

# Application of temporary immersion system RITA® for efficient biomass multiplication and production of somatic seeds for ex situ conservation of *Linnaea borealis* L.

**Małgorzata Anna Kikowska** (✉ [kikowska@ump.edu.pl](mailto:kikowska@ump.edu.pl))

Poznan University of Medical Sciences Faculty of Pharmacy: Uniwersytet Medyczny im Karola Marcinkowskiego w Poznaniu Wydział Farmaceutyczny <https://orcid.org/0000-0002-5939-5292>

**Kamil Danek**

Poznan University of Technology: Politechnika Poznanska

**Justyna Gornowicz\_Porowska**

Poznan University of Medical Sciences Faculty of Pharmacy: Uniwersytet Medyczny im Karola Marcinkowskiego w Poznaniu Wydział Farmaceutyczny

**Barbara Thiem**

Poznan University of Medical Sciences Faculty of Pharmacy: Uniwersytet Medyczny im Karola Marcinkowskiego w Poznaniu Wydział Farmaceutyczny

---

## Research Article

**Keywords:** Twinflower, Shoot multiplication, RITA® bioreactor, Encapsulated nodes, Storage of somatic seeds

**Posted Date:** June 30th, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1420220/v1>

**License:** © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

*Linnaea borealis* L., belonging to the *Linnaeaceae* family, in some European countries including Poland, is a protected species. Phytochemical studies indicate the presence of pharmacologically important secondary metabolites, which may indicate undiscovered biological activity and untapped therapeutic potential of this species. Harvesting the raw material from natural sites is impossible due to the protection status of this glacial relict in many countries. The aim of the current work was to develop an efficient protocol of *Linnaea borealis* L. var. *borealis* shoot multiplication via the development of axillary buds in an agitated culture and a temporary immersion system as well as to establish the production of somatic seeds via alginate encapsulated nodes for short-time storage. The highest results of growth parameters were obtained for shoots agitated in a liquid medium enriched with cytokinin, auxin and gibberellin ( $18.26 \pm 0.35$  shoots per explant and  $2184.95 \pm 98.12\%$  biomass increase, respectively). The growth of shoots in the RITA® bioreactor was highly efficient, especially when hormone supplementation in the medium was used, the amount of medium in the culture vessel was 100 or 150 ml, and the immersion time of the shoots was 1 or 2 min. The survival and recovery rates of *L. borealis* somatic seeds, regardless of encapsulation matrix used, subsequently decreased with the increased storage duration (from 100–60% at 4°C and from 100–54% at -18 °C, respectively). This is the first report on twinflower shoot multiplication in a temporary-immersion system and somatic seeds production of this species.

## Introduction

*Linnaea borealis* L. (Twinflower) is a creeping evergreen dwarf shrub assigned to the *Linnaeaceae* family. Twinflower typically associated with boreal forests, has a circumboreal distribution, across the Northern Hemisphere, occurring from Scotland and northern Europe through Russia to Siberia, northern Asia to Kamchatka and Japan, northern China and Mongolia, and from Alaska and Canada to Greenland (Alm 2006). In some countries and regions is critically endangered but in others is considered extinct. In Poland, as a relic of the Late Glacial period, this strictly protected species reaches the southern extent of its range (Ciosek et al. 2015; Zawadzka et al. 2017). In Norwegian traditional medicine, *L. borealis* has a long tradition as a cure for shingles (*Herpes zoster*). In the past, this species was also used in European countries to treat skin diseases and other kinds of rash, eczema, measles, hives, ringworm, scabies, water blisters, rheumatism, and finger infections (Brondegaard 1959; Thiem and Buk-Berge 2017).

The chemical composition of this species had been not well known (Glennie 1969), but thanks to the use of biomass multiplied with biotechnological methods, the knowledge about the presence of biologically active compounds has been significantly expanded (Thiem et al. 2021). In the late sixties of the last century, the following flavonoids were preliminary detected in ethanol extracts from the leaves collected from a ground plant of the American subspecies of *L. borealis*: glycosides of quercetin and kaempferol, as well as derivatives of apigenin and luteolin. Several compounds belonging to phenolic acids have been identified, including p-coumaric acid, p-hydroxybenzoic acid, caffeic acid, ferulic acid, protocatechic acid, vanillic acid, phloretic acid, and four chlorogenic acid isomers. Moreover, modern research

employing ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method indicated the presence of 30 phenolic and 19 flavonoid compounds, 13 iridoid compounds 2 organic acids, 14 triterpenoid saponins in ethanol extracts from shoots cultured in vitro from the European subspecies of *L. borealis*. For the first time, the presence of the following saponins was demonstrated: macranthoidin A, akebiasaponin D, Ioniceroside C, bourneioside B, cauloside C, alpha-hederin, and cauloside A. Iridoids and secoirridoids were annotated as loganic acid and epi-loganic acid, deoxyloganic acid, swertiamarin and geniposidic acid, loganin, secologanin and secoxyloganin. Due to the presence of valuable secondary metabolites – flavonoids, phenolic acids, iridoids, secoirridoids, and triterpenoid saponins (Glennie 1969, Thiem et al. 2021) this taxon can be interesting for the pharmaceutical and cosmetic industries.

Twinflower is rarely propagated generatively—seeds are often not produced, not many seeds of one-seeded capsules have been found to be viable, or seedlings are rarely observed. Moreover, the sexual method does not guarantee obtaining uniform true-to-type plants. Greenhouse propagation via stem cuttings has been unsatisfactory due to the frequent failure of forming roots. The plant itself is small, produces few shoots and these have leaves with small-sized blades, which makes it not possible to collect a sufficient amount of plant material for phytochemical and biological studies. Additionally, populations of twinflower are decreasing in several sites. Moreover, the availability of the quantity of plants growing in the wild is also significantly limited due to their strict or partial species protection (Thiem and Buk-Berge 2017). Shoot multiplication with an application of temporary-immersion bioreactor may be a tool adopted to produce a high amount of shoot biomass in a relatively short time according to the established protocol. On the other hand, somatic seeds, formed by encapsulating nodal parts, may constitute a propagule bank for subsequent shoot regeneration and preservation of continuous biomass production, and constitute a form of ex vitro protection of this valuable and protected species.

The aim of the current work was to develop an efficient protocol of *Linnaea borealis* L. var. *borealis* shoot multiplication via the development of axillary buds in an agitated culture and a temporary immersion system as well as to establish the production of somatic seeds via alginate encapsulated nodes for short-time storage of the endangered species.

## Materials And Methods

### Plant material and culture conditions

Shoot fragments from adult plants of *L. borealis* L. var. *borealis* were collected from the mixed coniferous forest in Wiselka, the Wolin National Park, Poland, in July 2017. The plant specimen was deposited in the Herbarium of the Department of Pharmaceutical Botany and Plant Biotechnology (now Laboratory of Pharmaceutical Biology and Biotechnology) of Poznan University of Medical Sciences. The micropropagation conditions were described in our previous article (Thiem et al. 2021). Briefly, fragments of shoots with nodes were surface disinfected with 4.28% of calcium hypochlorite for 15 min. Then, explants (nodal segments or shoot tips with apical meristems) were transferred to the solidified with agar

(7.2 g) basal medium consisting of MS medium (Murashige and Skoog 1962) enriched with plant growth regulators (PGRs), 6-benzylaminopurine (BAP, 1.0 mg/l); BAP (1.0 mg/l) + indole-3-acetic acid (IAA; 0.1 mg/l); BAP (1.0 mg/l) + IAA (0.1 mg/l) + gibberellic acid (GA<sub>3</sub>; 1.0 mg/l). Clusters of 2-3 shoots multiplied using the method of stimulation of new buds from pre-existing meristems on solid media were the source of explants for the research included in this work.

All the culture vessels were kept in a growth chamber with a 16 h photoperiod (55 µmol/m<sup>2</sup>s) provided by cool white fluorescent lamps, and a 21 °C day temperature.

### **Agitated cultures**

Double shoots about 2–3 cm long with 5–6 nodes, obtained from developed shoot cultures on the solid media, were transferred to the liquid MS medium without or with the selected concentrations of PGRs, namely (1) MS; (2) MS + BAP (1.0 mg/l); (3) MS + BAP (1.0 mg/l) + IAA (0.1 mg/l); (4) MS + BAP (1.0 mg/l) + IAA (0.1 mg/l) + GA<sub>3</sub> (1.0 mg/l). 100-cm<sup>3</sup> Erlenmeyer flasks with 10 cm<sup>3</sup> of medium were used for shoot biomass production. Cultures were maintained on a rotary shaker (110 rpm) in the same conditions as previously. After 6 weeks of culture, the number of new shoots per explant and the shoot growth index (GI) were measured. At least 10 explants were used for the multiplication of shoots. The initial (G<sub>0</sub>) and the final (G<sub>X</sub>) fresh weight of biomass of cultured shoots were measured. The biomass growth index [GI] was calculated according to the following formula:  $GI = [(GX - G0)/G0] \times 100\%$ . The correctness of the morphology and the possibility of necrosis, deformation or hyperhydricity were also assessed during the experiment period.

### **RITA® temporary immersion system**

The explants were cultivated in liquid medium in commercial containers of RITA® system following the instructions of the manufacturer (<http://www.vitropic.fr>). An agar-free proliferation medium was used, which was sterilized before adding it to the containers. Double shoots about 2–3 cm long with 5–6 nodes, obtained from developed shoot cultures on the solid media, were transferred to the RITA® vessels without or with the selected concentrations of PGRs, namely (1) MS; (2) MS + BAP (1.0 mg/l); (3) MS + BAP (1.0 mg/l) + IAA (0.1 mg/l); (4) MS + BAP (1.0 mg/l) + IAA (0.1 mg/l) + GA<sub>3</sub> (1.0 mg/l); (5) MS + BAP (1.0 mg/l) + IAA (0.1 mg/l) + GA<sub>3</sub> (1.0 mg/l) + adenine sulfate (AS, 80 mg/l). Each RITA® vessel contained 3-4 explants. 6-8 containers were used for each treatment, and all the experiments were repeated twice. Multiplication of shoots was done using 36-64 explants. At the end of the 4-week cycle, the following parameters were determined: percentage of response, the total number of shoots, fresh weight of shoots, and percentage of hyperhydricity. After 6 weeks of culture, the number of new shoots per explant and the shoot growth index (GI) were measured. At least 10 explants were used for the multiplication of shoots. The initial (G<sub>0</sub>) and the final (G<sub>X</sub>) fresh weight of biomass of cultured shoots were measured. The biomass growth index [GI] was calculated according to the following formula:  $GI = [(GX - G0)/G0] \times 100\%$ .

In previous experiments (Thiem et al. 2021) double and triple shoots gave better results in multiplication than single shoots. For this reason, double shoots were used in all experiments. The effect of medium hormonal supplementation - BAP/ IAA/ GA<sub>3</sub> (BAP 1.0 mg/l; BAP 1.0 mg/l + IAA 1.0 mg/l and BAP 1.0 mg/l + IAA 1.0 mg/l + GA<sub>3</sub> 1.0 mg/l) in comparison to the control (MS without phytohormones) was evaluated. In order to determine the optimum immersion frequency, the system was controlled using a programmer with one immersion (1 min, 2 min and 3 min) per 1h. Also, the effect of culture medium volume (80 ml, 100 ml, 150 ml, and 200 ml) was tested.

### **Somatic seeds production and maintenance**

Shoot fragments with nodes of 304 mm were excised from shoots multiplied under in vitro conditions. For encapsulation, explants were plunged into the solution of sodium alginate and then into calcium chloride (CaCl<sub>2</sub> × 2H<sub>2</sub>O) for complexation. Somatic seeds were washed with sterile distilled water. The encapsulated propagules were placed in 90 cm diameter Petri dishes at 4 °C in darkness and after the storage time, they were transferred to the growth chamber for recovery. MS medium supplemented with BAP 1.0 mg/l, IAA 1.0 mg/l, and GA<sub>3</sub> 1.0 mg/l was used for plant regeneration from somatic seeds.

Different concentrations of sodium alginate solution, 3% (w/v) or 4% (w/v), and calcium chloride, 200 mM and 300 mM, were tested. Time period (20 min) for Na<sup>+</sup>/Ca<sup>2+</sup> ion exchange in the calcium chloride solution was applied. In the next step, different storage times of somatic seeds (2, 4, 6 months) placed in 90 cm diameter Petri dishes at 4 °C and -18 °C in darkness were tested. Each experiment was repeated three times for c.a. 40 explants. Non-stored synthetic seeds were used as a control.

The following parameters were determined – the percentage of response and the total number of recovery shoots.

### **Statistical analysis**

Data are expressed as means and standard error (SE). The collected data were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's POST-HOC test. A two-sided p-value of 0.05 was applied to declare statistical significance. Statistical analysis was performed by using the Statistica software program (Statsoft, Kraków, Poland).

## **Results And Discussion**

The efficient micropropagation protocol of *Linnaea borealis* var. *borealis* was established previously using the method of stimulation of new buds from pre-existing meristems. The influence of the type of the plant explant (single, double and triple shoots), hormonal supplementation in the medium, and culture system on shoot multiplication were estimated (Thiem et al. 2021). On this basis, the most appropriate type of explant (double shoots), a medium variant (BAP 1.0 mg/l + IAA 1.0 mg/l + GA<sub>3</sub> 1.0 mg/l and controls) as well as a culture system (a temporary immersion bioreactor), which combine the advantages

of growing shoots on a solid or in liquid medium were selected in order to increase the scale of shoot cultivation.

In this study, the shoots of *L. borealis* were propagated by stimulating the division of meristematic cells located in the nodal parts of the stem and in the apical part of the shoot, using double shoots as explants.

All explants placed in the liquid medium on the rotary shaker gave a response, which proves the high morphogenic potential of the plant, regardless of the medium variant. However, the supplementation of the nutrient solution in PGRs significantly influenced the number of new shoots and the growth of fresh shoot mass. The highest results of growth parameters (more than 18 shoots per explant and more than 2000% biomass increase) for this type of culture system were obtained after 6 weeks for shoots agitated in a medium enriched with cytokinin and auxin ( $18.32 \pm 0.52$  and  $2225.46 \pm 91.81$  respectively) as well as cytokinin auxin and gibberellin ( $18.26 \pm 0.35$  and  $2184.95 \pm 98.12$  respectively) (Table 1).

Table 1

The influence of hormonal supplementation of MS medium on *L. borealis* shoot biomass growth parameters using a system of shoots agitated in a liquid medium

Medium Variant	Percentage of Response	Multiplication Rate	Fresh Biomass Growth Ratio (Mean $\pm$ SE)	Hyper-hydricity
MS	100%	$4.32 \pm 0.08^c$	$541.48 \pm 62.21^c$	NO (0%)
MS + BAP	100%	$12.21 \pm 0.13^b$	$1233.26 \pm 73.41^b$	NO (0%)
MS + BAP + IAA	100%	$18.32 \pm 0.52^a$	$2225.46 \pm 91.81^a$	NO (0%)
MS + BAP + IAA + GA <sub>3</sub>	100%	$18.26 \pm 0.35^a$	$2184.95 \pm 98.12^a$	NO (0%)
<b>MS</b> – Murashige & Skoog medium; <b>BAP</b> – 6-benzylaminopurine; <b>IAA</b> – indole-3-acetic acid; <b>GA<sub>3</sub></b> – gibberellic acid; Mean values within a column with the same letter are not significantly different at P = 0.05				
(Duncan's Multiple Range Test)				

Despite the use of liquid media, the shoots were characterized by the correct morphology and the vitrified shoots were not observed (Fig. 1,2), which is also due to the correctly selected volume of the medium in relation to the size of the culture vessel. The explants were not completely immersed in the medium and only the liquid rinsed them in the rhythm of shaking. Farahani and Majd (2012) concluded that the lack of oxygen in the liquid media containing small explants is the major limiting factor to growth.

An interesting article comparing the stationary and shaking system of running a shoot culture with the use of a liquid medium is the work on the medicinal species *Salvia officinalis* L. The use of the agitated culture system in vitro was not recommended in the case of sage cultivation. Shoots obtained by this method were verified and necrotic, while those growing in the stationary system on the medium with the addition of agar had the correct morphology (Grzegorzczak et al. 2008). Another article describing the comparison of the stationary and agitated system of shoot cultures is the work on the species *Eryngium alpinum* L. As shown by the results, plants propagated with the use of agar-solidified medium were characterized by correct morphology and intense green color, which distinguishes them from plants from the agitated system, in which verified shoots could be observed. The cultures conducted in the stationary system were also characterized by a greater number of new shoots compared to the agitated system. In the case of this species, it was probably an improper ratio of the volume of the medium to the culture vessel (Kikowska et al. 2020). On the other hand, the work of Merhotra team shows the many advantages of using the in vitro culture system in a liquid medium in the process of micro-propagation. The author states that the use of a liquid medium is associated with better uptake of nutrients and phytohormones by plant tissues during cultivation, which is responsible for improving the condition of the plant and generating proper development. Another advantage of using a liquid medium is the reduction of apical dominance, which affects the development of side buds and a greater increase in biomass (Merhotra et al. 2007).

As the research shows, the supplementation of the nutrient medium with phytohormones has a significant impact on the proper development of shoots in in vitro cultures. Experiments carried out on 3 subspecies of *Hypericum perforatum* L. using liquid MS medium in an agitated system indicated that the greatest increase in biomass could be observed when the ratio of cytokinins to auxins was 1: 1. Morphology of growing shoots in in vitro cultures changed with increasing concentration of phytohormones: at the lowest concentration formed normal, green shoots were observed; while at the highest concentration the shoots were underdeveloped and a developing callus was observed at their base (Kwiecień et al. 2018).

When analyzing the influence of nutrient supplementation, it can be concluded that full supplementation with BAP + IAA + GA<sub>3</sub> gives the best results both in terms of multiplied shoots, length of explants and biomass growth of *E. alpinum* (Kikowska et al. 2020). The literature shows that cytokinins such as BAP favorably influence the development of side buds in shoot cultures of many plant species e.g. *Rubus chamaemorus* L. (Thiem 2001), *Lychnis flos-cuculi* L. (Maliński et al. 2019), *Plantago media* L. (Budzianowska et al. 2019), *Chaenomeles japonica* L. (Kikowska et al. 2019) while gibberellin GA<sub>3</sub> lengthens the internode parts of shoots (Zhang et al. 2016; dos Santos et al. 2017).

All shoots grown in the RITA® bioreactors, regardless of the type of supplementation, were characterized by the correct morphology and viability. The presence of hyperhydricity and callus was not confirmed in any of the treatments (Fig. 3).

The supplementation of the nutrient solution in PGRs significantly influenced the number of new shoots and the enhancement of fresh shoot mass. The highest results of growth parameters (more than 10 shoots per explant and more than 600% biomass increase) for this type of culture system were obtained for shoots temporary immersed in a medium enriched with cytokinin, auxin and gibberellin ( $10.32 \pm 0.43$  and  $681.35 \pm 35.45$ , respectively) (Table 2).

Table 2

The influence of hormonal supplementation of MS medium on *L. borealis* shoot biomass growth parameters using RITA® system of shoots immersed in a liquid medium

Medium Variant	Percentage of Response	Multiplication Rate	Fresh Biomass Growth Ratio (Mean $\pm$ SE)	Hyper-hydricity
MS	80%	$3.52 \pm 0.18^d$	$214.49 \pm 37.52^c$	NO (0%)
MS + BAP	100%	$5.21 \pm 0.12^c$	$241.77 \pm 15.54^c$	NO (0%)
MS + BAP + IAA	100%	$8.62 \pm 0.22^b$	$483.98 \pm 67.40^b$	NO (0%)
MS + BAP + IAA + GA <sub>3</sub>	100%	$8.54 \pm 0.46^b$	$451.87 \pm 57.99^b$	NO (0%)
MS + BAP + IAA + GA <sub>3</sub> + AS	100%	$10.32 \pm 0.43^a$	$681.35 \pm 35.45^a$	NO (0%)
<b>MS</b> – Murashige & Skoog medium; <b>BAP</b> – 6-benzylaminopurine; <b>IAA</b> – indole-3-acetic acid; <b>GA<sub>3</sub></b> – gibberellic acid; <b>AS</b> – adenine sulfate. Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan's Multiple Range Test)				

The shoots have grown in 100 and 150 ml of liquid medium were characterized by viability, correct morphology and the highest growth parameters (multiplication rate  $10.23 \pm 0.24$  and  $12.92 \pm 0.33$ , respectively; biomass growth ratio  $849.83 \pm 76.21$  and  $894.31 \pm 18.02$ , respectively). On the other hand, the shoots immersed in 80 ml of the nutrient solution were drying out, therefore the fresh growth biomass ratio decreased during the culture. The shoots immersed in 200 ml of the medium were characterized by overgrowth and hyperhydricity, and therefore, more than as a result of the multiplication of shoots, the ratio of fresh growth biomass was relatively high (Table 3).



Table 3

The influence of medium volume in bioreactor vessels on *L. borealis* shoot biomass growth parameters using RITA® system of shoots immersed in a liquid medium

Medium Volume	Percentage of Response	Multiplication Rate	Fresh Biomass Growth Ratio (Mean ± SE)	Hyperhydricity
80 ml	50%	1.54 ± 0.04 <sup>d</sup>	-52.74 ± 21.24 <sup>c</sup>	NO (0%)
100 ml	100%	10.23 ± 0.24 <sup>b</sup>	849.83 ± 76.21 <sup>a</sup>	NO (0%)
150 ml	100%	12.92 ± 0.33 <sup>a</sup>	894.31 ± 18.02 <sup>a</sup>	NO (0%)
200 ml	100%	8.93 ± 0.32 <sup>c</sup>	632.49 ± 31.13 <sup>b</sup>	YES (75%)
Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan's Multiple Range Test)				

The shorter time of immersion (1 min and 2 min) turned out to be more favorable for the multiplication of shoots (8.92 ± 0.31 and 9.56 ± 0.22, respectively) and the production of twinflower biomass (721.15 ± 29.13 and 687.36 ± 36.32, respectively) (Table 4). Shoots showed no altered morphology.

Table 4

The influence of immersion frequency on *L. borealis* shoot biomass growth parameters using RITA® system of shoots immersed in a liquid medium

Immersion Frequency	Percentage of Response	Multiplication Rate	Fresh Biomass Growth Ratio (Mean ± SE)	Hyperhydricity
1 min. / 1h	100%	8.92 ± 0.31 <sup>a</sup>	721.15 ± 29.13 <sup>a</sup>	NO (0%)
2 min. / 1h	100%	9.56 ± 0.22 <sup>a</sup>	687.36 ± 36.32 <sup>a</sup>	NO (0%)
3 min. / 1h	100%	5.67 ± 0.13 <sup>b</sup>	281.67 ± 17.07 <sup>b</sup>	NO (0%)
Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan's Multiple Range Test)				

For *Linnaea borealis* L., the growth of shoots in the RITA® bioreactor was highly efficient, especially when hormone supplementation in the medium was used (Table 2), the amount of medium in the culture vessel was 100 or 150 ml (Table 3), and the immersion time of the shoots in the medium was 1 or 2 min (Table 4).

Similar observations with RITA® system have been demonstrated by other authors. The highest shoot number of *Stevia rebaudiana* Bertoni, a medicinal plant containing steviol glycosides, was obtained from the RITA® bioreactor filled with 300 ml of medium in the culture vessel and the application of the immersion frequency of 10 s per 1 hour. In general, as a result of the gradual increase in the immersion frequency from 1 per 8 hours to 1 per 1 hour and the average medium volume from 100 ml to 300 ml in the culture vessel, the number of shoots per explant increased. When employing more volume of culture medium, vitrified shoots were formed, which showed abnormalities in their morphology and anatomy (Bayraktar 2019). Another species, *Schisandra chinensis* (Turcz.) Baill, a rich source of therapeutically important lignans with anticancer, immunostimulatory and hepatoprotective properties, showed the highest growth of shoot biomass in the RITA® system compared to four other breeding systems, including a balloon-type bioreactor or a Platform system. Growth of shoots in the RITA® bioreactor using 200 ml of medium and 5 min dipping periods once every 90 minutes resulted in good growth of healthy and correctly developed shoots, which gives hope for an easy expansion of the culture scale in the future (Szopa et al. 2017). In turn, in the RITA® bioreactor, immersion in 200 ml culture media lasting 15 minutes every 4 hours resulted in an approx. 1.8-fold increase in the biomass of the exotic species used in the production of perfumes and traditional medicine of Asian countries, *Aquilaria malaccensis* Lamk. The shoots were healthy and were not producing callus (Esyanti et al. 2019). Studies on in vitro shoot multiplication of *Scutellaria alpine* L., a species rich in polyphenol metabolites, show the benefits of using a temporary-immersion bioreactor. Experimentally selected parameters – 60 ml of the liquid medium in the culture vessel and the immersion time – 40 seconds influenced the quality and number of multiplied shoots. The results of the conducted research show a 1.5-fold increase in the number of cultured shoots and a 4-fold increase in the shoot biomass compared to the cultivation of the same species carried out on a solidified agar medium (Grzegorzczak-Karolak et al. 2017).

The aim of this study was to determine the best system for propagating the twinflower shoots in the in vitro system - liquid media in an agitated system and in a temporary immersion bioreactor were used. *Linnaea borealis* L. produce lateral shoots of three kinds, vertical sexually reproductive shoots, non-sexually reproductive leafy shoots, and horizontal shoots (Niva et al. 2003). This species may develop efficiently new shoots in liquid media (in an agitated system and in a temporary immersion bioreactor), as the medium rinses the node fragments of all shoots, regardless of their location on the shoot. On a solid medium, there is no possibility of direct contact of media nutrients with nodes that do not straightway touch the medium (located on the higher parts of the stem), therefore they mainly multiply at the base node of the shoot. On the other hand, in liquid media, shoots also spread from higher placed nodes and horizontal shoots grow, which do not encounter any obstacles, and from them, new vertical shoots also grow. In horizontally spreading shoots, the nutrients may be resorbed by more parts simultaneously. Moreover, some observations argue that the population of this species during wet years clearly grows (Piękoś-Mirkowa and Mirek 2003). As it results from our observations of the wild population, this species prefers has been found in damp locations, within easy reach of running or dripping water (data not shown). For this reason, the system of shoots agitated or immersed in liquid

media is an efficient system for the multiplication of the shoot biomass of this species, and is more preferred than a stationary system on solidified media.

One of the technologies of conservation of rare and endangered plants is short cold storage of encapsulated propagules. The formation of beads with appropriate stability and hardness is of key importance for producing somatic seeds: very hard beads limit the regeneration ability, while soft beads dissolve without protecting the encapsulated propagules. The type of explant, the concentrations of sodium alginate (SA) and calcium chloride (CC), and complexation duration were studied for *L. borealis* somatic seeds. Out of the two different concentrations of SA (3%, 4%) and the two concentrations of CC (100, 200 mM) evaluated to develop the encapsulation matrix, 3% and 4% SA and 200 mM CC were the most appropriate for beads production (Table 5). Therefore these two options (3% SA + 200 mM CC and 4% SA + 200 mM CC) were selected to be used in the experiment of short-term storage of propagules at lower (4°C) and low (-18 °C) temperatures (Table 6).

Table 5  
The influence of bead composition on synthetic seeds formation

<b>SODIUM ALGINATE</b>	<b>CALCIUM CHLORIDE</b>	<b>BEAD CHARACTERISTIC</b>	<b>PURPOSE</b>
3%	100 mM	Too soft to handle, formed tails	Not used for research
3%	200 mM	Isodiametric beads	Used for research
4%	100 mM	Isodiametric beads	Not used for research
4%	200 mM	Isodiametric beads	Used for research

Table 6

The influence of beat composition and storage duration (4°C, -18°C) on the recovery of somatic seeds of *L. borealis*.

Sodium Alginate	Calcium Chloride	Storage Temperature	Storage Duration	Survival Percentage	Recovery Percentage ± SE
3%	200 mM	4°C	0 months	100%	100 ± 0.00%
3%	200 mM	4°C	2 months	80%	90 ± 3.7%
3%	200 mM	4°C	4 months	60%	73 ± 2.6%
3%	200 mM	4°C	6 months	60%	67 ± 2.6%
4%	200 mM	4°C	0 months	100%	98 ± 1.3%
4%	200 mM	4°C	2 months	100%	89 ± 2.7%
4%	200 mM	4°C	4 months	80%	78 ± 2.0%
4%	200 mM	4°C	6 months	70%	70 ± 2.6%
3%	200 mM	-18 °C	0 months	100%	100 ± 0.0%
3%	200 mM	-18 °C	2 months	80%	78 ± 2.0%
3%	200 mM	-18 °C	4 months	60%	62 ± 2.5%
3%	200 mM	-18 °C	6 months	60%	58 ± 2.9%
4%	200 mM	-18 °C	0 months	100%	98 ± 1.3%
4%	200 mM	-18 °C	2 months	100%	73 ± 1.5%
4%	200 mM	-18 °C	4 months	80%	59 ± 2.3%
4%	200 mM	-18 °C	6 months	70%	54 ± 2.2%
Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan's Multiple Range Test)					

The survival and recovery rates, regardless of the encapsulation matrix used, subsequently decreased with the increased storage duration (from 100–60% and from 100–54%, respectively). Plants regenerated more efficiently when the explants, from which they developed, were stored at a higher temperature (4°C). The highest percentage of survival (100%) and recovery (98–100%) were obtained for beads inoculated immediately after formation (Table 6).

Somatic seeds production of a wide range of important endangered and protected species is considered an effective way to support their conservation. The maximum regeneration rate of  $74\% \pm 2.72\%$  was observed for axillary buds of *Eryngium alpinum* L., a protected alpine species, encapsulated in 4% sodium–alginate complexed with 300 mM calcium chloride after 2 months of storage at low temperature (Kikowska et al. 2020). Axillary buds of *Rubus chamaemorus* L., a glacial relict occurring in a few protected reserves, encapsulated in alginate hydrogel (5% SA and 50  $\mu$ M CC) and stored at 4°C for 3 months survived and regenerated in almost 56% (Thiem 2002). The storage potential of artificial seeds of *Cymbidium aloifolium* protocorms, a Threatened Orchid of Nepal, up to 90 days found 83.33% viability at 4°C storage on MS media (Pradhan et al. 2016). The results indicated that synseeds (3% SA, 100 mM CC) obtained from shoot tips of *Taraxacum pinnatum* Pawł. can be stored at 4°C even for 12 months (Kamińska et al. 2017).

Somatic seeds possess immense potential for large-scale production of plants as an alternative option to true seeds, have the potential to regenerate elite genotypes, and preserve important plant genetic resources (Nandini and Giridhar 2019). Artificial seed production and storage protocols allow the continuous supply of plant material of medicinal importance (Kikowska and Thiem 2011). Moreover, collections of in vitro cultures in combination with the methods of storing plant material provide tools that guarantee the protection of genetic resources of valuable plant species (Mikuła et al. 2013).

## Conclusions

Thanks to the in vitro multiplication of *Linnaea borealis* L. biomass in liquid systems, unlimited production of plant material for phytochemical and biological research is possible. The development of new storage technologies including in vitro collection and somatic seeds remains of high priority for rare, endangered and protected species. Moreover, applied conditions for somatic seeds production provide a cost-effective and time-saving method for the active protection of this species.

## Abbreviations

**AS** Adenine sulfate

**BAP** 6-Benzylaminopurine

**CC** Calcium chloride

**GA<sub>3</sub>** Gibberellic acid

**G0** Initial fresh weight of biomass

**GI** Growth index

**GX** Final fresh weight of biomass

**IAA** Indole-3-acetic acid

**MS** Murashige & Skoog medium

**PGRs** Plant growth regulators

**SA** Sodium alginate

## **Declarations**

### **Data availability**

The data are available from the corresponding author on reasonable request.

### **Code availability**

Not applicable.

### **Funding**

Research supported by statutory funds of the Department of Pharmaceutical Botany and Plant Biotechnology at the Poznań University of Medical Sciences.

### **Conflict of interest**

The authors declare that they have no conflict of interest.

### **Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

### **Author Contributions**

MK and BT: study conception and design. MK and KD: material preparation, data collection and analysis. BT and JGP: supervision. MK: first draft of the manuscript. MK, KD, JGP, BT: writing—review and editing. All authors read and approved the final manuscript.

## References

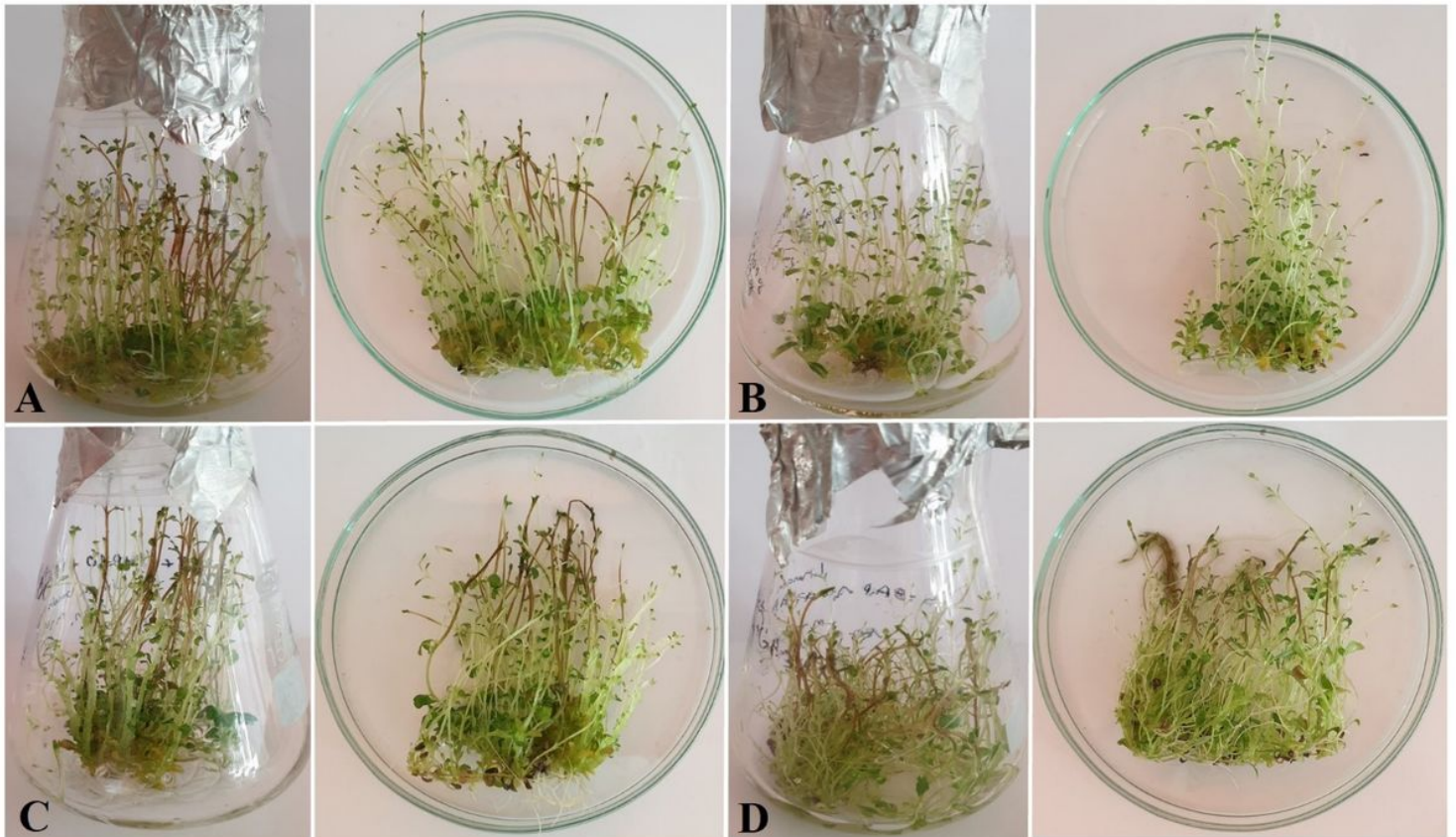
1. Alm T (2006) Ethnobotany of *Linnaea borealis* (Linnaeaceae) in Norway. Bot J Linn Soc 151:437–452. <https://doi.org/10.1111/j.1095-8339.2006.00516.x>
2. Bayraktar M (2019) Micropropagation of *Stevia rebaudiana* Bertoni Using RITA Bioreactor Hortscience 54(4):725–731. <https://doi.org/10.21273/HORTSCI13846-18>
3. Brondegaard VJ (1959) Linnaea i folkemedicine. Sven. Linnésällskapets Årsskrift 1959, 42, 89–98. (In Norwegian)
4. Budzianowska A, Kikowska M, Małkiewicz M, Karolak I, Budzianowski J (2019) Phenylethanoid glycosides in *Plantago media* L. organs obtained in in vitro cultures. Acta Biol Cracov Bot 61/1:75–86. <https://doi.org/10.24425/118060>
5. Ciosek MT, Krechowski J, Sikorski R, Trębicka A, Piórek K (2015) The Twinflower (*Linnaea borealis* L.) in the northern part of the Południowopodlaska Lowland. For Res Pap 76(2):113–121. <https://doi.org/10.1515/frp-2015-0011>
6. dos Santos DS, Cardoso-Gustavson P, Nievola CC (2017) Stem elongation of ornamental bromeliad in tissue culture depends on the temperature even in the presence of gibberellic acid. Acta Physiol Plant 39:230. <https://doi.org/10.1007/s11738-017-2536-0>
7. Esyanti R, Faizal A, Fadholi M (2019) Shoot multiplication and growth rates of *Aquilaria malaccensis* Lamk shoot cultures in temporary immersion system (TIS)-RITA and bubble column bioreactors. Pak J Bot 51(4). [https://10.30848/pjb2019-4\(36\)](https://10.30848/pjb2019-4(36))
8. Glennie CW (1969) Comparative Phytochemical Study of Caprifoliaceae. Ph.D. Thesis, The University of British Columbia, Vancouver, BC, Canada, pp. 3–96
9. Grzegorzczak I, Wysokińska H (2008) Liquid shoot culture of *Salvia officinalis* L. for micropropagation and production of antioxidant compounds; effect of triacontanol. Acta Soc Bot 77(2):99–104
10. Grzegorzczak-Karolak I, Rytczak P, Bielecki S, Wysokińska H (2017) The influence of liquid systems for shoot multiplication, secondary metabolite production and plant regeneration of *Scutellaria alpina*. Plant Cell Tiss Organ Cult 128:479–486. <https://10.1007/s11240-016-1126-y>
11. Kamińska M, Gołębiowski M, Tretyn A, Trejgell A (2017) Efficient long-term conservation of *Taraxacum pinnatum* synthetic seeds in slow growth conditions. Plant Cell Tiss Organ Cult 132:469–478. <https://doi.org/10.1007/s11240-017-1343-z>
12. Kikowska M, Thiem B, Szopa A, Ekiert H (2020) Accumulation of valuable secondary metabolites: phenolic acids and flavonoids in different in vitro systems of shoot cultures of the endangered plant species-*Eryngium alpinum* L. Plant Cell Tiss Organ Cult 141:381–391. <https://doi.org/10.1007/s11240-020-01795-5>
13. Kikowska M, Thiem B (2011) Alginate-encapsulated shoot tips and nodal segments in micropropagation of medicinal plants. A review. Herba Pol 57(4):45–57
14. Kwiecień I, Smolin J, Beerhues L, Ekiert H (2018) The impact of media composition on production of flavonoids in agitated shoot cultures of the three *Hypericum perforatum* L. cultivars „Elixir”, „Helos”,

- „Topas”. *Vitro Cell Develop Biol - Plant* 54:332–340. <https://doi.org/10.1007/s11627-018-9900-7>
15. Maliński MP, Kikowska M, Kruszka D, Napierała M, Florek E, Sliwinska E, Thiem B (2019) Various in vitro systems of Ragged Robin (*Lychnis flos-cuculi* L.): a new potential source of phytoecdysteroids? *Plant Cell Tiss Organ Cult* 139:39–52. <https://doi.org/10.1007/s11240-019-01660-0>
  16. Merhotra S, Goel MK, Kukreja AK, Mishra BN (2007) Efficiency of liquid culture systems over conventional micropropagation: A progress towards commercialization. *Afr J Biotech* 6(13):1484–1492
  17. Mikuła A, Makowski D, Tomiczak K, Rybczyński JJ (2013) In vitro culture and cryopreservation for biodiversity conservation – genebank standards. *Pol J Agron* 14:3–17
  18. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol Plant* 15:473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
  19. Nandini B, Giridhar P (2019) Insight View of Topical Trends on Synthetic Seeds of Rare and Endangered Plant Species and Its Future Prospects. In: Faisal M, Alatar A (eds) *Synthetic Seeds*. Springer, Cham. [https://doi.org/10.1007/978-3-030-24631-0\\_5](https://doi.org/10.1007/978-3-030-24631-0_5)
  20. Niva M, Svensson BM, Karlsson PS (2003) Nutrient resorption from senescing leaves of the clonal plant *Linnaea borealis* in relation to reproductive state and resource availability. *Funct Ecol* 17:438–444. <https://doi.org/10.1046/j.1365-2435.2003.00757.x>
  21. Piękoś-Mirkowa H, Mirek Z (2003) *Flora Polski. Atlas Roślin Chronionych*. Multico Oficyna Wydawnicza, Warszawa, ISBN 83-7073-256-9
  22. Pradhan S, Tiruwa B, Subedee B, Pant B (2016) Efficient Plant Regeneration of *Cymbidium aloifolium* (L.) Sw., a Threatened Orchid of Nepal through Artificial Seed Technology. *Am J Plant Sci* 7:1964–1974. <https://10.4236/ajps.2016.714179>
  23. Szopa A, Kokotkiewicz A, Luczkiewicz M, Ekiert H (2017) *Schisandra* lignans production regulated by different bioreactor type. *J Biotechnol* 247:11–17. <https://10.1016/j.jbiotec.2017.02.007>
  24. Thiem B (2001) Micropropagation of cloudberry (*Rubus chamaemorus* L.) by initiation of axillary shoots. *Acta Soc Bot Pol* 70(1):11–16. <https://doi.org/10.5586/asbp.2001.002>
  25. Thiem B (2002) In vitro culture techniques in conservation of *Rubus chamaemorus* L. *Acta Biol Cracov Bot* 44:181–188
  26. Thiem B, Buk-Berge E (2017) Twinflower (*Linnaea borealis* L.) – plant species of potential medicinal properties. *Herba Pol* 63(3):56–64. <https://10.1515/hepo-2017-0019>
  27. Thiem B, Kruszka D, Turowska N, Sliwinska E, Berge V, Kikowska M (2021) *Linnaea borealis* L. var. *borealis*—In Vitro Cultures and Phytochemical Screening as a Dual Strategy for Its Ex Situ Conservation and a Source of Bioactive Compounds of the Rare Species. *Molecules* 26:6823. <https://10.3390/molecules26226823>
  28. Zawadzka D, Zawadzki G, Bednarek J, Bednarek JB, Piechowska D, Mikitiuk A (2017) The twinflower in the Augustow Forest: Occurrence, condition and threats. *For Res Pap* 78:77–78. <https://10.1515/frp-2017-0008>



29. Zhang N, Xie YD, Guo HJ, Zhao LS, Xiong HC, Gu JY, Li JH, Kong FQ, Sui L, Zhao ZW, Zhao SR, Liu LX (2016) Gibberellins regulate the stem elongation rate without affecting the mature plant height of a quick development mutant of winter wheat (*Triticum aestivum* L.). *Plant Physiol Biochem* 107:228–236. <https://doi.org/10.1016/j.plaphy.2016.06.008>

## Figures



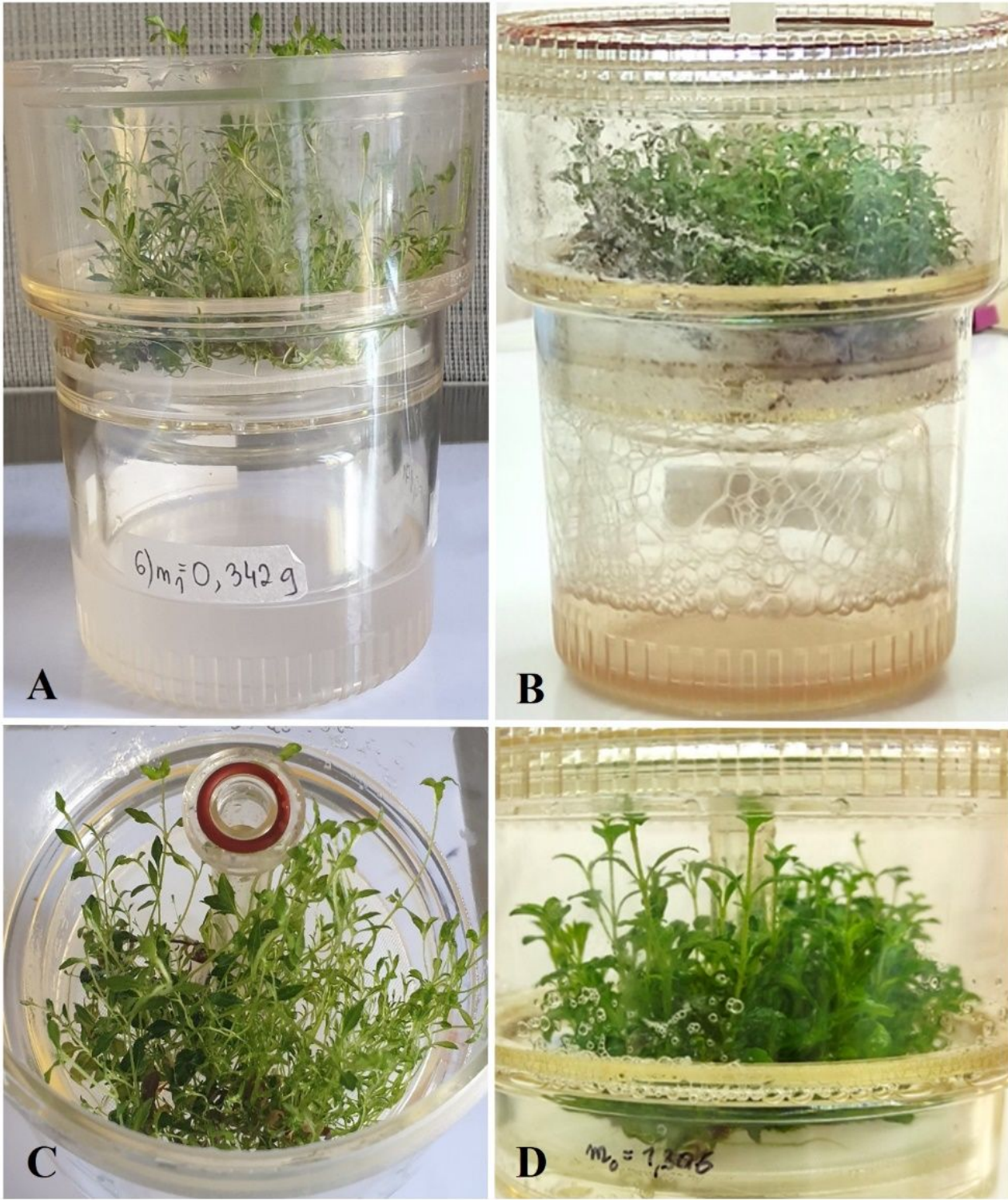
**Figure 1**

Multiplied shoots of *L. borealis* in the liquid medium obtained from double shoots A) MS, B) MS + BAP 1.0 mg/l, C) MS + BAP 1.0 mg/l + IAA 1.0 mg/l, D) MS + BAP 1.0 mg/l + IAA 1.0 mg/l + GA<sub>3</sub> 1.0 mg/l



**Figure 2**

Multiplied shoots of *L. borealis* in the liquid medium obtained from double shoots agitated on a rotary shaker



**Figure 3**

In vitro shoot cultures of *L. borealis* carried out in the temporary-immersion system RITA®