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*Research Article*

# *In Vitro* Micropropagation of *Galanthus plicatus* M. Bieb.: An Efficient *Ex Situ* Conservation Strategy for a Critically Endangered Species from the Republic of Moldova

# Mikrorozmnażanie *in vitro Galanthus plicatus* M. Bieb.: skuteczna strategia ochrony *ex situ* krytycznie zagrożonego gatunku z Republiki Mołdawii

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## Abstract

*Galanthus plicatus* M. Bieb. (*Amaryllidaceae*), a critically endangered geophytic species from the Republic of Moldova, has high ecological, ornamental, and pharmaceutical value as a source of biologically active alkaloids. However, excessive harvesting and habitat degradation have significantly reduced natural populations, creating an urgent need for effective *ex situ* conservation strategies. This study aimed to develop and optimize an *in vitro* micropropagation protocol for the rapid multiplication and conservation of the species' genetic resources. Bulb-derived explants were cultured on Murashige and Skoog (MS) basal medium supplemented with different concentrations of cytokinins (BAP and 2iP), auxin (IBA), and sucrose (45–60 g/L). The results showed that media supplemented with BAP (1.5–2.0 mg/L) and IBA (0.5 mg/L), combined with elevated sucrose concentrations, enhanced microbulb induction and proliferation, yielding up to 24 ± 0.43 microbulbs per explant. Increased sucrose levels also promoted reserve accumulation and microbulb maturation. An optimized sterilization protocol developed without mercuric chloride ensured a high

percentage of viable and contamination-free explants. These findings confirm the effectiveness of in vitro micropropagation as a sustainable *ex situ* conservation strategy for *G. plicatus* and support the reduction of pressure on natural populations.

**Keywords**

*Galanthus plicatus*, *in vitro* micropropagation, microbulb induction, *ex situ* conservation, plant growth regulators, plant genetic resources, *Amaryllidaceae,* critically endangered species

**Streszczenie**

*Galanthus plicatus* M. Bieb. (*Amaryllidaceae*), krytycznie zagrożony gatunek geofityczny występujący w Republice Mołdawii, ma dużą wartość ekologiczną, ozdobną i farmaceutyczną jako źródło biologicznie czynnych alkaloidów. Jednak nadmierne pozyskiwanie roślin oraz degradacja siedlisk doprowadziły do znacznego zmniejszenia liczebności naturalnych populacji rośliny, co stwarza pilną potrzebę opracowania skutecznych strategii ochrony *ex situ*. Celem niniejszego badania było opracowanie i optymalizacja protokołu mikrorozmnażania *in vitro* umożliwiającego szybkie namnażanie oraz ochronę zasobów genetycznych tego gatunku. Eksplantaty pozyskane z cebul hodowano na podstawowej pożywce Murashige'a i Skooga (MS), uzupełnionej różnymi stężeniami cytokinin (BAP i 2iP), auksyny (IBA) oraz sacharozy (45–60 g/l). Wyniki wykazały, że pożywki uzupełnione BAP (1,5–2,0 mg/l) i IBA (0,5 mg/l), w połączeniu ze zwiększonym stężeniem sacharozy, sprzyjały indukcji i proliferacji mikrocebul, których liczba osiągała do 24 ± 0,43 na eksplantat. Zwiększone stężenie sacharozy sprzyjało również gromadzeniu substancji zapasowych i dojrzewaniu mikrocebul. Zoptymalizowany protokół sterylizacji, opracowany bez użycia chlorku rtęci, zapewnił wysoki odsetek żywotnych eksplantatów wolnych od zanieczyszczeń. Wyniki te potwierdzają skuteczność mikrorozmnażania *in vitro* jako zrównoważonej strategii ochrony *ex situ G. plicatus* oraz wskazują, że metoda ta może przyczynić się do zmniejszenia presji na populacje naturalne.

**Słowa kluczowe**

*Galanthus plicatus*, mikrorozmnażanie *in vitro*, indukcja mikrocebul, ochrona *ex situ*, regulatory wzrostu roślin, zasoby genetyczne roślin, *Amaryllidaceae*, gatunek krytycznie zagrożony

## 1. Introduction

The family *Amaryllidaceae* represents one of the most important plant families from a phytochemical perspective, being included among the approximately twenty plant families known for their high content of biologically active alkaloids (Zhong 2013). This family comprises about 1100 species of perennial bulbous plants grouped into approximately 70 genera (POWO 2024), distributed mainly in tropical and warm temperate regions worldwide. Scientific interest in members of this family is largely driven by their ability to produce a wide range of alkaloids with significant pharmacological properties.

The genus *Galanthus* L. (*Amaryllidaceae*) currently includes approximately 25 species of perennial geophytes, naturally distributed in Europe and the Middle East. In the flora of the Republic of Moldova, two species are recorded: *Galanthus nivalis* L. and *G. plicatus* M. Bieb. (Pînzaru 2023). These plants are valued

both for their ornamental importance and their well-documented medicinal properties. The diversity of alkaloids represents one of the most distinctive phytochemical features of this genus. To date, approximately 127 secondary metabolites belonging to 16 structural types have been identified in *Galanthus* species, representing about 20% of the total alkaloids known in the *Amaryllidaceae* family. Among the compounds first identified in this genus are galanthindole, graciline, and plicamine. Although phytochemical research on *Galanthus* remains incomplete, existing studies highlight a remarkable structural diversity of alkaloids, many of which exhibit biological activities that are still insufficiently explored (Georgiev et al. 2024).

Alkaloids characteristic of the *Amaryllidaceae* family exhibit diverse biological properties, including anticholinesterase, antibacterial, antifungal, antiviral, and anticancer activities (Georgiev et al. 2020). A representative example is galanthamine, initially isolated from *Galanthus woronowii* Losinsk., which is currently used in the treatment of Alzheimer's disease symptoms as an approved acetylcholinesterase inhibitor. Other important compounds, such as lycorine, narciclasine, and pancratistatin, are known for their cytotoxic and antitumor activities. However, for many structural types of alkaloids identified in *Galanthus* species, including galanthindole and graciline, information on biological activity remains limited, highlighting the need for further research in this field.

Within the genus *Galanthus* L., some species have a wide geographical distribution, while others are restricted to narrow ranges. For example, *Galanthus nivalis* L. is widely distributed across Europe, from the Pyrenees to Italy, northern Greece, Ukraine, and European Turkey, whereas *Galanthus trojanus* is considered a rare species, being reported from a single location in western Turkey (Davis and Özhatay 2001).

*Galanthus plicatus* M. Bieb. (Fig. 1a), commonly known as the pleated snowdrop, is a mesophilic geophytic species belonging to the Dobrogean–Bessarabian–Tauric–Caucasian floristic element. It is distributed in Romania (Dobrogea), the Republic of Moldova (Cantemir) (Fig. 1b), Ukraine (Cherkasy region and Crimea), the northwestern Caucasus, and northern Turkey (Dihoru and Negrean 2009). The species typically grows in hilly and submontane deciduous forests, preferring humus-rich soils with moderate moisture.

The importance of this species is ecological, economic, and scientific in nature (Kong et al. 2021). From an ecological perspective, *G. plicatus* contributes to maintaining biodiversity in forest ecosystems, being one of the earliest flowering species in spring and providing an important source of nectar and pollen for pollinating insects at the beginning of the growing season. From an ornamental standpoint, the pleated

snowdrop is valued for its elegant white flowers and is widely used in horticulture and the ornamental plant trade. However, the increasing demand for this species has led to intensified pressure on natural populations, particularly through uncontrolled harvesting of bulbs and flowers from the wild.

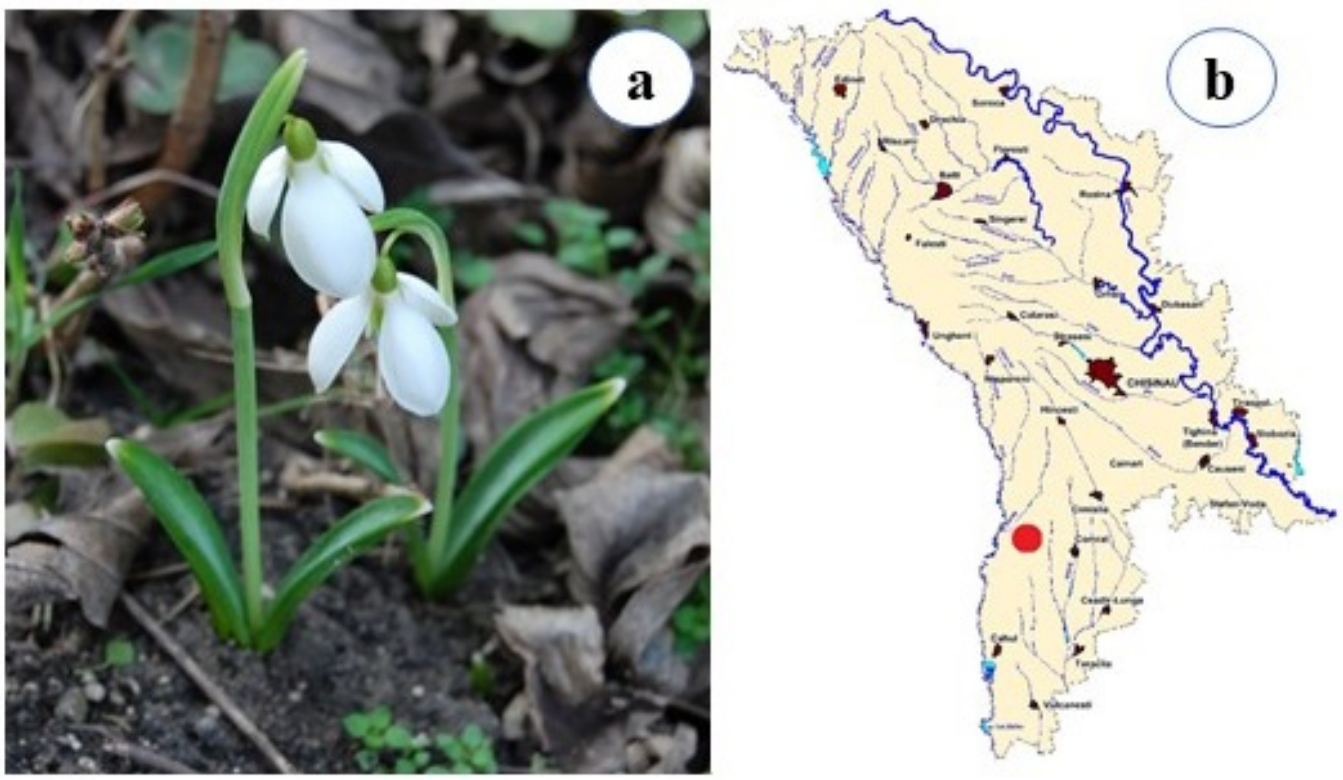


**Figure 1.** *Galanthus plicatus* M. Bieb.: (a) habit in natural habitat; (b) distribution of the species in the Republic of Moldova. Source: Prepared by the Authors.

Another important aspect is its medicinal value, as *G. plicatus* represents a natural source of galanthamine, an alkaloid used in the treatment of Alzheimer's disease and other neurological disorders. The exploitation of these biological resources has further increased the economic interest in this species, consequently raising the risk of population decline.

In general, natural populations of plant species are subject to a complex set of risk factors that contribute to reduced genetic diversity and diminished regenerative capacity. Major threats include habitat degradation caused by anthropogenic activities such as deforestation, urbanization, and agricultural expansion, as well as the effects of climate change on temperature and precipitation regimes. In addition, excessive collection from the wild, the introduction of invasive species, and ecosystem fragmentation significantly contribute to the decline of natural populations. These processes reduce genetic variability and increase species vulnerability to biotic and abiotic factors, highlighting the need for effective *in situ* and *ex situ* conservation strategies.

Currently, most species of the genus *Galanthus* L. are included in international protection lists and are

regulated under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), due to risks associated with overharvesting and uncontrolled bulb trade (International Union for Conservation of Nature 2018). Notably, according to CITES regulations, only rural communities in many countries are permitted limited harvesting and trade of three species (*G. nivalis*, *G. elwesii*, and *G. woronowii*) (Kong et al. 2021). *G. plicatus* is included in the Red Book of the Republic of Moldova, where it is classified as Critically Endangered (CR) (Red Book of the Republic of Moldova 2015).

In the Republic of Moldova, this species is recorded in a single natural location near Capaclia village (Cantemir district), where it grows in an oak forest with silver linden. The population occupies approximately 2.5 ha and is protected within the "Codrii Tigheci" Landscape Reserve at an altitude of 138 m. The forest canopy (70–80% cover) is dominated by *Quercus petraea* (Matt.) Liebl., mixed with *Tilia tomentosa* Moench, *Acer platanoides* L., *Carpinus betulus* L., *Cerasus avium*, and *Acer campestre* L. The shrub layer (30–50% cover) includes *Acer tataricum*, *Cornus mas*, *Cotinus coggygria* Scop., *Crataegus monogyna* Jacq., *Euonymus europaeus* L., *E. verrucosus* Scop, and *Viburnum lantana*. The herbaceous layer covers approximately 90% of the area. *Galanthus plicatus* M. Bieb. occurs sporadically or in small groups (abundance–dominance 1–2), accompanied by species such as *Adoxa moschatellina* L., *Aegopodium podagraria* L., *Anemonoides ranunculoides* (L.) Holub, *Anthriscus longirostris* Bertol., *Arum orientale* M.Bieb., *Asarum europaeum* L., *Asparagus tenuifolius* Lam., *Campanula rapunculoides* L., *Carex brevicollis* DC., *Carex pilosa* Scop., *Convallaria majalis* L., *Corydalis marschalliana* Pers., *C. solida* (L.) Clairv., *Euphorbia amygdaloides* L., *Ficaria verna* Huds., *Fritillaria montana* Hoppe, *Gagea lutea* (L.) Ker Gawl., *Geranium robertianum* L., *Geum urbanum* L., *Glechoma hirsuta* Waldst. and Kit., *Isopyrum thalictroides* L., *Lamium maculatum* (L.) L., *Mercurialis ovata* Sternb. et Hoppe, *Polygonatum hirtum* (Bosc. ex Poir.) Pursch, *P. multiflorum* (L.) All., *Scilla bifolia* L., *Stellaria holostea* L., *S. media* (L.) Vill., *Tulipa biebersteiniana* var. *biebersteiniana* L., *Veronica hederifolia* L., *Viola reichenbachiana* Jord., and *V. suavis* M. Bieb.

Given the restricted distribution of the species, the anthropogenic pressures on natural populations, and its ecological, ornamental, and pharmacological value, the development of efficient methods for controlled propagation becomes essential for its conservation. In this context, *in vitro* micropropagation techniques represent a promising biotechnological strategy for *ex situ* conservation of plant genetic resources and for obtaining uniform biological material required for both conservation programs (Călugăru-Spătaru et al. 2023; Brînză et al. 2025) and phytochemical research on secondary metabolites (Călugăru-Spătaru et al. 2013;

Lozinschii et al. 2025).

Therefore, the aim of this study was to develop and optimize an efficient *in vitro* propagation protocol for *Galanthus plicatus*, enabling the production of a large number of viable microbulbs and contributing to the sustainable conservation of this species of conservation concern.

The conservation of critically endangered plant species such as *Galanthus plicatus* should not be understood solely as a technical or biological challenge but also as a matter of environmental responsibility. The ongoing decline of natural populations is largely driven by anthropogenic pressures, including habitat disturbance and unsustainable harvesting, which raises important normative questions concerning the human–nature relationship. In this context, conservation efforts – particularly those aimed at reducing pressure on wild populations – can be interpreted as part of a broader ethical commitment to biodiversity protection. The development of *in vitro* propagation protocols therefore represents not only a methodological advancement but also a response to the need for more responsible and sustainable forms of interaction with fragile natural systems.

## 2. Materials and Methods

The research presented in this study was conducted in the laboratories of "Plant Biochemistry" at the Institute of Genetics, Physiology and Plant Protection of Moldova State University and "Genetics, Physiology and Plant Biochemistry" at the "Ion Creangă" State Pedagogical University of Chișinău.

The biological material used for micropropagation consisted of bulbs of *Galanthus plicatus* M. Bieb., obtained from mature and healthy plants. The bulbs were collected from the natural habitat of the species (oak forest near Capaclia village, Cantemir District, Republic of Moldova) in collaboration with the staff of the Cociulia Forestry District, Silva-Sud Cahul Forestry Enterprise, the authority responsible for the management of the area, in accordance with the national legislation on protected plant species and the principles of biodiversity conservation. This scientific collaboration was recently formalized through Cooperation Agreement No. 4 of 6 July 2026 between the Institute of Genetics, Physiology and Plant Protection of Moldova State University and the Moldsilva Agency, establishing an institutional framework for the scientific collection, *in vitro* propagation, and *ex situ* conservation of protected forest plant species. Sampling was restricted to the minimum number of bulbs required for establishing *in vitro* cultures in order to minimize any impact on the natural population. The selection of donor plants represented a critical stage of the study, with

only representative specimens exhibiting typical morphological traits and showing no signs of phytopathogenic infections or pest damage being chosen.

Considering the status of *G. plicatus* as a rare and protected species, as well as its restricted distribution, the amount of plant material used for the initiation of *in vitro* cultures was strictly limited to minimize the impact on natural populations. Under these conditions, the experimental design was adapted accordingly, with a limited number of culture media variants tested, selected based on literature data and previous experience in the *in vitro* culture of geophytic species.

The initiation of *in vitro* cultures was carried out under aseptic conditions in a laminar airflow cabinet. After sterilization, bulb explants of *G. plicatus* were inoculated onto Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962).

### 2.1. Sterilization of plant material

The sterilization of explants represented a critical step, considering the natural origin of the plant material and the high risk of contamination with epiphytic and endophytic microorganisms. In this context, a sterilization protocol was developed based on the sequential use of several disinfecting agents with complementary modes of action, avoiding the use of mercuric chloride ($HgCl_2$) due to its high toxicity.

The protocol included preliminary washing of the bulbs with detergent and running water, followed by successive treatments with amoxicillin prepared from commercially available pharmaceutical-grade tablets (250 mg/L, 30 min), folpet 800 WG fungicide (1 g/L; 30 min), a 1:1 (v/v) dilution of commercial sodium hypochlorite solution containing 5% available chlorine, 6% hydrogen peroxide (10 min), and 70% ethanol (1 min), with intermediate rinsing steps using autoclaved water. The sterilization agents used and their effects are presented in Table 1.

**Table 1.** Chemical sterilizing agents used for *G. plicatus* explants
This table presents the sterilizing agents used in the explant disinfection protocol, together with their duration of application and intended function. The protocol combines several agents with complementary effects in order to reduce microbial contamination while maintaining explant viability. The sequence shows that the procedure was designed to balance effective sterilization with the need to protect sensitive plant material of a critically endangered species.

| Sterilizing agent | Duration (min) | Effect |
|---|---|---|
| Detergent | 10 | Removes impurities and surface lipids from explants |
| 250 mg/L Amoxicillin (Antibiotic) | 30 | Inhibits bacterial growth |
| 1 g/L Folpet 800 WG (Fungicide solution) | 30 | Prevents fungal development |

| Sterilizing agent | Duration (min) | Effect |
|---|---|---|
| 2.5% available chlorine (prepared by 1:1 dilution of commercial bleach) | 15 | Acts as a strong disinfectant, destroying microorganisms |
| Autoclaved water | 5 | Removes chemical residues and reduces the risk of toxic effects on explants |
| 6% hydrogen peroxide | 10 | Degrades pathogens through oxidative action |
| 70% ethanol | 1 | Final surface sterilization |
| Autoclaved water | 5 | Removes chemical residues and reduces the risk of toxic effects on explants |

The sequential application of multiple sterilizing agents was justified by the fact that plant material collected from natural habitats is limited and valuable, and the loss of explants could compromise the entire source of donor plants. The combined use of chemical agents ensured effective disinfection through differentiated action against bacteria, fungi, and spores, maximizing explant survival and reducing the impact on natural populations.

### 2.2. Culture conditions

The inoculated explants were cultured under controlled conditions at a temperature of 20-25°C, with a photoperiod of 16 h light and 8 h dark, and a light intensity of 30-50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by fluorescent light sources.

These conditions promoted uniform culture development and the accumulation of reserve substances specific to bulbous organs.

### 2.3. Culture media for *in vitro* initiation

For the initiation of *in vitro* cultures, three variants of culture media based on Murashige and Skoog (MS) medium were tested: one standard medium and two modified variants (Table 2).

**Table 2.** Composition of standard and modified culture media for *in vitro* initiation of *G. plicatus*

This table compares the composition of the standard and modified culture media used for the initiation of *in vitro* cultures of *Galanthus plicatus*. The main differences concern the concentration of mineral salts, the addition of BAP, sucrose levels, and activated charcoal. These modifications were introduced to identify the medium most suitable for stabilizing explants and promoting the initiation of aseptic cultures.

| Nr. | Component | MS standard | MS modified (I) | MS modified (II) |
|---|---|---|---|---|
| 1. | MS: macro- and microelements, chelated Fe, vitamins | 100% | 100% | 50 % |
| 2. | Myo-inositol (mg/L) | 100 | 100.0 | 100.0 |
| 3. | Glycine (mg/L) | 2.0 | 2.0 | 2.0 |
| 4. | Nicotinic acid (mg/L) | 0.5 | 0.5 | 0.5 |

| Nr. | Component | MS standard | MS modified (I) | MS modified (II) |
|---|---|---|---|---|
| 5. | Pyridoxine HCl (mg/L) | 0.5 | 0.5 | 0.5 |
| 6. | Thiamine HCl (mg/L) | 0.1 | 0.1 | 0.1 |
| 7. | BAP (mg/L) | - | 0.5 | 0.5 |
| 8. | Sucrose (g/L) | 30 | 30 | 45 |
| 9. | Agar (g/L) | 6 | 6 | 6 |
| 10. | Activated charcoal (g/L) | - | 1 | 1 |

The pH of all media was adjusted to 5.8 prior to sterilization.

### 2.4. Culture media for microbulb induction

For microbulb induction, eight experimental variants of MS medium were tested, differing in the type and concentration of cytokinin, the presence of the auxin IBA, sucrose concentration, and supplementation with activated charcoal (Table 3). Two cytokinins were used: 6-benzylaminopurine (BAP) and N6-(2-isopentenyl)adenine (2iP), each at concentrations of 0.5, 1.0, 1.5, and 2.0 mg/L. In most variants, the auxin IBA was added at a concentration of 0.5 mg/L.

**Table 3.** Culture media used for microbulb induction and multiplication (media No. 1–8, ½ MS) and subsequent development and maturation (media No. 9–10, full-strength MS) in *G. plicatus*

This table shows the effect of different combinations of cytokinins, auxin, sucrose, and activated charcoal on microbulb induction in *Galanthus plicatus*. The results indicate a clear increase in the number of microbulbs per explant with higher concentrations of growth regulators and elevated sucrose content. The best response was obtained on medium No. 4, which produced the highest multiplication rate among all tested variants.

| No. | Medium No. | CK | CK dose (mg/L) | Auxine | Auxin dose (mg/L) | Sucrose g/L | Activated charcoal, g/L | Number of microbulbs per explant | CV (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1. | No. 1 | BAP | 0.5 | - | - | 45 | 1.0 | 2.9 ± 0.05 | 2.99 |
| 2. | No. 2 | BAP | 1.0 | IBA | 0.5 | 45 | 1.0 | 5.0 ± 0.06 | 2.08 |
| 3. | No. 3 | BAP | 1.5 | IBA | 0.5 | 45 | 1.0 | 15.0 ± 0.11 | 1.27 |
| 4. | No. 4 | BAP | 2.0 | IBA | 0.5 | 60 | 1.0 | 24.0 ± 0.43 | 3.10 |
| 5. | No. 5 | 2iP | 0.5 | -- | - | 45 | 1.0 | 5.0 ± 0.02 | 0.69 |
| 6. | No. 6 | 2iP | 1.0 | IBA | 0.5 | 45 | 1.0 | 11.0 ± 0.09 | 1.42 |
| 7. | No. 7 | 2iP | 1.5 | IBA | 0.5 | 45 | 1.0 | 19.0 ± 0.14 | 1.28 |
| 8. | No. 8 | 2iP | 2.0 | IBA | 0.5 | 60 | 1.0 | 21.0 ± 0.21 | 1.73 |
| 9. | No. 9 | - | - | IBA | 0.5 | 45 | 1.0 | Not evaluated | - |
| 10. | No. 10 | - | - | - | - | 45 | 1.0 | Not evaluated | - |

Values are presented as mean ± standard error (SE), calculated from three independent biological replicates. The coefficient of variation (CV%) was calculated as (SD/mean) × 100 and was used as a

descriptive indicator of biological variability among independent biological replicates. Variants 9 and 10 were used exclusively for the development and maturation of microbulbs and were therefore not evaluated for multiplication efficiency.

This experimental design allowed a comparative evaluation of the effects of the two cytokinins, as well as their interaction with IBA and the effects of sucrose levels on microbulb induction and development.

In modified variant II, the reduction of mineral salt concentration to half-strength MS (½ MS) aimed to decrease osmotic stress on explants, while supplementation with BAP stimulated cell division and microbulb initiation. Increased sucrose concentration provided an enhanced energy supply, and the addition of activated charcoal contributed to the adsorption of phenolic compounds and excess growth regulators, thereby reducing tissue browning.

### 2.5. Statistical analysis

Experimental data were expressed as mean ± standard error (SE), calculated from three independent biological replicates for each experimental treatment. Each biological replicate consisted of one independent culture vessel containing explants established under identical culture conditions.

The evaluated parameter was the number of microbulbs produced per explant. Descriptive statistics, including the standard deviation (SD) and the coefficient of variation (CV%), were calculated to characterize the biological variability among treatments.

Owing to the protected status of *Galanthus plicatus* and the limited availability of donor material collected under conservation constraints, the number of biological replicates was intentionally restricted. Consequently, formal inferential statistical analyses (e.g., one-way ANOVA followed by multiple comparison tests) were not performed, as the experimental design did not provide sufficient statistical power for robust hypothesis testing. Therefore, the results are presented as descriptive biological trends and interpreted in relation to the observed morphogenetic responses.

The calculated coefficients of variation (CV%) ranged from 0.69 to 3.10%, indicating very low biological variability among biological replicates and supporting the reproducibility of the developed protocol.

## 3. Results and Discussion

Micropropagation of species belonging to the genus *Galanthus* L. has been extensively investigated in previous studies, demonstrating the efficiency of *in vitro* cultures as a reliable method for rapid plant

multiplication and *ex situ* conservation of genetic resources. Tipirdamaz, Ellialtıoğlu, and Çakırlar (1999) highlighted the influence of explant type, carbohydrate source, and medium pH on regeneration in *G. ikariae*. Subsequently, Tilly-Mandy et al. (2006) and Staikidou et al. (2006) emphasized the essential role of growth regulators and sucrose concentration in stimulating bulblet formation. Efficient protocols for *G. woronowii* were developed by Malyarovskaya et al. (2018), while Asadi et al. (2021) investigated the stability of regenerated material and the risk of somaclonal variation. Overall, these studies confirm that the success of micropropagation depends on the optimization of initiation, multiplication, and regeneration stages, a conclusion also supported by the results obtained in the present study for *G. plicatus*.

To optimize the micropropagation protocol, several variants of Murashige and Skoog (MS) medium were evaluated, differing in the type and concentration of growth regulators and in sucrose levels. The response of explants was analyzed based on the number of microbulbs generated per explant and the morphological characteristics of the regenerated structures.

### 3.1. Initiation of *in vitro* cultures and the importance of explant sterilization

The limitation in the number of experimental variants tested during the initiation stage was determined by the need for responsible use of plant material, given the conservation status of *G. plicatus*. The adopted approach aimed to minimize the impact on natural populations while maintaining experimental relevance through the selection of variants based on data from scientific literature.

For species of the genus *Galanthus* L., explants commonly used in *in vitro* cultures include whole bulbs, bulb scales, and apical or lateral meristems. These types of explants are characterized by the presence of active meristematic tissues with high cell division and regeneration capacity, which favors the induction of organogenesis and the formation of bulbous structures under controlled culture conditions. The use of bulbs or bulb scales as initial material has been reported in micropropagation studies of *Galanthus* species due to their high regenerative potential and their capacity for efficient bulblet formation under *in vitro* conditions (Staikidou et al. 2006; Tilly-Mandy et al. 2006; Malyarovskaya et al. 2018).

In the present study, the bulbs introduced into culture were monitored after inoculation to evaluate explant viability and adaptation to the artificial medium. The initiation of *in vitro* cultures required the application of a rigorous sterilization protocol, as plant material originating from natural habitats often carries a high microbial load due to epiphytic and endophytic microorganisms. Microbial contamination is one of the

main causes of failure in *in vitro* cultures, necessitating the use of effective decontamination procedures (George et al. 2008).

The developed protocol included the sequential application of several disinfecting agents (Table 1), ensuring efficient elimination of microorganisms while maintaining tissue viability. The effectiveness of the sterilization protocol was evaluated before the comparative assessment of the initiation media, based on the percentage of explants that remained both sterile and viable after inoculation. Across all three initiation media, 80–95% of the explants remained free from contamination and viable during the culture establishment phase, indicating that this response primarily reflected the efficiency of the sterilization procedure rather than differences among the culture media. These results confirm the suitability of the developed sterilization protocol for the successful establishment of *G. plicatus in vitro* cultures.

An important aspect of the developed protocol is the exclusion of mercuric chloride ($HgCl_2$), a commonly used sterilant in plant tissue culture but characterized by high toxicity and phytotoxic potential. Its replacement with less aggressive disinfecting agents contributes to reducing the toxicological impact of the procedure and improving the sustainability of the method, which is particularly relevant for rare or protected species (Cassells 2012).

Following successful establishment, the responses of explants cultured on the different initiation media were subsequently evaluated according to their morphogenetic development and culture stabilization. After inoculation, the explants exhibited a whitish-yellow coloration characteristic of the initial stages of cellular proliferation (Fig. 2).

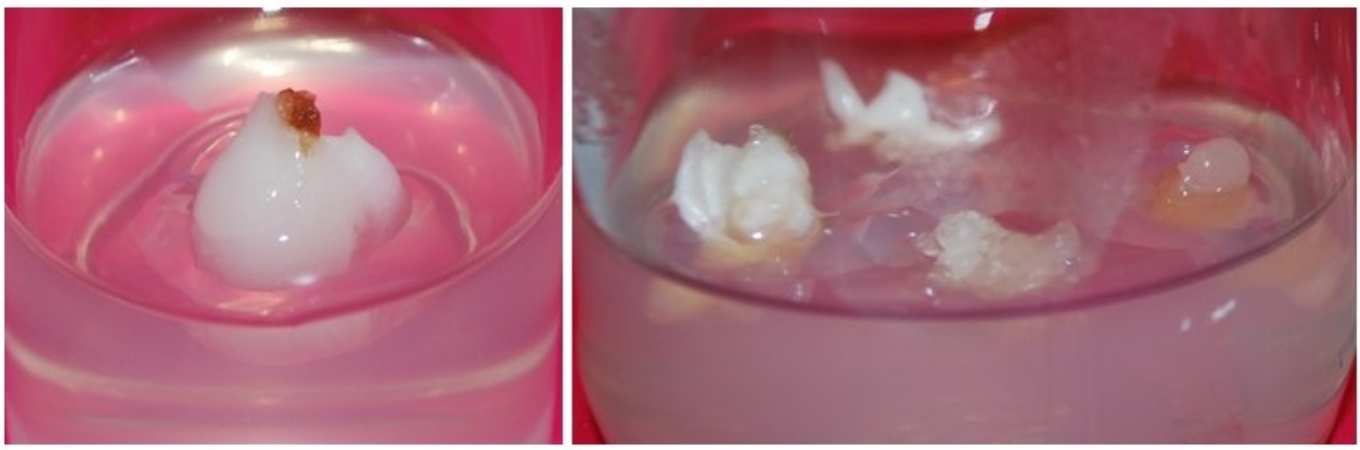

**Figure 2.** Bulb explants of *Galanthus plicatus* cultured on modified half-strength MS (½ MS) medium (variant II) during the initiation stage.

In some cases, slight browning was observed at the level of apical tissues, a phenomenon associated

with the oxidation of phenolic compounds released by explants in response to mechanical and chemical stress generated during sterilization and transfer to artificial culture media. Phenolic oxidation is a common occurrence in *in vitro* cultures of plants originating from natural habitats and may inhibit explant growth if not properly controlled (Thomas 2008).

To reduce this effect, the culture media were supplemented with activated charcoal (Table 2), an additive known for its role in stabilizing *in vitro* cultures. Activated charcoal has the ability to adsorb phenolic compounds released by explants, as well as excess growth regulators from the culture medium (Thomas 2008), thereby contributing to the maintenance of favorable conditions for explant development. As a result, the use of this additive reduced tissue browning and maintained culture viability during the initiation phase.

Comparative analysis showed that half-strength MS (½ MS) medium supplemented with 0.5 mg/L BAP, 45 g/L sucrose, and 1 g/L activated charcoal provided optimal conditions for culture stabilization. The reduction in mineral salt concentration decreased osmotic stress, while BAP stimulated meristematic activity.

Overall, the obtained results highlight the importance of optimizing both the sterilization protocol and the composition of the culture medium for the successful initiation of *in vitro* cultures of *G. plicatus*. The application of an efficient decontamination procedure, combined with the use of phenolic compound adsorbents, represents a key factor in culture stabilization and the subsequent development of an efficient micropropagation system.

### 3.2. Microbulb induction and multiplication

Microbulb induction represents a critical stage in the micropropagation of geophytes, as these bulbous structures serve as storage organs capable of supporting plant regeneration and subsequent adaptation to *ex vitro* conditions. Microbulb formation is directly influenced by the composition of the culture medium, particularly the type and concentration of growth regulators, as well as the level of the carbon source available in culture.

In the present study, several variants of Murashige and Skoog (MS) medium were evaluated, differing in the type of cytokinin used (BAP or 2iP), the presence of the auxin IBA, and the sucrose concentration added to the culture medium. The aim of these experiments was to identify the optimal combination of factors that stimulate microbulb induction and proliferation in *G. plicatus*.

Within the series of media supplemented with 6-benzylaminopurine (BAP), a progressive increase in

the number of microbulbs produced per explant was observed with increasing cytokinin concentration (Fig. 3). At a concentration of 0.5 mg/L BAP, an average of 2.9 ± 0.05 microbulbs per explant was obtained (medium No. 1) (Fig. 3a), indicating moderate meristematic activity in the absence of auxin. The addition of indole-3-butyric acid (IBA) at 0.5 mg/L promoted the morphogenetic response. Thus, the medium supplemented with 1.0 mg/L BAP and 0.5 mg/L IBA produced 5.0 ± 0.06 microbulbs per explant (medium No. 2). A further increase in BAP concentration to 1.5 mg/L (medium No. 3), while maintaining the same auxin level, led to enhanced proliferation, generating 15.0 ± 0.11 microbulbs per explant (Fig. 3b). The highest multiplication rate was recorded on medium supplemented with 2.0 mg/L BAP + 0.5 mg/L IBA and 60 g/L sucrose (medium No. 4), where 24.0 ± 0.43 microbulbs per explant were obtained (Fig. 3c), indicating intensified meristematic activity and organogenetic processes.

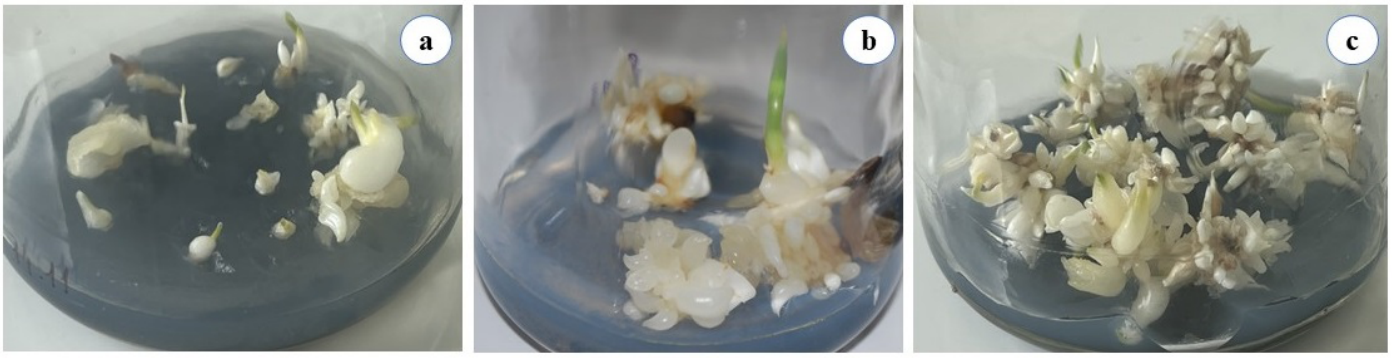


**Figure 3.** Microbulb induction of *Galanthus plicatus* on MS media supplemented with BAP: (a) 0.5 mg/L; (b) 1.5 mg/L; (c) 2.0 mg/L.

A comparable response was observed in media supplemented with N6-(2-isopentenyl)adenine (2iP). Thus, the medium containing 0.5 mg/L 2iP generated 5.0 ± 0.02 microbulbs per explant (medium No. 5), while increasing the concentration to 1.0 mg/L 2iP + 0.5 mg/L IBA resulted in the formation of 11.0 ± 0.09 microbulbs per explant (medium No. 6). At a concentration of 1.5 mg/L 2iP + 0.5 mg/L IBA, 19.0 ± 0.14 microbulbs per explant were obtained (medium No. 7), whereas on medium supplemented with 2.0 mg/L 2iP + 0.5 mg/L IBA and 60 g/L sucrose, 21.0 ± 0.21 microbulbs per explant were formed (medium No. 8). Although the highest overall multiplication rate was obtained with 2.0 mg/L BAP, 2iP produced higher microbulb numbers than BAP at the lower and intermediate cytokinin concentrations tested.

The presented data reveal clear trends of increasing multiplication rates with rising cytokinin concentrations and in the presence of the auxin IBA, as well as a favorable effect of elevated sucrose concentrations on microbulb formation. Although inferential statistical analysis was not the primary objective

of this study, the observed differences among variants reflect distinct biological responses depending on the composition of the culture medium.

The differences between the two cytokinins can be explained by their distinct physiological mechanisms. BAP is known for its pronounced mitogenic effect on meristematic cells, stimulating cell division and the initiation of organogenetic structures. In contrast, 2iP exhibits an action closer to the natural hormonal balance of plants, which may promote more uniform tissue differentiation, albeit with a slightly lower numerical rate (George et al. 2008).

The role of the auxin IBA in stimulating organogenesis was evident in most experimental variants. A comparison between media without auxin and those supplemented with 0.5 mg/L IBA suggests that the cytokinin–auxin interaction promoted a higher rate of microbulb proliferation. This observation is consistent with previous studies, which identify the balance between cytokinins and auxins as a key factor regulating organogenesis and regeneration processes (George et al. 2008; Cassells 2012).

Another major factor influencing microbulb induction was sucrose concentration. Increasing its level from 45 g/L to 60 g/L was associated with the highest multiplication rates. Sucrose plays an essential role in *in vitro* cultures of geophytes, as it represents the primary carbon source for explant metabolism and acts as an osmotic factor promoting carbohydrate accumulation and the formation of storage organs (Thomas 2008).

Comparison of the present results with those reported for other *Galanthus* species indicates that the observed trends are consistent with previous studies. Tipirdamaz, Ellialtıoğlu, and Çakırlar (1999) demonstrated that high sucrose concentrations (approximately 60 g/L) significantly stimulated *in vitro* bulblet formation in *G. ikariae*. Similarly, Staikidou, Selby, and Hanks (2006) reported that activated charcoal played an important role in promoting bulblet growth in *Galanthus* species cultured *in vitro*, and that its combination with elevated sucrose concentrations significantly enhanced the development of bulbous structures.

Comparable results were also reported for *G. woronowii*, where Malyarovskaya et al. (2018) showed that media supplemented with BAP can stimulate explant regeneration, although the reported multiplication rate was lower than that obtained in the present study. This difference may reflect species-specific biological characteristics of *G. plicatus*, as well as the optimization of the hormonal combinations used.

Similar trends have been observed in other ornamental bulbous geophytes. Studies on the genus *Narcissus* showed that culture media supplemented with cytokinins in combination with auxins promoted

meristematic proliferation and *in vitro* regeneration of vegetative structures (Santos et al. 2002). Comparable findings were reported for *Hyacinthus orientalis*, where the interaction between auxins and cytokinins in MS medium significantly influenced morphogenesis and the development of regenerated plantlets (El-Naggar et al. 2023). Likewise, studies on other bulbous geophytes, such as species of *Lilium*, demonstrated that high sucrose concentrations (45-60 g/L) stimulate bulbil formation and development *in vitro* by promoting carbohydrate accumulation required for storage organ formation (Azadi and Khosh-Khui 2007).

An important role in culture stability was also attributed to activated charcoal, which can adsorb phenolic compounds released by explants and excess growth regulators from the culture medium. Through this action, activated charcoal contributes to culture stabilization and prevents tissue browning, a phenomenon frequently encountered in *in vitro* cultures of geophytes (Thomas 2008).

Overall, the obtained results indicate that microbulb formation in *G. plicatus* is optimally stimulated by hormonal combinations based on 2.0 mg/L cytokinin (BAP or 2iP), 0.5 mg/L IBA, and high sucrose concentrations (60 g/L). Compared to other species of the genus *Galanthus* L. and other bulbous geophytes cultivated *in vitro*, the multiplication rate obtained in the present study can be considered high, confirming the efficiency of the developed protocol and its relevance for rapid multiplication and *ex situ* conservation of the studied species.

### 3.3. Microbulb maturation stages

Following the multiplication stage, the microbulbs were transferred to culture media intended for bulb development and physiological maturation. This phase aimed to promote bulb enlargement and the accumulation of reserve compounds required for subsequent plantlet development. Explants were cultured for approximately 8 weeks on each medium variant.

The regenerated microbulbs were first transferred to full-strength Murashige and Skoog (MS) medium supplemented with 0.5 mg/L indole-3-butyric acid (IBA) and 45 g/L sucrose (medium No. 9), which promoted further bulb development. Subsequently, the microbulbs were cultured on hormone-free MS medium containing 45 g/L sucrose (medium No. 10), where bulb maturation and reserve accumulation were completed. Because these media were designed to promote development and maturation rather than further proliferation, the number of microbulbs was not recorded for variants No. 9 and No. 10.

Morphological observations performed after approximately eight weeks of culture revealed that the

regenerated microbulbs developed a compact ovoid-to-spherical morphology with creamy-white storage tissues (Fig. 4). Most microbulbs produced one or two green leaf primordia, while root initiation or early root development was observed in several individuals. The regenerated bulbs remained morphologically uniform and showed no obvious symptoms of hyperhydricity or tissue necrosis, indicating successful maturation under the applied culture conditions.

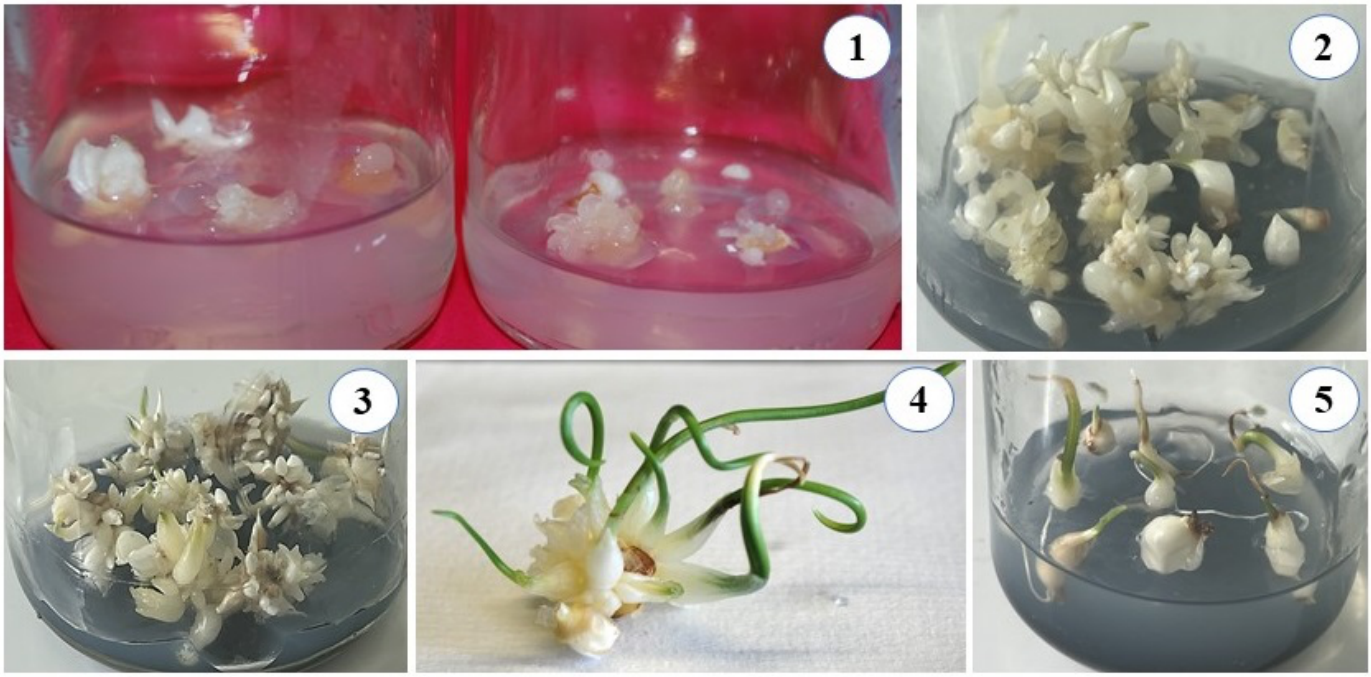


**Figure 4.** Successive stages of *in vitro* micropropagation of *Galanthus plicatus*: (1) initiation and establishment of bulb explants on ½ MS medium supplemented with 0.5 mg/L BAP; (2) early microbulb induction; (3) microbulb proliferation; (4) microbulb development and maturation; (5) mature *in vitro*-derived microbulbs ready for acclimatization and *ex vitro* establishment.

The relatively high sucrose concentration (45 g/L) was considered to support carbohydrate accumulation and the development of storage tissues, whereas the gradual reduction and subsequent elimination of plant growth regulators facilitated physiological maturation of the microbulbs. The sequential culture strategy, consisting of multiplication on cytokinin-containing media followed by development and maturation on media with reduced or no growth regulators, enabled the production of vigorous microbulbs suitable for subsequent acclimatization.

This approach allows the rapid production of planting material from a limited number of initial explants and is therefore particularly valuable for the *ex situ* conservation of rare and protected plant species.

### 3.4. Genetic fidelity of regenerated material

Although the present protocol is based predominantly on direct organogenesis from bulb explants,

which is generally associated with a lower risk of somaclonal variation than regeneration through a callus phase, the possibility of genetic or epigenetic alterations during prolonged *in vitro* culture cannot be completely excluded. Cytokinins, particularly 6-benzylaminopurine (BAP), are known to promote intensive cell division and microbulb proliferation, but prolonged exposure or repeated subculturing may increase the likelihood of somaclonal variation. Since the regenerated material is intended for *ex situ* conservation, long-term germplasm preservation, and potential future reintroduction into natural populations, verification of genetic fidelity is recommended before its practical application. Future studies should therefore assess the genetic stability of regenerated plants using molecular markers such as ISSR or SSR, complemented, where appropriate, by flow cytometry to confirm genome stability and clonal uniformity. Such analyses would provide additional validation of the protocol for conservation-oriented applications.

### 3.5. Significance of the results for species conservation

The results obtained in the present study indicate that *in vitro* micropropagation provides a promising biotechnological tool for the multiplication and *ex situ* conservation of *Galanthus plicatus*. Given the restricted distribution of the species, its ecological, ornamental, and pharmaceutical value, and the pressures affecting its natural populations, the development of an efficient protocol for controlled propagation is of considerable practical importance for the conservation and sustainable use of its genetic resources.

Beyond its technical value, the proposed protocol should be viewed within a broader framework of environmental responsibility. *Ex situ* conservation is not intended to replace the protection of natural habitats or the ecological processes that sustain wild populations. Rather, it constitutes a complementary conservation strategy that may reduce anthropogenic pressure on endangered species while supporting their long-term survival. In this perspective, plant biotechnology serves not merely as a technological tool but as an instrument for implementing responsible stewardship of biodiversity. Its application should therefore remain closely linked to the protection of ecosystem integrity and to the ethical obligation to preserve biological diversity for present and future generations.

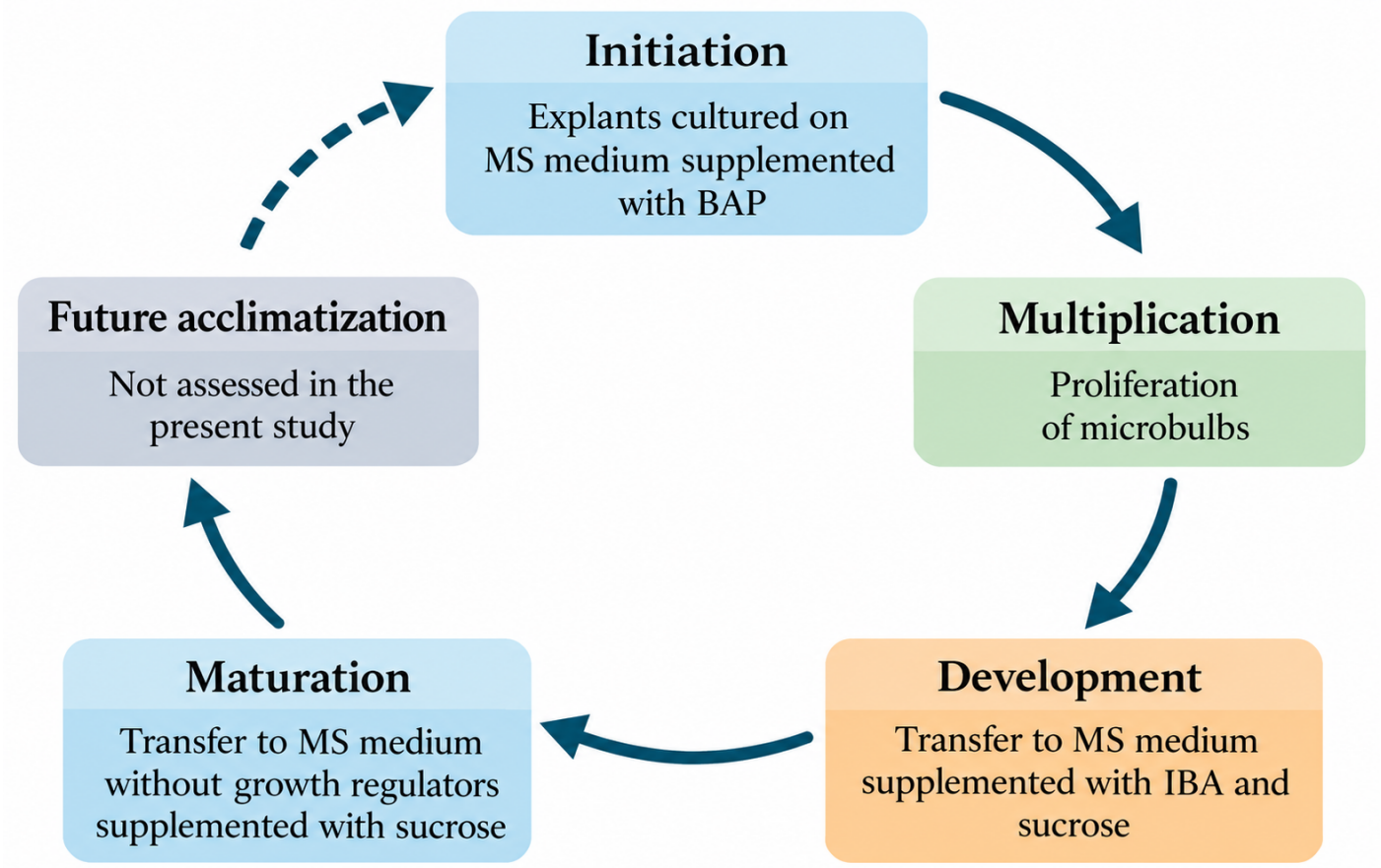


**Figure 5.** Schematic overview of the *in vitro* micropropagation protocol developed for *Galanthus plicatus*, based on the experimental results of the present study. The final acclimatization stage is shown as a future application and was not assessed in this study. Source: Prepared by the Authors; adapted for clarity, accessibility, and visual quality by the SEeB Editorial Team with the assistance of artificial intelligence.

The proposed protocol enables the rapid production of a large number of viable microbulbs from a limited amount of initial plant material. These microbulbs may provide a valuable source of biological material for *ex situ* conservation collections, phytochemical investigations, and studies of secondary metabolites, including alkaloids with therapeutic potential. The sequence of multiplication, development, and maturation stages summarized in Figure 5 demonstrates the capacity of the culture system to produce well-differentiated microbulbs of *G. plicatus* under controlled conditions.

By reducing the need to collect additional plant material from natural habitats, this approach may contribute to limiting pressure on wild populations and supporting the conservation of the species' genetic resources. Although acclimatization and *ex vitro* establishment were not evaluated in the present study, the regenerated microbulbs represent promising material for future acclimatization experiments, horticultural cultivation, phytochemical research, and, following appropriate validation, controlled reintroduction programmes.

Before the regenerated material is used in long-term conservation, gene banking, or restoration programmes, its genetic fidelity should be verified using suitable molecular markers, such as ISSR or SSR, and, where appropriate, flow cytometry. Such analyses are required to confirm clonal uniformity and to exclude relevant genetic or genomic alterations potentially associated with repeated subculturing and cytokinin exposure.

Considering that *G. plicatus* is represented by a single confirmed natural population in the Republic of Moldova and is classified nationally as Critically Endangered, the present protocol provides a valuable scientific and practical basis for future *ex situ* conservation, ecological restoration, and sustainable propagation programmes aimed at safeguarding the genetic resources of this species.

The study also contributes to the objectives of the United Nations Sustainable Development Goal 15—Life on Land—by supporting the conservation of threatened plant diversity and the long-term protection and sustainable management of plant genetic resources.

## 4. Conclusions

The present study demonstrates that *in vitro* micropropagation provides an efficient, reproducible, and environmentally responsible approach for the rapid multiplication and *ex situ* conservation of the critically endangered species *Galanthus plicatus* M. Bieb.

The optimized sterilization protocol, based on the sequential application of multiple disinfecting agents and the exclusion of mercuric chloride, ensured a high percentage of sterile and viable explants (80–95%), providing a reliable basis for culture establishment while reducing the environmental impact associated with conventional sterilization procedures.

Among the culture media evaluated, half-strength MS medium supplemented with 2.0 mg/L BAP, 0.5 mg/L IBA, and 60 g/L sucrose proved to be the most effective for microbulb induction, yielding up to 24.0 ± 0.43 microbulbs per explant. The results also confirmed the importance of cytokinin–auxin interactions and elevated sucrose concentrations for successful organogenesis, bulb development, and physiological maturation.

The developed protocol enables the production of vigorous and morphologically uniform microbulbs from a limited amount of starting material, an important advantage for species with restricted natural populations where destructive sampling must be minimized.

Although acclimatization and genetic fidelity were beyond the scope of the present study, these aspects represent essential next steps before large-scale conservation or reintroduction programmes can be implemented. Future studies should evaluate the genetic fidelity of regenerated plants using appropriate molecular markers (e.g., ISSR or SSR) and, where appropriate, flow cytometry to confirm clonal fidelity before their application in conservation programmes.

Although the present study focuses on the development of a biotechnological propagation protocol, its broader significance lies in supporting conservation practices that reconcile scientific innovation with environmental responsibility. The preservation of critically endangered plant species cannot rely exclusively on technological solutions; it also requires a long-term commitment to protecting natural habitats, maintaining ecological processes, and promoting responsible human interaction with the living world. In this context, *ex situ* propagation should be understood as one component of an integrated conservation strategy rather than an alternative to in situ protection.

Overall, the protocol developed in this study provides a practical biotechnological framework for the conservation, sustainable propagation, and future restoration of *Galanthus plicatus*. Beyond its application to this species, the proposed methodology may also serve as a useful framework for the *ex situ* conservation of other rare and threatened geophytic species.

## Statements

### Author Contributions:

Conceptualization, M.L. and T.C.S.; methodology, M.L., T.C.S. and L.B.; investigation, M.L., T.C.S., L.B., E.C.; resources, P.P., E.C.; data curation, M.L., T.C.S., L.B.; writing-original draft preparation, M.L., T.C.S., L.B.; writing-review and editing, M.L., T.C.S., L.B., E.C., N.M.; visualization, M.L., T.C.S. and L.B. All authors have read and agreed to the published version of the manuscript.

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### Institutional Review Board Statement:

Not applicable.

**Permits for Collection of Plant Material**

Bulbs of *Galanthus plicatus* M. Bieb., a protected species in the Republic of Moldova, were collected from the natural population near Capaclia village, Cantemir District, in collaboration with the Cociulia Forestry District of the Silva-Sud Cahul Forestry Enterprise. Collection was conducted in accordance with applicable national legislation and under the authorization of Moldova State University. Sampling was limited to the minimum amount of plant material necessary to establish *in vitro* cultures and to minimize any impact on the natural population. The research was conducted within the institutional cooperation framework established by Cooperation Agreement No. 4 of 6 July 2026 between the Institute of Genetics, Physiology and Plant Protection of Moldova State University and the Moldsilva Agency.

**Data Availability Statement:**

Not applicable.

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**AI Tools Declaration:**

The authors declare that no AI tools were used in the preparation of this manuscript.

**Conflicts of Interest:**

The authors declare no conflicts of interest.

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