Optimization of conditions for microclonal propagation of *SALVIA PRATENSIS* L.

I.E. Buchenkov[1], *V.O.* Lemiasheuski[1,*], *A.G.* Sysa[1], *Sui* Xin[2], *Yang* Liu [2], *Song* Zengyi[3]

[1]Polessky State University, Pinsk, Republic of Belarus
[2]Heilongjiang University, Harbin, China
[3]International Sakharov Environmental Institute of Belarusian State University, Minsk, Belarus

Abstract. The possibility of introducing *Salvia pratensis*, a protected medicinal plant of the Republic of Belarus, into in vitro culture for mass reproduction and return to natural ecosystems, as well as practical use as medicinal plant raw materials, has been studied. A sterilization regime for *Salvia pratensis* when introducing it into culture has been identified, which provides the best ratio of the number of sterile plant explants and the number of viable seeds. The optimal sterilizing agent is 0.1% silver nitrate solution with an exposure time of 20 minutes. The effect of various concentrations of gibberellic acid over a certain period of time on *Salvia pratensis* seeds has been identified. It has been established that a 10% solution of gibberellic acid for one hour has the greatest effect on seed germination for *Salvia pratensis*. Nutrient media have been selected for in vitro cultivation of *Salvia pratensis* plants. The optimal nutrient medium for *Salvia pratensis* is MR_{20}.

1 Introduction

Restoring populations of rare and protected plant species remains a priority in modern ecology and biotechnology, particularly under increasing anthropogenic pressure, habitat fragmentation, and climate change. For many endangered species, traditional propagation and reintroduction methods prove insufficient due to low seed productivity, stringent growth requirements, or physiological barriers such as seed dormancy. In this context, biotechnological approaches, particularly *in vitro* microclonal propagation, play a pivotal role, enabling not only biodiversity conservation but also the creation of depository collections and scalable production of bioactive compounds with pharmacological potential [1, 2].

One such species requiring urgent conservation measures is meadow sage (*Salvia pratensis* L.), a relict forest-steppe species listed in the Red Book of Belarus (NT category) and located at the northern edge of its range. Its primary populations in southeastern regions of Belarus (Gomel Oblast) inhabit dry sparse forests, coastal slopes, and anthropogenic landscapes, where they face threats from agricultural activities and ecosystem degradation [8]. Beyond its ecological role, *S. pratensis* is of significant interest as a source of

* Corresponding author: lemeshonak@mail.ru

secondary metabolites, including flavonoids (apigenin, rutin, quercetin), phenolic acids (chlorogenic, ferulic, gallic), and coumarins, which exhibit antioxidant, anti-inflammatory, antibacterial, and hepatoprotective activities [3, 4]. These properties position the species as a promising candidate for medical, pharmaceutical, and breeding biotechnology.

Despite its value, *S. pratensis* micropropagation remains understudied. Unlike other *Salvia* species such as *S. officinalis* [5], *S. miltiorrhiza* [6], and *S. sclarea* [7], which have well-established *in vitro* protocols, research on *S. pratensis* is fragmented. An analysis of Scopus and Web of Science publications (2015–2023) confirms a lack of systematic data on key aspects of its biotechnological cultivation, including:

1. Optimization of seed sterilization, complicated by a dense seed coat and essential oils that inhibit germination.
2. Overcoming physiological and mechanical seed dormancy, requiring the selection of effective growth regulators such as gibberellic acid.
3. Development of nutrient media that balance shoot growth and root formation while accounting for species-specific traits of *S. pratensis*.

The aim of this study is to develop and optimize a protocol for *in vitro* microclonal propagation of *Salvia pratensis* L., applicable for both species conservation and industrial-scale production of bioactive compounds. To achieve this goal, the following objectives were addressed:

– Comparative analysis of sterilizing agents (silver nitrate, Lysoformin 3000, Belizna, Chloramine B, Biocide-S) for obtaining sterile explants.

– Investigation of the effects of gibberellic acid (GA) on seed germination, varying concentration (1%, 10%, 50%, 100%) and exposure time (1, 3, 6, 12 h).

– Evaluation of plant growth and morphogenesis on modified Murashige-Skoog media (MR20, MR1/2, MR0) supplemented with phytohormones.

This work advances methods for conserving rare *Salvia* species by demonstrating the unique biotechnological approaches required for *S. pratensis*, considering its eco-physiological traits, chemical composition, and pharmacological potential.

2 Materials and Methods

2.1 Study Object

The study object comprised seeds of meadow sage (*Salvia pratensis* L.), collected during mass ripening (July–August 2022) from natural populations in the Gomel Region, Belarus (coordinates: 52°24′34″N, 30°58′21″E; Buda-Koshelevo district). To minimize genetic heterogeneity, the sample included 500 seeds selected from 10 populations.

2.2 Collection and Preparation of Plant Material

Seeds were collected in dry, clear weather between 10:00 and 14:00. Mature seed-bearing inflorescences were carefully separated from plants, placed in sterile paper bags, and labeled. After collection, seeds were cleaned of plant debris and dried in a climate chamber (Binder KBWF 240) at 25 ± 1°C, 40 ± 5% relative humidity, and diffused light for 72 hours. The raw material was spread in a 1–2 cm layer on filter paper and stirred every 6 hours to ensure uniform drying. Dried seeds were stored in sealed glass containers at 4°C in darkness until experiments commenced.

2.3 Sterilization of Explants

All manipulations were performed in a Lamsystems A2 laminar flow hood (Class II). Sterilization followed a five-step protocol:

1. Pre-cleaning: Seeds were washed in a 1% liquid soap solution (Neutral, Merck) for 10 minutes, followed by three rinses with distilled water.
2. Degreasing: Immersion in 70% ethanol for 1 minute.
3. Primary sterilization: Treatment with one of five agents for 20 minutes at room temperature (Table 1).
4. Rinsing: Three washes with sterile distilled water (5 minutes each).
5. Sterility control: 10% of seeds from each group were incubated on LB (Luria-Bertani) agar medium at 28°C for 7 days. Explants with no visible microbial growth were deemed sterile.

Table 1. Sterilizing Agents and Their Concentrations

Sterilizing Agent	Concentration	Manufacturer
Lysoformin 3000	10%	LLC "Gigiena Plus"
Belizna	5–15%	JSC "BSC"
Biocide-S	3%	LLC "Yugo-Zapad-Khimprom"
Chloramine B	5%	LLC "Biochim Rus"
Silver nitrate	0.1%	Sigma-Aldrich

2.4 Gibberellic Acid (GA) Treatment

A stock solution of GA (1000 mg/L) was prepared by dissolving gibberellic acid (Sigma-Aldrich ≥ 90%) in sterile distilled water, followed by filtration through a 0.22 μm membrane (Millipore). Working concentrations (1%, 10%, 50%, 100%) were obtained via serial dilution. Sterile seeds were soaked in GA solutions at 23 ± 1°C in darkness. Exposure times were 1, 3, 6, and 12 hours. The control group was treated with sterile distilled water. Each experimental variant was replicated five times (biological replicates), with 20 seeds per replicate (technical replicates).

2.5 *In Vitro* Cultivation

Sterile seeds were placed in Petri dishes (Ø 90 mm) containing 30 mL of Murashige-Skoog (MS) agar medium without phytohormones for germination. Seedlings were transferred to modified MS media:

1. MR20: Full-strength MS + 0.5 mg/L IAA (indole-3-acetic acid) + 1 mg/L BAP (6-benzylaminopurine) + 20 g/L sucrose.
2. MR1/2: Half-strength macro- and microelements of MS + 0.5 mg/L IAA + 1 mg/L BAP + 10 g/L sucrose.
3. MR0: MS without phytohormones + 20 g/L sucrose.

Cultivation Conditions:

1. Germination stage: 14 days in darkness at 23 ± 1°C.
2. Growth stage: 16/8-hour light/dark photoperiod, 50 μmol/m²/s light intensity (LED lamps), 24 ± 1°C temperature, and 60–70% humidity. Subculturing to fresh medium was performed every 40 days.

2.6 Growth Parameter Evaluation

After 30 days of cultivation, the following parameters were measured:
1. Shoot and root length (mm): Measured using a digital caliper.
2. Leaf and root count: Number of fully developed leaves and roots per plantlet.
3. Vitrification frequency [14]: Percentage of plants exhibiting hyperhydric (glassy) tissues.
4. Survival rate (%): Proportion of viable plantlets relative to initial explants.

3 Results and discussion

3.1 Efficacy of Sterilizing Agents

The results of treating *Salvia pratensis* seeds with various sterilizing agents are presented in Table 2. The highest proportion of sterile explants (93.63 ± 1.35%) while retaining viability (54.25 ± 1.07%) was achieved using a 0.1% silver nitrate ($AgNO_3$) solution. This aligns with findings for other *Salvia* species, such as *S. plebeia* [9] and *S. officinalis* [5], where silver nitrate effectively suppressed pathogens without significant toxicity to embryos. The efficacy of $AgNO_3$ is likely due to its ability to penetrate the dense seed coat of *S. pratensis* without damaging the meristem. However, the exposure time (20 minutes) proved critical, as exceeding it reduced viability.

Lysoformin 3000 (10%) achieved the highest sterility (96.85 ± 1.27%), but all seedlings died, likely due to the toxicity of glutaraldehyde, which disrupts cell membranes, as demonstrated for *S. miltiorrhiza* [10]. Belizna (5–15%), Chloramine B (5%), and Biocide-S (3%) showed moderate sterility (70.25 ± 1.06%, 59.75 ± 1.55%, and 61.13 ± 1.35%, respectively) but low viability (13.50 ± 0.92%, 24.47 ± 0.74%, and 35.74 ± 0.86%, respectively), likely due to residual chlorine inhibiting seed metabolism.

Table 2. Effect of Sterilizing Agents on Sterility and Viability of *S. pratensis* Explants

Sterilizing Agent	Sterile Explants, %	Viable Explants, %
Lysoformin 3000 (10%)	96.85 ± 1.27	0.00 ± 0.00
Belizna (5–15%)	70.25 ± 1.06	13.50 ± 0.92
Chloramine B (5%)	59.75 ± 1.55	24.47 ± 0.74
Biocide-S (3%)	61.13 ± 1.35	35.74 ± 0.86
Silver nitrate (0.1%)	93.63 ± 1.35	54.25 ± 1.07

3.2 Effect of Gibberellic Acid (GA) on Seed Germination

GA treatment significantly increased the germination rate of *S. pratensis* seeds (Table 3). The optimal regimen – 10% GA with a 1-hour exposure-yielded 91.88 ± 2.94% germination, 1.7 times higher than the control (54.75 ± 1.85%). This effect is likely due to the activation of *GA3ox* genes, which encode enzymes responsible for synthesizing active gibberellins that accelerate endosperm degradation and overcome physiological seed dormancy [11]. However, extending the exposure time to 12 hours reduced efficacy to 67.63 ± 1.59% (10% GA), consistent with reports of GA phytotoxicity under prolonged exposure, which induces oxidative stress, as observed in *S. miltiorrhiza* [12].

The 1% GA concentration exhibited a non-linear response: germination was inhibited at 1 hour (31.13 ± 3.53%) but stimulated at 3–12 hours (78.62 ± 3.98%–82.25 ± 2.12%). This may reflect dose-dependent amylase activation at low concentrations and suppression at higher levels, as described for *Arabidopsis thaliana* [11]. The native GA solution (100

µg/mL) showed minimal efficacy (32.50 ± 3.87% at 12 hours), underscoring the need for standardized GA concentrations in future studies.

Table 3. Effect of GA on Germination of *S. pratensis* Seeds (1 Hour Exposure)

GA Concentration	Germinated Seeds, %
Control (Water)	54.75 ± 1.85
1%	31.13 ± 3.53
10%	91.88 ± 2.94
50%	72.13 ± 3.55
100%	51.75 ± 2.62

3.3 Growth and Morphogenesis on Nutrient Media

The growth and morphogenesis of *S. pratensis* seedlings on modified media are presented in Table 4 and Figures 1–2. The MR20 medium yielded the best growth: after 30 days, shoot length reached 3.2 ± 0.5 cm, leaf count was 6 ± 1, and root length was 4.5 ± 0.7 cm. The high sucrose concentration (20 g/L) and presence of phytohormones (0.5 mg/L IAA and 1 mg/L BAP) promoted active morphogenesis, consistent with findings for *S. officinalis*, where sucrose stimulated phenolic compound synthesis [2].

Table 4. Growth and Morphogenesis of *S. pratensis* Seedlings on Modified Media (30 Days)

Medium	Shoot Length, cm	Leaf Count	Root Length, cm	Vitrification Frequency, %
MR20	3.2 ± 0.5	6 ± 1	4.5 ± 0.7	5 ± 2
MR1/2	1.8 ± 0.3	4 ± 1	5.1 ± 0.6	30 ± 5
MR0	0.9 ± 0.2	2 ± 1	1.2 ± 0.3	10 ± 3

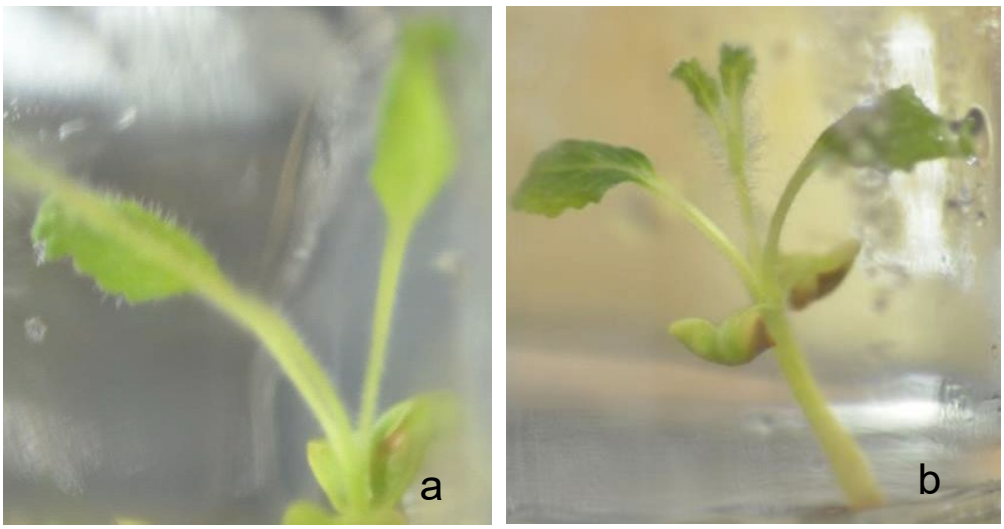

Fig. 1. Development of *S. pratensis* under *in vitro* conditions on MR20 medium:(a) Second week of cultivation; (b) First month of cultivation

The MR1/2 medium, with reduced macro- and micronutrient concentrations, slowed shoot growth (1.8 ± 0.3 cm) but enhanced root system development (5.1 ± 0.6 cm). However, 30% of plants exhibited vitrification, likely due to macronutrient imbalances, particularly reduced Ca^{2+} and NO_3^- levels, as reported for other *Salvia* species [10]. The

MR0 medium (phytohormone-free) proved unsuitable for long-term cultivation, with minimal shoot (0.9 ± 0.2 cm) and root (1.2 ± 0.3 cm) growth, confirming the essential role of phytohormones in morphogenesis, as observed in *S. miltiorrhiza* [13].

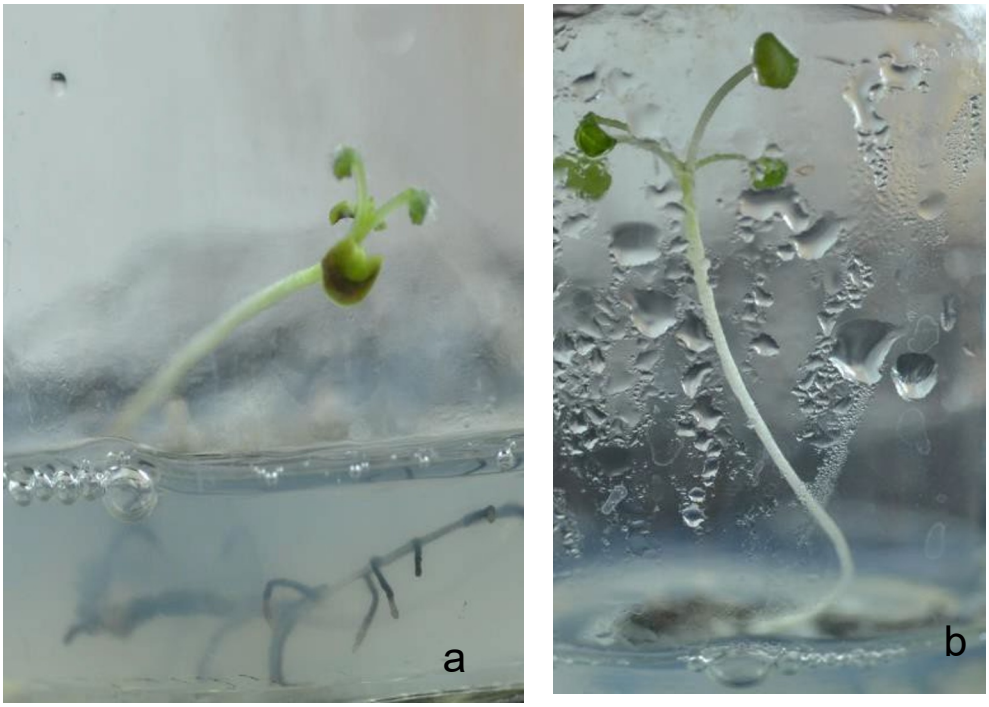

Fig. 2. Development of *S. pratensis* under *in vitro* conditions on MR1/2 medium: (a) Second week of cultivation; (b) First month of cultivation

After transferring sterile seedlings to different media, robust growth occurred only on MR20 and MR1/2. While growing on MR20 active plant development was observed. The third leaf formed within the first week, followed by root thickening and fourth-leaf emergence by the third week. After one month, shoots reached 3 cm, with an average of six leaves. This medium is optimal for *in vitro* preservation of *S. pratensis*, ensuring stable growth and development.

While growing on MR1/2 shoot growth rate remained high, with the third leaf forming by the second to third week. Root thickening and elongation occurred by the third to fourth week. After one month, shoots reached 5 cm, with an average of four leaves.

The 0.1% $AgNO_3$ protocol achieved high sterility without meristem damage, consistent with findings for *S. plebeia* [9]. However, exceeding the 20-minute exposure caused toxicity, highlighting the need for precise timing.

The 10% GA (1-hour exposure) peak efficacy aligns with gibberellin-mediated dormancy-breaking pathways in *S. miltiorrhiza* [10]. Prolonged exposure induced oxidative stress, as seen in other *Salvia* species [7].

MR20's superiority over MR1/2 and MR0 mirrors results for *S. miltiorrhiza* [13], where full-strength nutrients and phytohormones enhanced shoot growth. MR1/2 may still be valuable for root explant production despite vitrification risks.

The optimized protocol (0.1% $AgNO_3$ + 10% GA, 1 h + MR20 medium) enables mass propagation of *S. pratensis* for reintroduction into natural ecosystems, critical for conservation under anthropogenic pressure, and biotechnological production of bioactive

compounds, such as ferulic acid, for pharmaceutical and cosmetic industries.

4 Conclusion

This study optimized key stages of *in vitro* micropropagation for *Salvia pratensis* L., contributing significantly to the advancement of biotechnological methods for conserving rare species and establishing a foundation for their practical application. The main results, scientific novelty, and practical implications are summarized as follows:

1. A 0.1% silver nitrate ($AgNO_3$) solution with a 20-minute exposure achieved an optimal balance between sterility (93.63 ± 1.35%) and seed viability (54.25 ± 1.07%), outperforming data for closely related species such as *S. officinalis* [5]. This protocol, developed for *S. pratensis* for the first time, is recommended for other *Salvia* species with dense seed coats. Lysoformin 3000 (10%), despite high sterility (96.85 ± 1.27%), was unsuitable due to complete loss of viability, attributed to glutaraldehyde toxicity [9].

2. The optimal treatment – 10% gibberellic acid (GA) with a 1-hour exposure – increased germination to 91.88 ± 2.94%, 1.7 times higher than the control (54.75 ± 1.85%). This effect is linked to the activation of hydrolases that degrade seed coats [10]. However, prolonged exposure (12 hours) reduced efficacy to 67.63 ± 1.59% due to oxidative stress, as observed in *S. miltiorrhiza* [12]. The non-linear response of 1% GA (inhibition at 1 hour vs. stimulation at 3–12 hours) suggests dose-dependent amylase activation, as in *Arabidopsis thaliana* [11], warranting further investigation.

3. The MR20 medium (full-strength MS + 0.5 mg/L IAA + 1 mg/L BAP + 20 g/L sucrose) yielded optimal growth, with shoots reaching 3.2 ± 0.5 cm, roots 4.5 ± 0.7 cm, and 6 ± 1 leaves, alongside minimal vitrification (5 ± 2%). This confirms the necessity of high mineral and phytohormone concentrations for slow-growing species like *S. pratensis* [10]. In contrast, MR1/2 caused 30% vitrification due to Ca^{2+} and NO_3^- imbalances but showed potential for root development. The MR0 medium (phytohormone-free) proved unsuitable, underscoring the critical role of cytokinins and auxins in morphogenesis.

This work advances biotechnological strategies for rare species conservation and industrial utilization. The optimized protocols for sterilization, germination, and cultivation not only address key challenges in *S. pratensis* micropropagation but are also adaptable to other vulnerable *Lamiaceae* species. These findings open new avenues for biodiversity conservation and sustainable use of plant resources.

References

1. R.G. Butenko, *Biology of Higher Plant Cells in Vitro and Biotechnology Based on Them* (Moscow: FBK-Press, 1999) 160 p. (In Russian).
2. A.M. Nosov, Use of Cellular Technologies for Industrial Production of Biologically Active Substances of Plant Origin, Biotechnology, **5**, 8–28 (2010) (In Russian).
3. M.V. Gavrilin, Phenolic Compounds of the Aboveground Part of Clary Sage (*Salvia sclarea* L.) Cultivated in the Stavropol Territory, Chemistry of Plant Raw Materials, **4**, 99–104 (2010). (In Russian).
4. A.P. Levitsky, E.K. Vertikova, I.A. Selivanskaya, Chlorogenic Acid: Biochemistry and Physiology, Microbiology and Biotechnology, **2**, 6–20 (2010) (In Ukrainian).
5. M. Papafotiou, G. Vlachou, A.N. Martini, Investigation of the Effects of the Explant Type and Different Plant Growth Regulators on Micropropagation of Five Mediterranean *Salvia* spp. Native to Greece, Horticulturae, **9(1),** 96 (2023). https://doi.org/10.3390/horticulturae9010096.

6. C. Han, X. Dong, X. Xing, et al. Gibberellin-Induced Transcription Factor SmMYB71 Negatively Regulates Salvianolic Acid Biosynthesis in *Salvia miltiorrhiza*, *Molecules,* **29(24),** 5892 (2024). https://doi.org/10.3390/molecules29245892.
7. K. Grigoriadou, F.A. Trikka, G. Tsoktouridis, et al. Micropropagation and Cultivation of *Salvia sclarea* for Essential Oil and Sclareol Production in Northern Greece, In Vitro Cellular & Developmental Biology - Plant, **56,** 51–59 (2020). https://doi.org/10.1007/s11627-019-10040-4.
8. Red Book of the Republic of Belarus (2015) https://minpriroda.gov.by/ru/red_book-ru/ (Date of access: 30.01.2025) (In Russian).
9. S. Ihsan, H. Gul, N. Jamila, et al. Biogenic *Salvia* Species Synthesized Silver Nanoparticles with Catalytic, Sensing, Antimicrobial, and Antioxidant Propertiesб Heliyon, **10(4),** e25814 (2024).
10. Y. Miao, X. An, Q. Han, L. Li, S. Luo, Effects of Different Treatments on Seed Germination of *Salvia miltiorrhiza*, Advances in Engineering Technology Research, **8(1),** 312–318 (2023).
11. S. Teshome, M. Kebede, Analysis of Regulatory Elements in GA2ox, GA3ox, and GA20ox Gene Families in *Arabidopsis thaliana*: An Important Trait, Biotechnology & Biotechnological Equipment, **35(1),** 1603–1612 (2021). https://doi.org/10.1080/13102818.2021.1995494.
12. Z. Liang, Y. Ma, T. Xu, et al., Effects of Abscisic Acid, Gibberellin, Ethylene and Their Interactions on Production of Phenolic Acids in *Salvia miltiorrhiza* Bunge Hairy Roots, PLoS ONE, **8(9),** e72806 (2013). https://doi.org/10.1371/journal.pone.0072806.
13. M. Krzemińska, A. Owczarek, M.A. Olszewska, I. Grzegorczyk-Karolak, In Vitro Strategy for the Enhancement of the Production of Bioactive Polyphenols in Transformed Roots of *Salvia bulleyana*, International Journal of Molecular Sciences, **23(14),** 7771 (2022). https://doi.org/10.3390/ijms23147771.
14. I.K. Sorokina, N.I. Starichkova, T.B. Reshetnikova, *Fundamentals of Plant Biotechnology* (Saratov: Saratov State University Publishing House, 2002) 34 p. (In Russian).
15. T. Murashige, F. Skoog, A Revised Medium for Rapid Growth and with Tobacco Tissue Cultures, Physiologia Plantarum, **15,** 397–473 (1962).