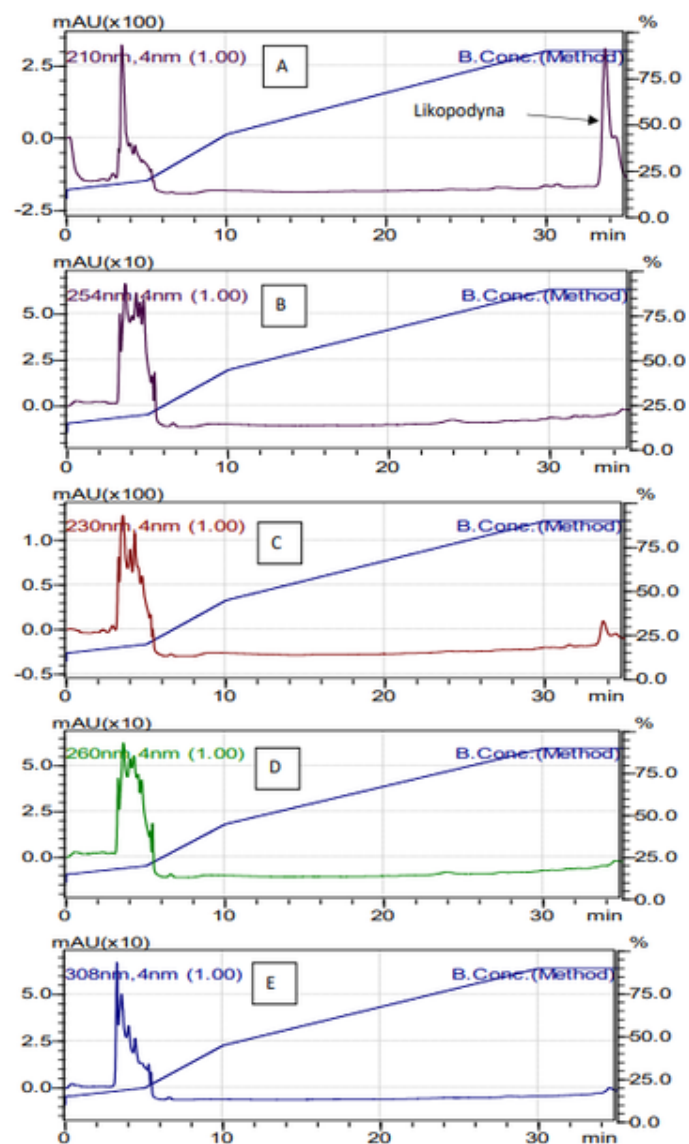


Fig. 3. Chromatograms for a gametophyte exposed to 10 μmol ACh, collected at 10 weeks. For lycopodin, $R_f = 34.29$; A – $\lambda = 210 \text{ nm}$, B – $\lambda = 254 \text{ nm}$, C – $\lambda = 230 \text{ nm}$, D – $\lambda = 260 \text{ nm}$, E – $\lambda = 308 \text{ nm}$.

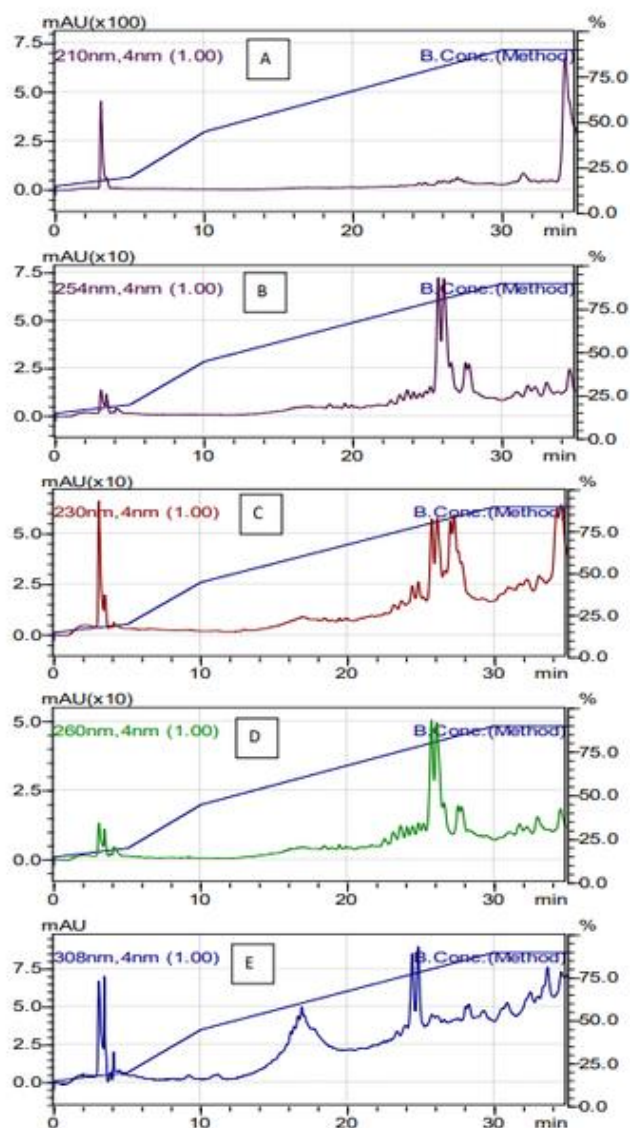


Fig. 4. Chromatograms for gametophyte exposed to 10 μmol ACh, collected at 4 weeks; A – $\lambda = 210$ nm, B – $\lambda = 254$ nm, C – $\lambda = 230$ nm, D – $\lambda = 260$ nm, E – $\lambda = 308$ nm.

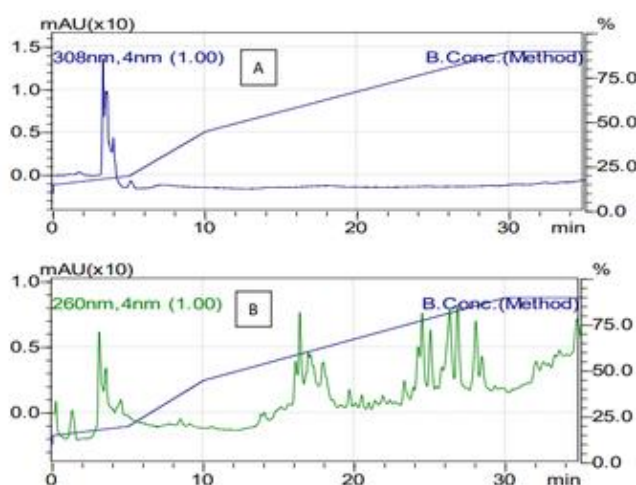


Fig. 5. Chromatograms with extreme numbers of peaks: A – lowest number of peaks (6), B – highest number of peaks (60).

Table 1. Content of HupA, HupB and lycopodine in dry mass of H. selago gametophyte and dry mass of extract at different acetylcholine concentrations.

Zawartość ACh	Tydzień zbioru	Zawartość HupA w 1g s.m. gametofitu $\pm$ SD (n=4) [mg]	Zawartość HupB w 1g s.m. gametofitu $\pm$ SD (n=4) [mg]	Zawartość lycopodyny w 1g s.m. gametofitu $\pm$ SD (n=4) [mg]	Zawartość HupA w 1g s.m. ekstraktu $\pm$ SD (n=4) [mg]	Zawartość HupB w 1g s.m. ekstraktu $\pm$ SD (n=4) [mg]
Próba Kontrolna 0 μ mol	2	3,17 $\pm$ 0,63	Brak	3,97 $\pm$ 1,26	27,63 $\pm$ 1,75	Brak
	4	3,07 $\pm$ 0,95	Brak	3,25 $\pm$ 0,99	28,30 $\pm$ 3,20	Brak
	6	3,01 $\pm$ 0,76	Brak	3,12 $\pm$ 0,43	40,14 $\pm$ 4,22	Brak
	8	4,22 $\pm$ 0,30	0,44 $\pm$ 0,18	3,03 $\pm$ 0,03	49,91 $\pm$ 3,95	44,51 $\pm$ 3,51
	10	3,15 $\pm$ 0,25	Brak	3,31 $\pm$ 0,29	42,93 $\pm$ 3,53	Brak
1 μ mol	2	Brak	Brak	3,96 $\pm$ 1,99	Brak	Brak
	4	Brak	Brak	3,10 $\pm$ 0,07	Brak	Brak
	6	Brak	Brak	3,67 $\pm$ 0,51	Brak	Brak
	8	Brak	Brak	2,19 $\pm$ 0,14	Brak	Brak
	10	Brak	Brak	3,07 $\pm$ 0,25	Brak	Brak
10 μ mol	2	Brak	Brak	7,04 $\pm$ 1,40	Brak	Brak
	4	Brak	Brak	11,23 $\pm$ 2,98	Brak	Brak
	6	Brak	Brak	13,61 $\pm$ 3,79	Brak	Brak
	8	Brak	Brak	16,72 $\pm$ 0,68	Brak	Brak
	10	Brak	Brak	10,98 $\pm$ 0,08	Brak	Brak
100 μ mol	2	Brak	Brak	2,86 $\pm$ 0,64	Brak	Brak
	4	Brak	Brak	4,32 $\pm$ 0,08	Brak	Brak
	6	Brak	Brak	4,26 $\pm$ 0,80	Brak	Brak
	8	Brak	Brak	5,06 $\pm$ 0,09	Brak	Brak
	10	Brak	Brak	3,16 $\pm$ 0,28	Brak	Brak
1000 μ mol	2	Brak	Brak	2,54 $\pm$ 0,67	Brak	Brak
	4	Brak	Brak	3,83 $\pm$ 0,11	Brak	Brak
	6	Brak	Brak	3,08 $\pm$ 0,86	Brak	Brak
	8	Brak	Brak	6,43 $\pm$ 0,54	Brak	Brak
	10	Brak	Brak	3,80 $\pm$ 0,08	Brak	Brak

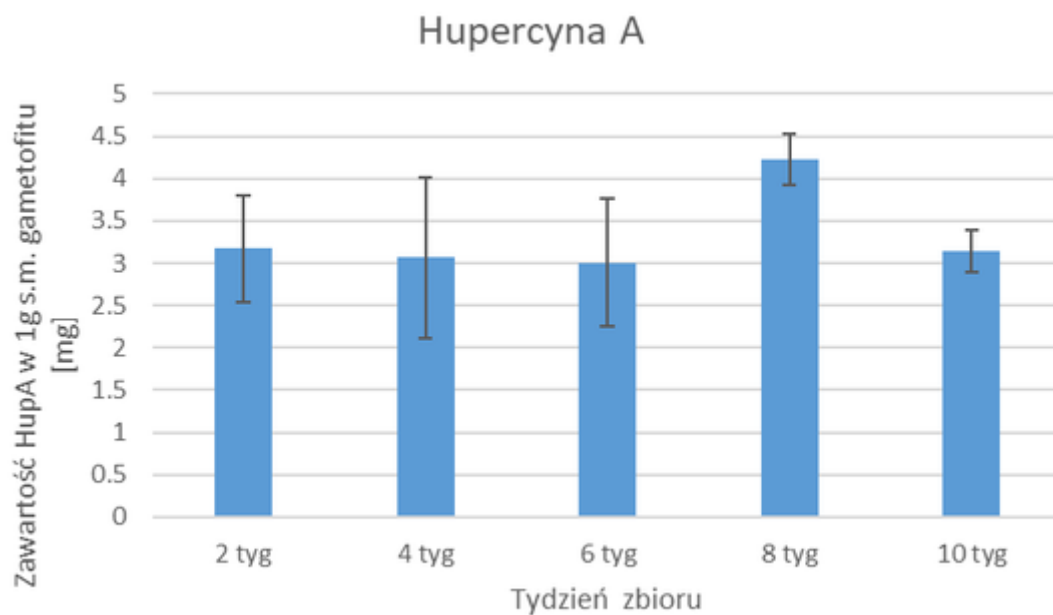


Fig. 6. Content of huperzine A in dry mass of gametophyte in the control sample over 10 weeks of culture.

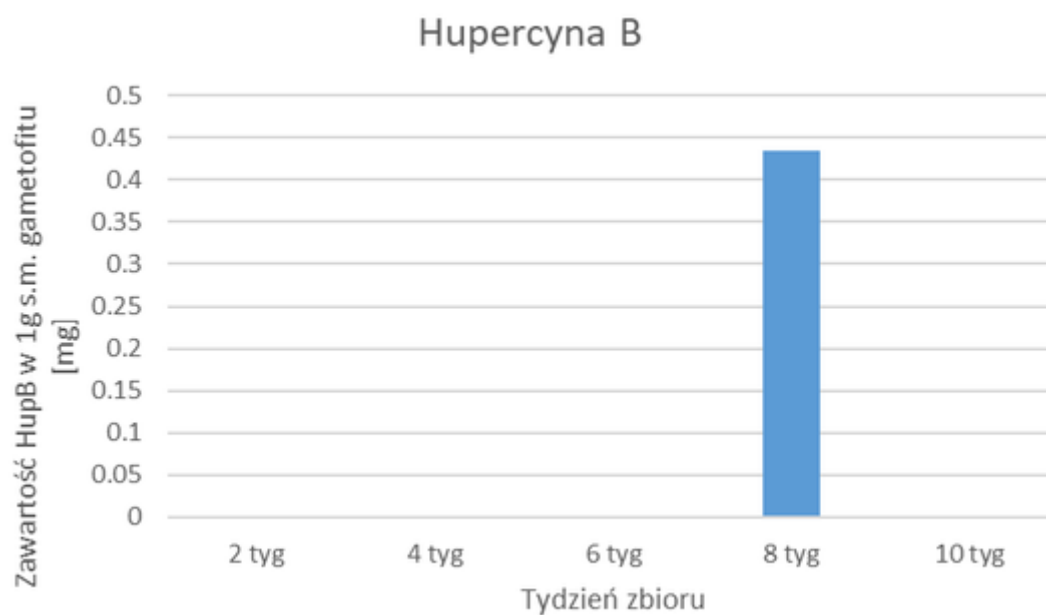


Fig. 7. Content of huperzine B in dry mass of *H. selago* gametophyte in the control sample over the weeks.

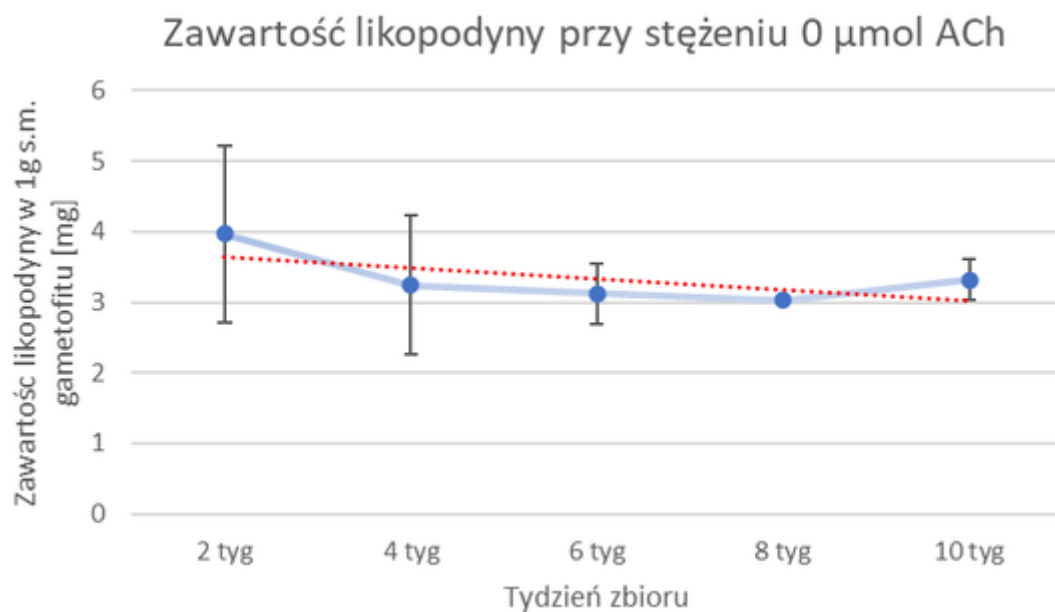


Fig. 8. Mean content of lycopodine in 1 g d.m. of prothallus in individual weeks of collection, obtained on media supplemented with 0 μmol ACh, together with the trend line.

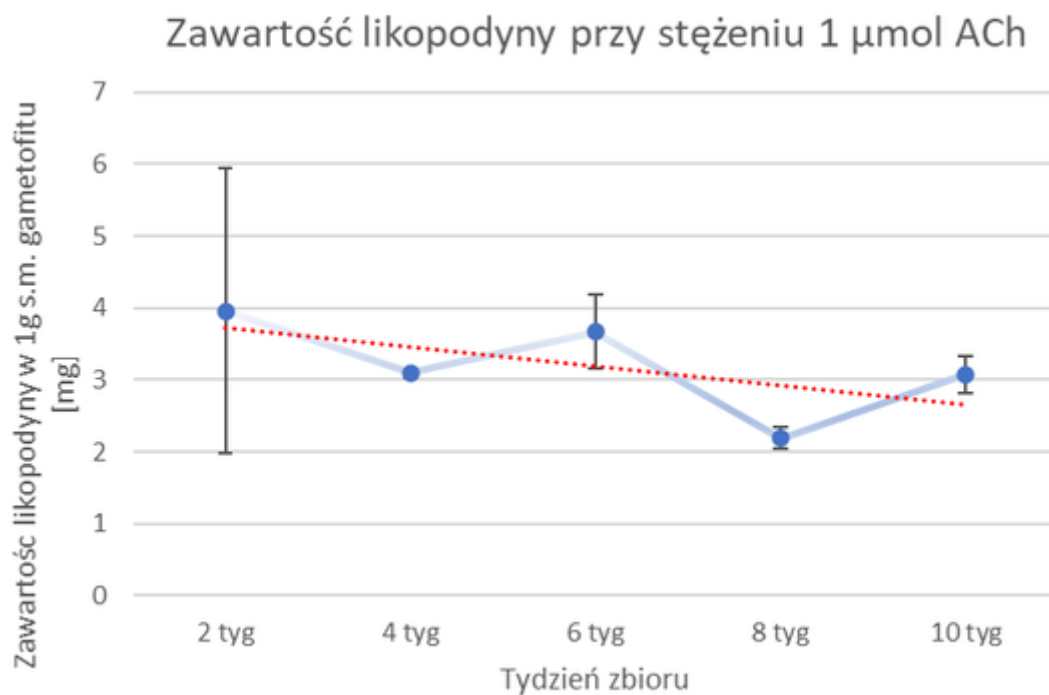


Fig. 9. Mean content of lycopodine in 1 g d.m. of prothallus in individual weeks of collection, obtained on media supplemented with 1 μmol ACh, together with the trend line.

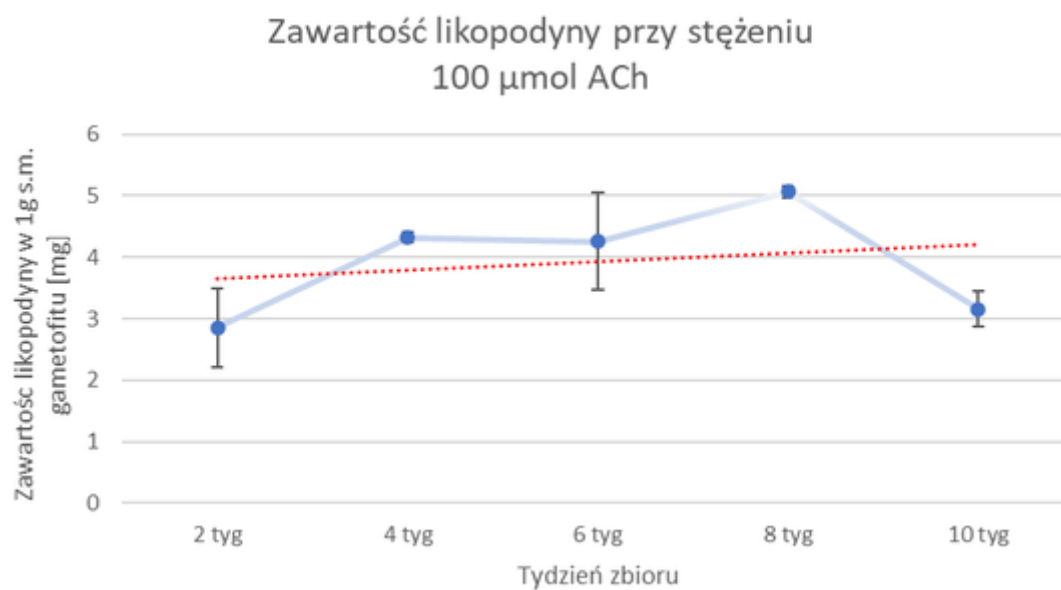


Fig. 10. Mean content of lycopodine in 1 g d.m. of prothallus in individual weeks of collection, obtained on media supplemented with 100 μmol ACh, together with the trend line.

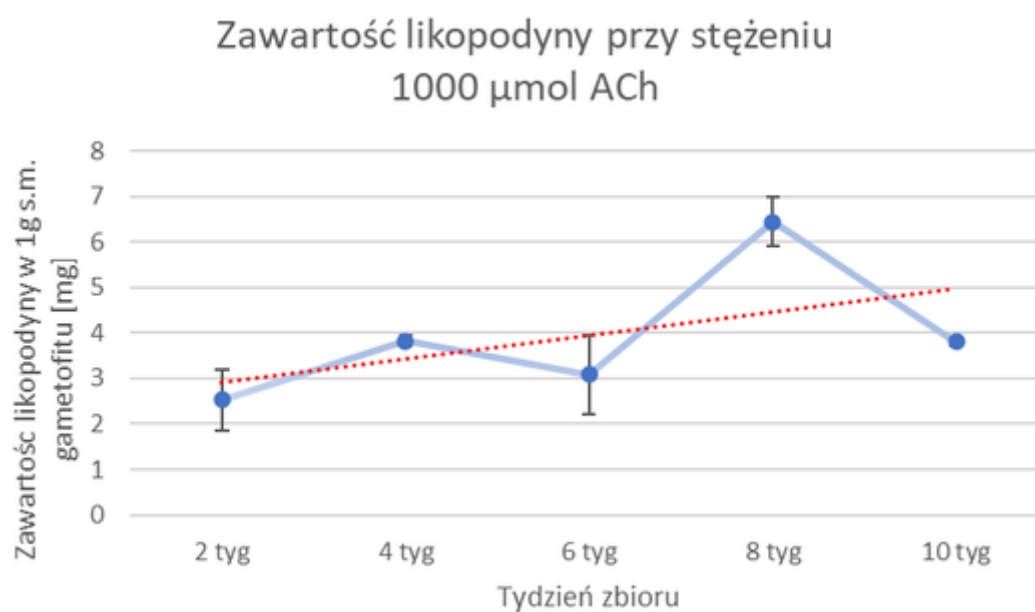


Fig. 11. Mean content of lycopodine in 1 g d.m. of prothallus in individual weeks of collection, obtained on media supplemented with 1000 μmol ACh, together with the trend line.

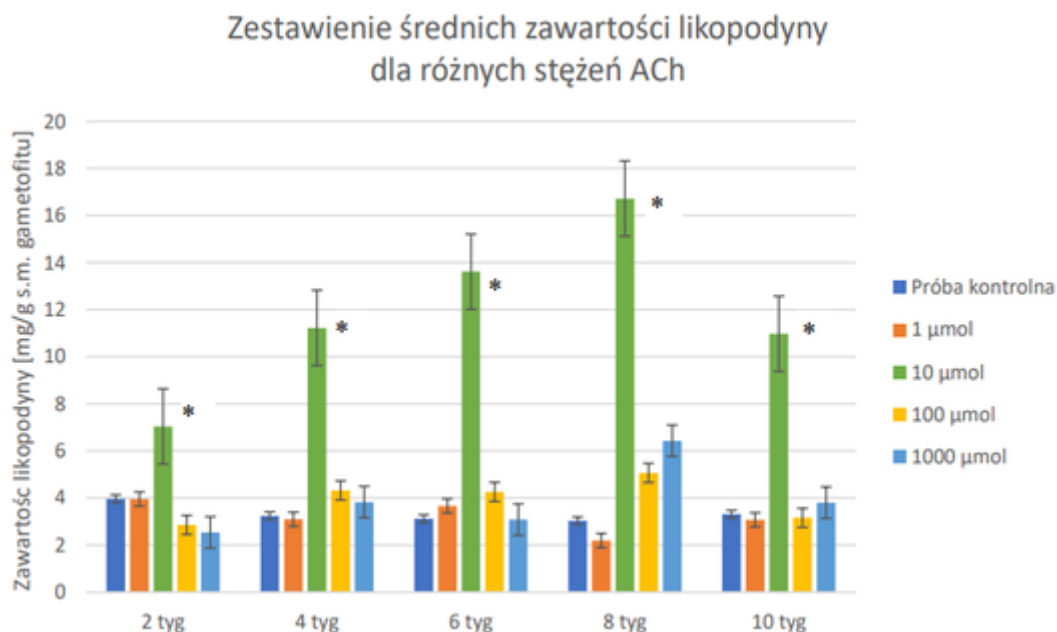


Fig. 12. Total content of *HupA* (A), *HupB* (B) [83] and lycopodine (C) in *in vitro* cultures of *H. selago* gametophytes, over 24 weeks (A and B) and 10 weeks (C).

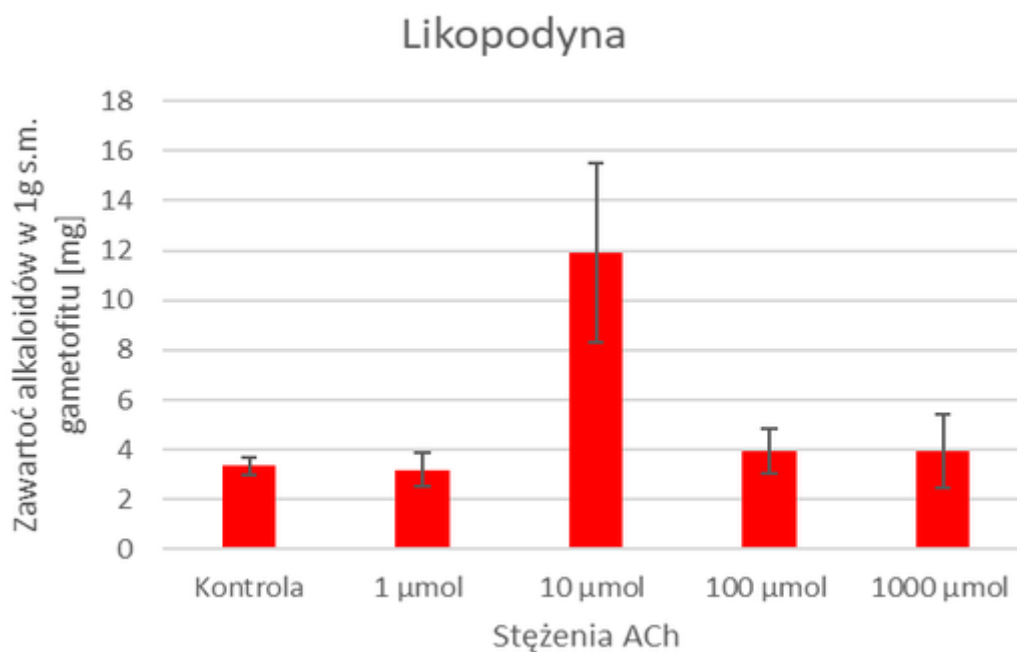


Fig. 13. Mean lycopodine content per 1 g of gametophyte dry matter [mg] across all weeks studied, at a given acetylcholine concentration.

Table 2. Data obtained using the Statistica program for a lycopodine concentration of 0 μmol .

Zmienne	Testy dla prób niezależnych										
	Uwaga: Zmienne traktowane są jako niezależne próby.										
	Srednia Grupa 1	Srednia Grupa 2	t	df	p	Nważnych Grupa 1	Nważnych Grupa 2	Odch. std Grupa 1	Odch. std Grupa 2	iloraz F Wariancje	p
Próba kontrolna vs. Stężenie 1	3.336057	3.19714	0.40234	8	0.697970	5	5	0.371134	0.677008	3.32756	0.271025
Próba kontrolna vs. Stężenie 2	3.336057	11.91598	-5.33822	8	0.000696	5	5	0.371134	3.574734	92.77392	0.000677
Próba kontrolna vs. Stężenie 3	3.336057	3.93289	-1.36081	8	0.210670	5	5	0.371134	0.907783	5.98277	0.111306
Próba kontrolna vs. Stężenie 4	3.336057	3.93738	-0.87257	8	0.408324	5	5	0.371134	1.495618	16.23977	0.019407

Table 3. Data obtained using the Statistica program for a lycopodine concentration of 10 μmol .

Zmienne	Testy dla prób niezależnych (Arkuszt1)										
	Uwaga: Zmienne traktowane są jako niezależne próby.										
	Srednia Grupa 1	Srednia Grupa 2	t	df	p	Nważnych Grupa 1	Nważnych Grupa 2	Odch. std Grupa 1	Odch. std Grupa 2	iloraz F Wariancje	p
Stężenie 1 vs. Stężenie 2	3.19714	11.91598	-5.35856	8	0.000679	5	5	0.677008	3.574734	27.88045	0.007027
Stężenie 1 vs. Stężenie 3	3.19714	3.93289	-1.45280	8	0.184348	5	5	0.677008	0.907783	1.79794	0.583814
Stężenie 1 vs. Stężenie 4	3.19714	3.93738	-1.00824	8	0.342858	5	5	0.677008	1.495618	4.88038	0.153845
Stężenie 2 vs. Stężenie 1	11.91598	3.19714	5.35856	8	0.000679	5	5	3.574734	0.677008	27.88045	0.007027
Stężenie 2 vs. Stężenie 3	11.91598	3.93289	4.83996	8	0.001288	5	5	3.574734	0.907783	15.50686	0.021131
Stężenie 2 vs. Stężenie 4	11.91598	3.93738	4.60405	8	0.001746	5	5	3.574734	1.495618	5.71276	0.119929
Stężenie 3 vs. Stężenie 1	3.93289	3.19714	1.45280	8	0.184348	5	5	0.907783	0.677008	1.79794	0.583814
Stężenie 3 vs. Stężenie 2	3.93289	11.91598	-4.83996	8	0.001288	5	5	0.907783	3.574734	15.50686	0.021131
Stężenie 3 vs. Stężenie 4	3.93289	3.93738	-0.00574	8	0.995563	5	5	0.907783	1.495618	2.71442	0.356827
Stężenie 4 vs. Stężenie 1	3.93738	3.19714	1.00824	8	0.342858	5	5	1.495618	0.677008	4.88038	0.153845
Stężenie 4 vs. Stężenie 2	3.93738	11.91598	-4.60405	8	0.001746	5	5	1.495618	3.574734	5.71276	0.119929
Stężenie 4 vs. Stężenie 3	3.93738	3.93289	0.00574	8	0.995563	5	5	1.495618	0.907783	2.71442	0.356827

Discussion

The subterranean prothalli of clubmosses are holosaprophytic organisms and remain in close symbiosis with fungi. They usually have a conical shape and lack chlorophyll, in contrast to above-ground gametophytes equipped with chloroplasts [8, 16]. Their size typically does not exceed 8 mm in length and 4 mm in width, although some may reach up to 20 mm in length and 5 mm in width (ibid.). Archegonia and antheridia are usually located at the apex of the gametophyte, with forms containing only antheridia being less common. The vegetative part of the gametophyte consists of four layers: an outer, cutinized epidermis with rhizoids; beneath it lies a cortical layer composed of several rows of ovoid cells; next is a palisade layer serving as a storage tissue; and centrally, a parenchymatous medulla (ibid.). In the present study, the development of gametophytes proceeded in accordance with descriptions found in the cited sources [8, 10].

Aside from the publications mentioned above, there is still little data on clubmoss prothalli originating from natural habitats. Likewise, in vitro cultures have yielded prothalli for only a small number of the approximately 300 clubmoss species [10, 16]. Freeborg and Wetmore [10] cultured *H. selago*, *L. complanatum* (= *Diphasiastrum complanatum*), and *L. cernuum*, demonstrating that their morphology, anatomy, and development are similar. The first stage of *H. selago* ontogeny—the development of the gametophyte—is an exceptionally long process dependent on the timing of spore germination and may last up to 15 years (ibid.). In natural conditions, the formation of a mature prothallus requires symbiosis with a fungus, which begins at the stage of only a few cells. In in vitro cultures, germination and growth of gametophytes do not require fungal presence. In laboratory conditions, prothallus maturation takes only a few weeks, which results from the use of substances that accelerate spore germination (e.g., growth regulators) and phytohormones that shorten the cell cycle, such as cytokinins (ibid.) [8, 10, 16].

The gametophytes obtained in these studies, however, were not used as material for secondary metabolite biosynthesis [10, 16]. According to the literature, at least 13 alkaloids occur in the tissues of *H. selago*, including acrofoline, 12-epilycodoline (pseudoselagine), lycodoline, lycopodine, selagoline, serratidinine, 6 β -hydroxyhuperzine A, α - and β -obscurine, huperzine B, serratine, and lucidoline [17, 18]. These data, however, refer to sporophytes. Previous phytochemical studies of gametophytes included only *H. selago* and *Phlegmariurus squarrosus*. Analyses by Szypuła et al. [18] using HPLC-HR-MS/MS showed that

extracts from prothalli contain alkaloids not previously described in *H. selago* sporophytes, including deacetylfaucettine, faucettimine, 16-hydroxyhuperzine B, deacetyllycoclavine, annopodine, lycopercurine, N-methylfastigioline, and flabeline (ibid.). Among alkaloids characteristic of *H. selago* sporophytes, gametophytes—besides HupA and HupB—also contained 6 β -hydroxyhuperzine A, serratinidine, lycopodine, and selagoline [18–20]. Notably, these compounds have not been detected in *H. serrata*, despite nearly 90 alkaloids being isolated from that species (ibid.).

To date, no effective methods have been developed for obtaining HupA and HupB from clubmoss tissue cultures on a scale larger than laboratory production [17, 20]. However, reports on in vitro cultures of sporophytes and gametophytes suggest that they may represent simpler and cheaper sources of alkaloids than chemical synthesis or extraction from wild plants (ibid.). The use of sporophytes, however, requires complex sterilization procedures involving both surface and internal disinfection, including antibiotics that eliminate only part of the endophytes. The only method enabling the establishment of axenic sporophyte cultures is somatic embryogenesis, described so far only for *Lycopodiella inundata* and *Huperzia selago* [10, 17, 20]. Among these species, only *H. selago* synthesizes huperzine A (ibid.). Although somatic embryogenesis is difficult, time-consuming, and inefficient, sporophytes regenerated by this method reached the highest HupA concentrations reported to date—3.33 mg/g DW of tissue [12, 17].

Studies have shown that *H. selago* gametophytes also possess the ability to biosynthesize alkaloids, including HupA, HupB, and lycopodine. Secondary metabolites of *H. selago* gametophytes were first described by Szypuła et al. (ibid.). One attempt was also made to use *Phlegmariurus squarrosus* gametophytes for HupA production, yielding 0.312–0.675 mg/g DW after 6–12 months [10, 13, 20]. According to Szypuła et al. [12], *H. selago* gametophytes maintain the ability to biosynthesize HupA and HupB for approximately 6 months of in vitro culture. Chromatographic analyses (HPLC-DAD and LCMS-IT-TOF) showed that the qualitative alkaloid profile in gametophytes is similar to that of sporophytes [11, 12], although HupA biosynthesis efficiency in gametophytes remains lower. The biosynthetic efficiency of HupA in gametophytes was on average 42% higher than in sporophytes of various clubmoss species collected from natural habitats (ibid.). Compared with the average HupA content in *H. serrata* sporophytes (0.0047–0.025%), *H. selago* gametophytes exhibit a 35-fold greater biosynthetic capacity [2, 12]. In in vitro gametophyte cultures, up to 4.73 mg (0.47%) HupA per 1 g DW has been reported. In the present study, the highest HupA content was recorded in the week-8 control sample, reaching 4.44 mg/g DW.

According to Szypuła et al. [12], during a 24-week experiment, HupA content remained high, ranging from 2.20 ± 0.11 to 2.67 ± 0.25 mg/g DW. In the present study, these values were even higher—the control HupA content ranged from 3.01 ± 0.76 to 4.22 ± 0.30 mg/g DW. In the experimental samples treated with various ACh concentrations, however, no HupA was detected. This result differs from previous reports, which indicated that gametophytes synthesized HupA at levels up to 0.47%. The complete inhibition of HupA biosynthesis observed in this experiment must therefore be considered unusual.

Data on HupB content in plant material are scarce. Information on the proportions of HupA to HupB in clubmoss tissues is also lacking. To date, HupB has been detected in only a few species, and its quantities remain poorly characterized. Xu et al. [124] showed that in Icelandic populations of *H. selago* sporophytes, HupB content ranged from 0.006 to 0.200 mg/g DW, with no correlation between HupA and HupB levels. It is known, however, that sporophytes contain on average several hundred times less HupB than HupA.

In gametophytes obtained in vitro by Szypuła et al. [21], HupB content ranged from 0.10 to 0.52 mg/g DW, representing a very high biosynthetic level compared with sporophytes. In the present study, HupB was detected only in the week-8 control sample, at 0.44 mg/g DW, consistent with Szypuła's findings. In ACh-treated samples, no HupB was detected, which also diverges from literature data.

The study also analyzed lycopodine content. According to the literature, sporophytes contain on average about 1.5 mg/g DW of this alkaloid [9]. Its levels in gametophytes had not been previously investigated. In the control sample, lycopodine averaged 3.34 ± 0.37 mg/g DW, ranging from 3.03 ± 0.03 to 3.97 ± 1.26 mg/g DW, comparable to HupA levels in sporophytes. In the experimental samples, mean lycopodine content was four times higher, reaching 11.92 mg/g DW. Values ranged from 7.04 ± 1.40 to 16.72 ± 0.68 mg/g DW, with the highest concentrations observed in cultures treated with 10 μ mol ACh and collected at week 8. In cultures treated with 1, 100, and 1000 μ mol ACh, lycopodine levels were similar to control values.

Statistically significant differences in lycopodine content were found between gametophytes grown on medium with 10 μ mol ACh and all other variants, including the control. Cultures treated with 1, 100, and 1000 μ mol ACh did not differ significantly from the control, although—similar to HupA and HupB—they did not show the presence of these alkaloids.

This study is the first to examine the effect of acetylcholine on alkaloid biosynthesis in *H. selago* gametophytes. It was hypothesized that ACh supplementation would stimulate HupA and HupB synthesis. The results did not confirm this hypothesis. In the experimental samples, neither huperzine was detected, while very high amounts of lycopodine—one of the major *H. selago* alkaloids whose biological function remains unknown—were observed. The absence of HupA and HupB alongside increased lycopodine suggests that ACh may inhibit the synthesis of these alkaloids or redirect metabolism toward enhanced lycopodine production.

The highest lycopodine values were recorded at week 8, resembling the dynamics of HupA synthesis described by Szypuła et al. [21], where maximum alkaloid levels also occurred at week 8 before declining. This may indicate that acetylcholine acts as a modulator of alkaloid biosynthesis—stimulating or inhibiting the formation of specific compounds. A comparison of HupA and HupB data from Szypuła's studies with lycopodine data from the present work (Fig. 36) may support this relationship.

Available publications on the effects of acetylcholine on plant growth and development do not explain its mechanisms of action. However, it is possible that ACh influences signaling pathways associated with key proteins regulating morphogenesis, such as phytochrome and cryptochrome. As indicated by Fijałkowska [22], light may affect the efficiency of HupA and HupB biosynthesis. The highest HupA levels were obtained in gametophyte cultures illuminated with light dominated by red and blue wavelengths (ibid.). Literature also suggests that acetylcholine may act on plant cells in a manner similar to red light, stimulating morphogenetic processes [23].

In the present experiment, gametophytes were grown in darkness, suggesting that ACh may have acted as a light substitute, stimulating lycopodine synthesis while inhibiting HupA production—the strongest acetylcholinesterase inhibitor. According to scientific reports, compounds blocking AChE activity increase tissue sensitivity to acetylcholine [23]. It is therefore possible that inhibition of HupA biosynthesis in the presence of ACh was an adaptive response reducing cellular sensitivity to this molecule.

In the experimental samples, lycopodine was synthesized in the greatest amounts, and it is also the weakest AChE inhibitor among the alkaloids studied. The results are statistically significant and indicate a clear relationship between ACh supplementation and changes in alkaloid biosynthesis profiles. Further research is necessary to fully understand these relationships.

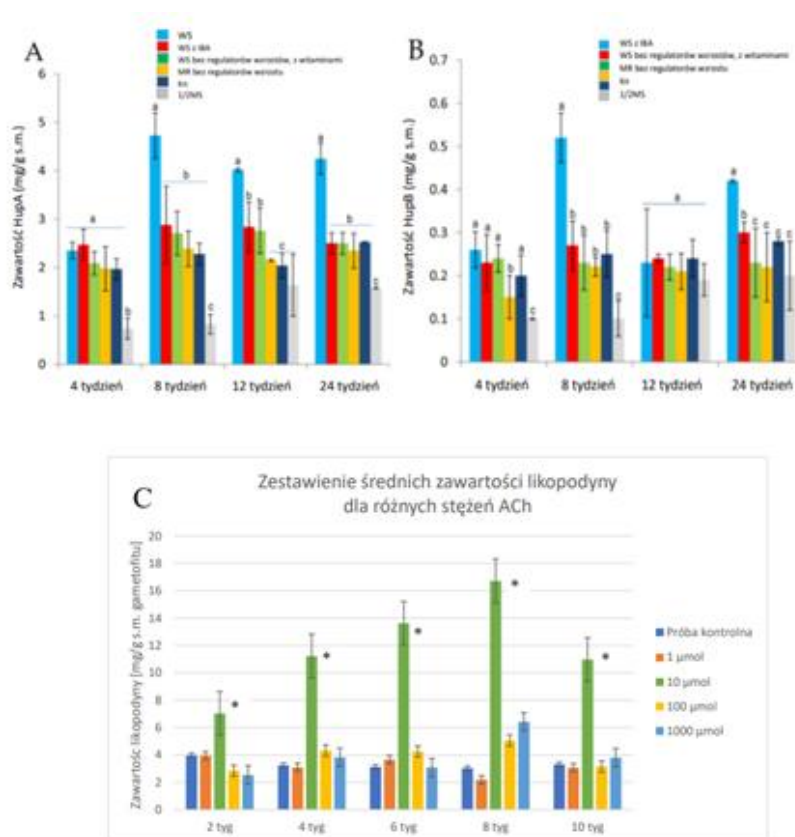


Fig. 14. Total content of HupA (A), HupB (B) [83], and lycopodine (C) in *H. selago* gametophyte in vitro cultures over a period of 24 weeks (A and B) and 10 weeks (C).

Conclusions

- *Huperzia selago* gametophytes grown in vitro confirm their high efficiency as a source of huperzine A and huperzine B.
- Acetylcholine added to the culture medium completely inhibits the biosynthesis of huperzine A and huperzine B in *H. selago* gametophytes.
- Acetylcholine at a concentration of 10 μ M significantly stimulates lycopodine biosynthesis, whereas the other tested concentrations do not show this effect.
- The duration of culture (8 weeks) is crucial for achieving maximum levels of the alkaloids studied.
- Acetylcholine exhibits strong and selective regulatory effects on alkaloid biosynthetic pathways in *H. selago*, opening new possibilities for research on the control of secondary metabolism in clubmosses.

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