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

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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 artificial seeds via alginate encapsulated nodes for short-time storage. The highest results of growth parameters were obtained for shoots agitated in MS (Murashige & Skoog) liquid medium enriched with cytokinin, auxin and gibberellin (18.3 ± 0.4 shoots per explant and 2185 ± 98% biomass increase, respectively). The growth of shoots in the RITA® bioreactor was highly efficient, especially when hormone supplementation in MS 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* artificial seeds, regardless of encapsulation matrix used, subsequently decreased with the increased storage duration up to 6 months (from 100 to 60% at 4 °C and from 100 to 54% at -18 ºC, respectively). This is the first report on twinflower shoot multiplication in a temporary-immersion system and artificial seeds production of this species.

Key message

The development of new storage technologies including in vitro collection and somatic seeds is of high priority for an endangered species *L. borealis* L. var. *borealis*.

Keywords Twinflower · Shoot multiplication · RITA® bioreactor · Encapsulated nodes · Storage of artificial seeds

**Abbreviations**

| Abbreviation | Description                      |
|--------------|----------------------------------|
| AS           | Adenine sulfate                  |
| BAP          | 6-Benzylaminopurine               |
| CC           | Calcium chloride                 |
| GA3          | 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                  |

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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 has been 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). Due to the presence of valuable secondary metabolites—flavonoids, phenolic acids, iridoids, secoiridoids, 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, artificial 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.

RITA® bioreactor, as well as other temporary-immersion systems, was intended for economically feasible and unlimited production of plant biomass by providing optimal nutrients, air transfer and lower mechanical stress. Periodically immersed into a liquid medium plant biomass secure the growth and proper physiology and morphology of the cultured organs. The advantages of RITA® system include simple operation, controlled environment, full separation of biomass and liquid medium (Georgiev et al. 2014). The goal of the presented work was to develop an efficient protocol of *Linnaea borealis* L. var. *borealis* shoot multiplication via the development of axillary buds a temporary immersion system to increase the scale of shoot biomass production.

Artificial 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 (Mikula et al. 2013). The goal of the presented work was to establish the production of artificial seeds via alginate encapsulated nodes for short-time storage of the endangered species.

The aim of the current project is to study the application of in vitro techniques in collecting the sufficient amount of high quality biomass of *Linnaea borealis* L. var. *borealis* dedicated for phytochemical and biological research.

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, 4.44 µM); BAP (4.44 µM)+indole-3-acetic acid (IAA; 0.45 µM); BAP (4.44 µM)+IAA (0.45 µM)+gibberellic acid (GA3; 2.89 µM). 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/m²s) provided by cool white fluorescent lamps, and a 21 °C day temperature.

Agitated shoots

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 (4.44 µM); (3) MS + BAP (4.44 µM) + IAA (0.45 µM); (4) MS + BAP (4.44 µM) + IAA (0.45 µM) + GA3 (2.89 µM). 100-cm³ Erlenmeyer flasks with 10 cm³ 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 (G0) and the final (GX) fresh weight of biomass of cultured shoots were measured. The biomass growth index was calculated according to the following formula: GI = [(GX – G0)/G0] × 100%.

The effect of hormonal supplementation, medium volume and immersion frequency were tested.

Shoots in 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 (4.44 µM); (3) MS + BAP (4.44 µM) + IAA (0.45 µM); (4) MS + BAP (4.44 µM) + IAA (0.45 µM) + GA3 (2.89 µM); (5) MS + BAP (4.44 µM) + IAA (0.45 µM) + GA3 (2.89 µM) + adenine sulfate (AS, 434 µM). 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 (G0) and the final (GX) fresh weight of biomass of cultured shoots were measured. The biomass growth index was calculated according to the following formula: GI = [(GX – G0)/G0] × 100%.

The effect of hormonal supplementation, medium volume and immersion frequency were tested.

Artificial seeds production and maintenance

Shoot fragments with nodes of 3–4 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 (CaCl₂·2H₂O) for complexation. Artificial 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 (4.44 µM), IAA (0.45 µM) and GA3 (2.89 µM) was used for plant regeneration from artificial 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⁺/Ca²⁺ ion exchange in the calcium chloride solution was applied. In the next step, different storage times of artificial 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 ca. 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

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,
The most appropriate type of explant (double shoots), a medium variant (BAP 4.44 µM + IAA 0.45 µM + GA$_3$ 2.89 µM 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.

Agitated shoots

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 (18.3 ± 0.5 and 2225 ± 92, respectively) as well as cytokinin auxin and gibberellic acid (18.3 ± 0.4 and 2185 ± 98, respectively) (Table 1).

Despite the use of liquid media, the shoots were characterized by the correct morphology and the vitrified shoots were not observed (Fig. 1A, 1B).

Shoots in RITA® temporary immersion system

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. 1C, 1D, 1E).

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 on shoot multiplication were estimated (Thiem et al. 2021). In this study, on the basis of the previous observations regarding the use of the appropriate explant and the possibility of liquid media application, different culture systems were compared and aligned biotechnological parameters applied to assess the biomass productivity. The most appropriate type of explant (double shoots), a medium variant (BAP 4.44 µM + IAA 0.45 µM + GA$_3$ 2.89 µM 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.

Table 1 The influence of hormonal supplementation of MS medium on *L. borealis* shoot biomass growth parameters using a system of shoots (at least 10 explants per treatment) agitated in a liquid medium for six weeks

| Medium Variant          | Percentage of Response | Multiplication Rate (Mean ± SE) | Fresh Weight Biomass Ratio (Mean % ± SE) | Hyperhydricity |
|-------------------------|------------------------|---------------------------------|----------------------------------------|----------------|
| MS                      | 100%                   | 4.3 ± 0.1<sup>a</sup>           | 541 ± 62<sup>a</sup>                  | NO (0%)        |
| MS + BAP                | 100%                   | 12.2 ± 0.1<sup>b</sup>          | 1233 ± 73<sup>b</sup>                 | NO (0%)        |
| MS + BAP + IAA          | 100%                   | 18.3 ± 0.5<sup>a</sup>          | 2226 ± 92<sup>a</sup>                 | NO (0%)        |
| MS + BAP + IAA + GA$_3$ | 100%                   | 18.3 ± 0.4<sup>a</sup>          | 2185 ± 98<sup>a</sup>                 | NO (0%)        |

MS – Murashige & Skoog medium; BAP – 6-benzylaminopurine; IAA – indole-3-acetic acid; GA$_3$ – gibberellic acid; Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan’s Multiple Range Test)
system were obtained for shoots temporary immersed in a medium enriched with cytokinin, auxin and gibberellin (10.3 ± 0.4 and 681 ± 36, respectively) (Table 2).

The shoots growing in 100 and 150 ml of liquid medium were characterized by viability, correct morphology and the highest growth parameters (multiplication rate 10.2 ± 0.2 and 12.9 ± 0.3, respectively; biomass growth ratio 850 ± 76 and 894 ± 18, 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).

The shorter time of immersion (1 and 2 min) turned out to be more favorable for the multiplication of shoots (8.9 ± 0.3 and 9.6 ± 0.2, respectively) and the production of twinflower biomass (721 ± 29 and 687 ± 36, respectively) (Table 4). Shoots showed no altered morphology.

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).

### Artificial seeds production and maintenance

The type of explant, the concentrations of sodium alginate (SA) and calcium chloride (CC), and complexation duration were studied for L. borealis artificial 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).

The survival and recovery rates, regardless of the encapsulation matrix used, subsequently decreased with the increased storage duration (from 100 to 60% and from 100 to 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

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**Table 2** The influence of hormonal supplementation of MS medium on L. borealis shoot biomass growth parameters using RITA® system (at least 36 explants per treatment) for 6 weeks of culture

| Medium Variant | Percentage of Response | Multiplication Rate (Mean ± SE) | Fresh Weight Biomass Ratio (Mean ± SE) | Hyperhydricity |
|----------------|------------------------|--------------------------------|----------------------------------------|---------------|
| MS            | 80%                    | 3.5 ± 0.2^a                  | 214 ± 38^c                            | NO (0%)        |
| MS + BAP      | 100%                   | 5.2 ± 0.1^b                  | 242 ± 16^c                            | NO (0%)        |
| MS + BAP + IAA| 100%                   | 8.6 ± 0.2^b                  | 484 ± 67^b                            | NO (0%)        |
| MS + BAP + IAA+ GA_3 | 100%          | 8.5 ± 0.5^b                  | 452 ± 58^b                            | NO (0%)        |
| MS + BAP + IAA+ GA_3+ AS | 100%      | 10.3 ± 0.4^c                  | 681 ± 35^a                            | NO (0%)        |

MS – Murashige & Skoog medium; BAP – 6-benzylaminopurine; IAA – indole-3-acetic acid; GA_3 – gibberellic acid; AS – adenine sulfate. Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan’s Multiple Range Test)

**Table 3** The influence of medium volume in bioreactor vessels on L. borealis shoot biomass growth parameters using RITA® system (at least 36 explants per treatment) for 6 weeks of culture

| Medium Volume | Percentage of Response | Multiplication Rate (Mean ± SE) | Fresh Weight Biomass Ratio (Mean ± SE) | Hyperhydricity |
|---------------|------------------------|--------------------------------|----------------------------------------|---------------|
| 80 ml         | 50%                    | 1.5 ± 0.0^d                  | -52 ± 21^e                             | NO (0%)        |
| 100 ml        | 100%                   | 10.2 ± 0.2^b                 | 850 ± 76^c                            | NO (0%)        |
| 150 ml        | 100%                   | 12.9 ± 0.3^a                 | 894 ± 18^b                            | NO (0%)        |
| 200 ml        | 100%                   | 8.9 ± 0.3^c                  | 633 ± 31^b                            | YES (75%)      |

Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan’s Multiple Range Test)

**Table 4** The influence of immersion frequency on L. borealis shoot biomass growth parameters using RITA® system (at least 36 explants per treatment) for 6 weeks of culture

| Immersion Frequency | Percentage of Response | Multiplication Rate (Mean ± SE) | Fresh Weight Biomass Ratio (Mean ± SE) | Hyperhydricity |
|---------------------|------------------------|--------------------------------|----------------------------------------|---------------|
| 1 min. / 1 h        | 100%                   | 8.9 ± 0.3^a                  | 721 ± 29^b                            | NO (0%)        |
| 2 min. / 1 h        | 100%                   | 9.6 ± 0.2^b                  | 687 ± 36^c                            | NO (0%)        |
| 3 min. / 1 h        | 100%                   | 5.7 ± 0.1^b                  | 282 ± 17^b                            | NO (0%)        |

Mean values within a column with the same letter are not significantly different at P = 0.05 (Duncan’s Multiple Range Test)

**Table 5** The influence of bead composition on L. borealis artificial seeds formation

| SODIUM ALGINATE | CALCIUM CHLORIDE | BEAD CHARACTERISTIC | PURPOSE |
|----------------|-----------------|---------------------|---------|
| 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 |

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).
survival (100%) and recovery (98–100%) were obtained for beads inoculated immediately after formation (Table 6).

**Discussion**

In our studies, two culture systems applying liquid media – agitated shoots culture and temporary immersion system were compared on the basis of *L. borealis* biomass increment calculated with the use of selected parameters – percentage of response, multiplication rate and fresh biomass growth ratio.

An interesting comparison between the different culture system are the studies on the medicinal species, e.g. *Salvia officinalis* L., *Hypericum perforatum* L. and *Eryngium alpinum* L. The use of the agitated culture system with total immersion was not recommended in the case of sage, St John’s wort and eryngo cultivation. Shoots obtained by this method often cause malformations, undesirable physiological conditions, and the cultures shoots are verified and necrotic. In these cases, it was probably an improper ratio of the volume of the medium to the culture vessel, which generated situation when shoots were completely submerged in the medium (Grzegorczyk and Wysokińska 2008; Savio et al. 2012; Kikowska et al. 2020b). On the other hand, despite the use of liquid media, the vitrified shoots were not observed for *L. borealis*, 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, which not resulted in the hyperhydricity of plant tissues. The lack of oxygen in the liquid media containing small explants is the major limiting factor to growth. 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).

RITA® system have been applied by other authors for the beneficial production of shoot biomass, e.g. *Stevia rebaudiana* Bertoni, a medicinal plant containing steviol glycosides (Bayraktar 2019), *Schisandra chinensis* (Turcz.) Baill. rich source of therapeutically important lignans with anticancer, immunostimulatory and hepatoprotective properties (Szopa et al. 2017), *Aquilaria malaccensis* Lamk. the exotic species used in the production of perfumes and traditional medicine of Asian countries (Esyanti et al. 2019), *Scutellaria alpina* L., a species rich in polyphenol metabolites (Grzegorczak-Karolak et al. 2017). In general, as a result of the gradual increase in the immersion frequency and the average medium volume in the culture vessel, the number of shoots per explant increased. However, when employing more volume of culture medium, vitrified shoots may formed, which showed abnormalities in their morphology and anatomy (Bayraktar 2019).

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*

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**Table 6** The influence of heat composition and storage duration (4 °C, -18°C) on the recovery of artificial 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%                 |
| 3%              | 200 mM           | 4 °C                | 2 months         | 80%                | 90 ± 4%                  |
| 3%              | 200 mM           | 4 °C                | 4 months         | 60%                | 73 ± 3%                  |
| 3%              | 200 mM           | 4 °C                | 6 months         | 60%                | 67 ± 3%                  |
| 4%              | 200 mM           | 4 °C                | 0 months         | 100%               | 98 ± 1%                  |
| 4%              | 200 mM           | 4 °C                | 2 months         | 100%               | 89 ± 3%                  |
| 4%              | 200 mM           | 4 °C                | 4 months         | 80%                | 78 ± 2%                  |
| 4%              | 200 mM           | 4 °C                | 6 months         | 70%                | 70 ± 3%                  |
| 3%              | 200 mM           | -18 °C              | 0 months         | 100%               | 100 ± 0%                 |
| 3%              | 200 mM           | -18 °C              | 2 months         | 80%                | 78 ± 2%                  |
| 3%              | 200 mM           | -18 °C              | 4 months         | 60%                | 62 ± 3%                  |
| 3%              | 200 mM           | -18 °C              | 6 months         | 60%                | 58 ± 3%                  |
| 4%              | 200 mM           | -18 °C              | 0 months         | 100%               | 98 ± 1%                  |
| 4%              | 200 mM           | -18 °C              | 2 months         | 100%               | 73 ± 2%                  |
| 4%              | 200 mM           | -18 °C              | 4 months         | 80%                | 59 ± 2%                  |
| 4%              | 200 mM           | -18 °C              | 6 months         | 70%                | 54 ± 2%                  |
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 (Piekoś-Mirkowa and Mirek 2003). As it results from our observations of the wild population, this species 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 artificial seeds: very hard beads limit the regeneration ability, while soft beads dissolve without protecting the encapsulated propagules (Kikowska and Thiem 2011). Artificial seeds production of a wide range of important endangered and protected species is considered an effective way to support their conservation, e.g. Eryngium alpinum L., a protected alpine species (Kikowska et al. 2020a), Rubus chamaemorus L., a glacial relic occurring in a few protected reserves (Thiem 2002), Cymbidium aloifolium, a Threated Orchid of Nepal (Pradhan et al. 2016), Taraxacum pienenicum Pawl. (Kamińska et al. 2017). 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 (Mikula 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 artificial seeds remains of high priority for rare, endangered and protected species. Moreover, applied conditions for artificial seeds production provide a cost-effective and time-saving method for the active protection of this species.

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.

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

Code Availability Not applicable.

Declarations

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.

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