OPTIMIZATION OF SEED STERILIZATION AND IN VITRO CULTIVATION METHODS FOR EFFECTIVE MICROPROPAGATION OF *ARNICA MONTANA* L.

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Abstract

Arnica montana is an invaluable medicinal herb, but its wild populations are declining. To aid conservation as well as propagation, the present work investigated methods for sterilizing seeds and culture media for initiating in vitro growth. Seeds sourced in the Ukrainian region surrounding the Krasna mountain range were sterilized by employing different chemicals; sodium hypochlorite gave the most regular and healthy-looking explants. Of the nutrient media tested, Murashige and Skoog (MS) furnished the highest initial development. In IAA-BAP combinations, the best formation was tested under different concentrations. Balanced levels, especially 1.0 mg/L of both growth regulators, resulted in robust as well as regular shooting. While high hormone levels decreased the viability, growth suppression was evident. The results point towards an easy and efficient protocol for starting cultures of *A. montana* applicable for both research purposes as well as practical cultivation.

Keywords: medicinal plants, seed sterilization, micropropagation, hormone balance, tissue culture

Introduction

Micropropagation is one of the most effective biotechnological approaches for the conservation and reproduction of valuable ornamental, medicinal and rare plant species (Cachita-Cosma et al., 2008;Petrus-Vancea – Cachiță 2008; Petrus-Vancea et al., 2009; Cristea et al., 2010; Jarda et al., 2010; Csabai et al., 2011; Taleie et al., 2012; Petrus-Vancea – Cachiță 2011;Cristea et al., 2013a; Cristea et al., 2013b; Jarda et al., 2014; Petruș-Vancea 2018; Cristea et al., 2019; Petruș-Vancea et al., 2021; Cseh et al., 2023; Tytarenko et al., 2023; Petrus-Vancea et al., 2024; Szarvas et al., 2025; Farkas et al., 2025; Kolesnyk et al., 2025). This method provides genetically stable and virus-free planting material with a high multiplication rate (Kushnir & Sarnatska, 2005; Uspanova & Chukanova, 2008; Marchuk, 2008). Plants propagated in vitro are characterized by genetic uniformity, which contributes to the standardization of the chemical composition of medicinal raw materials and the reproducibility of their pharmacological effects.

The use of biotechnological methods is particularly relevant for *Arnica montana* L. (*Asteraceae*), a rare species listed in the Red Data Book of Ukraine (Red Data Book of Ukraine, 2021), as well as in the European Red List of Vascular Plants (category LC). This species is threatened in many European countries and is protected under Annex D of EU Council Regulation No. 338/1997 concerning the control of trade in endangered species (Lange, 1998; Falniowski et al., 2013). Natural populations of *A. montana* are declining due to habitat degradation and overharvesting. The species is naturally distributed mainly in alpine regions, while its cultivation under lowland conditions is inefficient due to specific ecological requirements. Tissue culture techniques help avoid excessive harvesting of wild populations, provide large-scale propagation of planting material without requiring extensive cultivation areas, and create a scientific basis for the conservation of the species' gene pool (Wojciechowicz et al., 2007; Jurica et al., 2021).

The pharmacological value of *A. montana* is attributed to its high content of bioactive compounds, particularly sesquiterpene lactones, thymol derivatives, phenolic acids, and flavonoids (Lyss et al., 1997; Klaas et al., 2002; Douglas et al., 2004; Grdiša et al., 2012; Krasteva et al., 2013; Georgiev et al., 2014), which are responsible for anti-inflammatory, wound-healing, antiseptic, and antioxidant activities (Willuhn, 1998; Brinkhaus et al., 2006; Sharma et al., 2016; Nieto-Trujillo et al., 2021; Kryvtsova et al., 2019; Kryvtsova & Koščová, 2020). Therefore, the application of in vitro technologies is an optimal strategy for the propagation of *A. montana*, ensuring a stable raw material base for the pharmaceutical industry while preserving natural populations.

Materials and methods

Healthy, undamaged seeds of *A. montana* collected from a natural population in the Krasna mountain meadow, Mizhhirya district, Zakarpattia region, were used in the study. The seeds were surface sterilized, then rinsed three times with sterile distilled water. To improve germination and activate physiological processes, stratification with gibberellic acid solution (0.1 mg/L) was applied. Seeds were subsequently sown onto agar-solidified hormone-free nutrient media: Murashige and Skoog (MS) (Murashige & Skoog, 1962), Gamborg, and White (Kushnir & Sarnatska, 2005).

The pH of the media was adjusted to 5.5–5.8 with 10% KOH or NaOH prior to autoclaving, considering potential acidification caused by sugar degradation during sterilization. Media were supplemented with myo-inositol (100 mg/L), sucrose (30 g/L), and agar (9 g/L).

Segments of aseptically grown seedlings (approximately 1 cm long, including meristematic tips and hypocotyls) were used as explants for microshoot induction. Explants were cultured on modified MS media supplemented with 17 different combinations of indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP). Hormone-free MS served as the control medium.

Cultivation was carried out in a growth chamber at 25 ± 1 °C, light intensity 2,000–3,000 lux, with a 16-hour photoperiod and relative humidity of 70–75%. Each treatment included 25 culture tubes, and experiments were performed in triplicate. Observations were recorded on day 28–30, analyzing survival rate (%), average number of shoots per explant, and average shoot length (cm). Sterilization efficiency was assessed by the percentage of non-contaminated explants relative to the total sterilized explants. Experimental data were statistically processed using MS Excel.

Results

Seed Sterilization

The study evaluated the effect of different sterilizing agents (hydrogen peroxide, sodium hypochlorite, mercuric chloride) and exposure times on the survival of *A. montana* explants cultured on three hormone-free media (MS, Gamborg, White) (Table 1).

Among the tested treatments, the highest efficiency was observed after seed sterilization with sodium hypochlorite for 10 minutes (80.3 ± 7.3%). Shorter exposure (5 min) significantly reduced efficiency (41.5 ± 5.8%), likely due to insufficient disinfection. Hydrogen peroxide showed relatively low effectiveness (40.1 ± 2.8% and 35.6 ± 4.2% after 10 and 20 min, respectively). Mercuric chloride ensured a high survival rate under short exposure (65.7 ± 5.0%), but its toxicity reduced survival drastically with longer treatments (33.9 ± 5.1%).

On Gamborg medium, the overall survival rate was lower. Sodium hypochlorite treatment for 10 minutes gave the highest result (43.7 ± 5.1%), while hydrogen peroxide showed the lowest (15.0

± 2.1% and 21.6 ± 3.5%). Mercuric chloride had moderate efficiency after short exposure (33.8 ± 6.1%), but prolonged treatments reduced viability to 25.2 ± 5.9%.

On White medium, similar trends were observed. The best results were achieved with sodium hypochlorite (63.1 ± 7.9% at 5 min, 48.5 ± 6.3% at 10 min). Mercuric chloride showed relatively high effectiveness only under short treatment (45.3 ± 5.1%), while longer exposure halved the result (21.7 ± 3.9%). Hydrogen peroxide demonstrated the lowest effectiveness across all conditions (23.2 ± 3.4% and 11.8 ± 2.9%).

These findings agree with previous studies (Zelenina, 2005; Kushnir, 2005), which identified sodium hypochlorite as the most effective and safe sterilizing agent for seeds of Asteraceae species (Petrova et al., 2011; Shatnawi, 2013; Trejgell et al., 2018). In our case, its optimal effect was recorded on MS medium after 10 min exposure, ensuring the highest survival rate (80.3%). Mercuric chloride may be suitable for short-term treatments, but its toxicity and environmental hazards limit its desirability. Hydrogen peroxide proved to be the least effective in all tested conditions.

As a control, *A. montana* seeds were germinated without sterilization in moist filter paper cultures at 20 ± 2 °C. On day 10, germination reached 47.8% of sown seeds. This suggests that sterilizing agents, especially NaClO, act not only as disinfectants but also as chemical stratification agents. Penetration of active components through the seed coat may alter the embryo's physiological state, release dormancy, and activate metabolic processes necessary for germination. Thus, sterilization treatments exhibit dual effects—ensuring asepsis and enhancing germination beyond control levels.

For subsequent in vitro culture, sodium hypochlorite treatment for 10 minutes combined with MS medium can be recommended as the most effective approach, providing an optimal balance between sterilization and explant viability.

In vitro Cultivation of Arnica montana

The effect of different IAA/BAP ratios on microshoot growth and development was also studied (Table 2).

Explants cultured on hormone-free MS medium showed low viability (27.1%), producing on average only 1.52 shoots per explant with a mean height of 2.35 cm. This indicates that phytohormones are necessary for morphogenesis induction, as salts and vitamins of MS alone cannot effectively stimulate shoot formation.

The addition of IAA and BAP significantly improved survival. The highest viability (90.8%) was recorded at 1.0 mg/L IAA + 0.5 mg/L BAP. Most combinations resulted in >70% survival, whereas high concentrations (2.0 mg/L IAA + 2.0 mg/L BAP) drastically reduced survival (36.2%).

Shoot number varied with hormone ratios. The control formed 1.52 shoots per explant, while the highest values (4.14 and 3.98 shoots per explant) were obtained at 1.0 mg/L IAA + 1.5 mg/L BAP and 1.5 mg/L IAA + 1.0 mg/L BAP, respectively. Similar trends were observed by Petrova et al. (2012), where maximum multiplication rates were achieved at moderate cytokinin concentrations, while higher levels had no positive effect.

Auxin-enriched treatments mainly stimulated shoot elongation. Shoot height in the control averaged 2.35 cm, while maximum elongation was observed at 1.5 mg/L IAA + 1.0 mg/L BAP (5.11 cm) and 2.0 mg/L IAA + 1.0 mg/L BAP (4.92 cm). Moderate cytokinin levels combined with elevated auxins enhanced shoot formation, consistent with previous reports (Lê, 1998; Surmacz-Magdziak & Sugier, 2012; Petrova et al., 2021). In contrast, excessive concentrations of both regulators reduced survival (to 36.2%) and suppressed morphogenesis, aligning with inhibitory effects described in earlier studies (Sujatha & Reddy, 1998; Kim et al., 2001).

Conclusion

Sodium hypochlorite (10 min) on MS medium proved to be the most effective sterilization method, yielding up to 80% viable explants of *A. montana*.
Optimal shoot proliferation (up to 4.1 shoots/explant, 90% survival) was achieved under moderate IAA–BAP combinations, while excessive doses inhibited morphogenesis.
In vitro micropropagation represents a reliable strategy for conservation of the endangered *A. montana* and for obtaining standardized raw material rich in pharmacologically valuable compounds.

Table 1. Sterilization conditions and viability of *A. montana* explants

Culture medium	Sterilizing agent	Exposure time, min	Viability of explants (mean ± standard error), %
MS	Hydrogen Peroxide (H_2O_2)	10	40.1±2.8
		20	35.6±4.2
	Sodium Hypochlorite (NaClO)	5	41.5±5.8
		10	80.3±7.3
	Mercuric Chloride ($HgCl_2$)	5	65.7±5.0
		10	33.9±5.1
Gamborg	Hydrogen Peroxide	10	15.0±2.1
		20	21.6±3.5
	Sodium Hypochlorite	5	37.4±4.2
		10	43.7±5.1
	Mercuric Chloride	5	33.8±6.1
		10	25.2±5.9
White	Hydrogen Peroxide	10	23.2±3.4
		20	11.8±2.9
	Sodium Hypochloride	5	63.1±7.9
		10	48.5±6.3
	Mercuric Chloride	5	45.3±5.1
		10	21.7±3.9

Table 2. Effect of growth regulators on the growth and morphogenesis of A.montana

№	IAA, mg/l	BAP, mg/l	Explant survival on day 30, %	Number of shoots per explant	Shoot height, cm
1	0	0	27.1±3.6	1.52±0.11	2.35±0.91
2	0.5	0.5	70.8±5.0	1.99±0.35	2.86±0.65
3	1.5	1.5	78.1±8.5	2.95±0.76	3.04±0.90
4	1.0	1.0	82.6±6.3	1.93±0.18	3.85±0.86
5	0	1	66.5±5.1	2.28±0.31	2.51±0.35
6	1	0	67.2±7.5	1.52±0.65	2.92±0.82
7	0.5	1	87.9±8.1	2.08±0.82	2.85±0.79
8	1	0.5	90.8±6.3	2.86±0.81	3.72±1.01

9	1	1.5	81.7±4.1	4.14±1.3	4.75±1.24
10	1.5	1	76.5±8.9	3.98±1.01	5.11±1.15
11	0	1.5	61.7±4.5	2.71±0.46	2.97±0.72
12	1.5	0	71.3±9.0	1.88±0.28	4.02±1.03
13	0.5	1.5	69.5±5.6	2.73±0.91	3.32±0.91
14	1.5	0.5	70.8±5.9	2.47±1.76	4.37±1.31
15	1	2	66.3±8.5	3.48±0.95	3.58±0.94
16	2	1	59.4±6.4	2.56±0.61	4.92±1.20
17	2	2	36.2±4.1	3.14±1.02	4.42±0.56

The table presents mean values ± standard error

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