Multiplication and Conservation of Threatened Medicinal Plant *Arnica montana* L. by *in vitro* Techniques

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Summary

An efficient and reproducible *in vitro* protocol for mass production of the threatened medicinal plant *Arnica montana* L. (Asteraceae) was developed. The effectiveness of various combinations of plant growth regulators on *A. montana* clonal multiplication was assessed, using seedlings' stems as initial explants. Among 12 tested nutrient media, the optimum one (MS supplemented with 1.0 mg/l BAP and 0.1 mg/l IAA) increased the organogenesis frequency up to 95% in the best origin, with mean number of shoots per explant 4.25 for 5 weeks. Sub-cultivations on this medium every 4 weeks led to increase of the propagation rate as in the fifth subculture the average number of shoots per explant reached 12.32±0.82. Rooting of uniform *in vitro* shoots was 100% successful on half strength MS medium supplemented with 0.5 mg/l IBA. The *ex vitro* adapted plants showed 90% survival, and were further acclimatized to two mountain *ex situ* collections. Plants looked healthy and true-to-type and began to bloom in the second or the third year. In addition, a successful protocol for slow-growth storage of *in vitro A. montana* cultures was elaborated, after testing 8 media with mannitol or sorbitol. The medium ½ MS containing 3% sorbitol and 2% sucrose was chosen as the best one, efficiently retarding the growth of the *in vitro* plantlets, thus allowing 6-month maintenance without sub-cultivation. The developed *in vitro* protocols could be of great value for commercial propagation and sustainable conservation of this threatened medicinal plant.

Key words

in vitro culture, plant growth regulators, multiplied shoots, slow-growth storage

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INTRODUCTION

Arnica montana L. (Asteraceae) is a valuable medicinal plant species used since ancient times in the folk medicine. Nowadays, it is widely applied in pharmacy, homeopathy and cosmetics due to its antiseptic, anti-inflammatory and antioxidant activity (Willuhn, 1998; Brinkhaus et al., 2006; Sharma et al., 2016). Phytochemical research revealed that its most biologically active compounds are sesquiterpene lactones, thymol derivatives, flavonoids and phenolic acids (Lyss et al., 1997; Klaas et al., 2002; Douglas et al., 2004). The species is strongly anthropogenically affected, although it is widespread in the high mountains, and some of its populations are within protected natural territories. Due to loss of habitats and intensive picking for commercial and medicinal purposes *A. montana* is included in the European Red List of Vascular Plants (LC), threatened in many European countries and included in the Annex D of the Council Regulation (EC) No 338/1997 on conservation of plant species through control of their trade (Lange, 1998; Falniowski et al., 2013). The increased demand of *A. montana* plant material requires development of methods for rapid multiplication. Plant tissue cultures are being widely used for large-scale plant production, as they provide new tools for preservation of valuable and endangered species and play a major role in the search for alternatives to the production of pharmaceutically important secondary metabolites, moreover, product quality and yield are usually established (Rao and Ravishankar, 2002; Okršlar et al., 2007; Karuppusamy, 2009; Oseni et al., 2018). A number of protocols for *in vitro* propagation of *A. montana* have been applied with various effectiveness depending on the applied culture media (Conchou et al., 1992; Lê, 1998; Butiuc-Keul and Deliu, 2001; Surmacz-Magdziak and Sugier, 2012). It is well known that various factors are influencing the micropropagation process, such as explant type, combination and concentration of plant growth regulators supplemented in the medium, and genotype.

One approach to the conservation of *in vitro* plant germplasm is micropropagation under optimal conditions which requires subcultures to fresh medium every few weeks; the other, which is storage-based, using slow growth procedures, allows longer intervals between subcultures, and is therefore preferred as cheaper and less labor-intensive and time-consuming (Moges et al., 2003; Trejgell et al., 2015; Chauhan et al., 2019). The long-term storage of *in vitro* cultures can be achieved by incubation at reduced temperature, changes of light period and light intensity, low concentration of nutrients in the culture media, addition of osmotic agents and growth retardants (Shibli et al., 2006; Thakur et al., 2015; Rodrigues et al., 2018). The literature survey revealed that up to now the protocol for *in vitro* conservation of *A. montana* under slow growth conditions has not been developed except for our observations on the osmotic regulator mannitol (Petrova et al., 2011).

The aim of the present study was to investigate the influence of different modified MS nutrient media on the *in vitro* clonal propagation of *A. monatna*, the acclimation of the obtained plants to mountain regions, as well as the slow growth storage of the *in vitro* cultures, in order to ensure long-term maintenance of valuable genotypes of this medicinal plant species as a source for secondary metabolite productions.

MATERIALS AND METHODS

Initial Plant Material

A. montana seeds were collected from three different origins: plants from a natural population in the Carpathian Mountains, Ukraine (AMU); German cultivar 'Arbo' (AMG), and plants growing in the botanical garden in Vienna, Austria (AMA). Seeds were disinfected after standard procedure, using commercial bleach, as previously described (Petrova et al., 2008), and germinated on basal MS medium (Murashige and Skoog, 1962) free of plant growth regulators (PGRs). Two-month old *in vitro* seedlings were used as a source of stem segments, which were the initial explants for the *in vitro* clonal multiplication.

In vitro Shoot Multiplication

Nutrient media and culture conditions for in vitro cultivation and rooting

Twelve different MS based media (Murashige and Skoog, 1962) containing 3% sucrose, modified for shoot formation and development, were evaluated. The effect of cytokinins: 6-benzylaminopurine (BAP), Zeatin (Zea), or N^6[2-isopentenyl]-adenine (2-iP) at concentrations 0.5 or 1.0 mg/l combined with two auxins: α-naphthalene acetic acid (NAA) or indole-3-acetic acid (IAA) at concentration 0.1 mg/l was tested. The medium pH was adjusted to 5.8 before autoclaving at 121 °C for 20 minutes at a pressure of 1.1 kg cm^{-2}. First trials were done with origin AMG. Forty stem explants were placed on each of the 12 medium variants, and each treatment was repeated twice. The organogenesis frequency (percentage of explants forming shoots), the propagation coefficient (PC) evaluated as an average number of shoots induced per explant, and the mean shoot height were assessed after 5 weeks of culture, and used as criteria for media comparison.

To test the plant origin impact, 40 explants per origin were cultivated on each of the best two media, and cultures were compared for their organogenesis frequency, propagation coefficient, and shoot height.

The multiple shoot-clumps were separated and single shoots were sub-cultured on media for root induction. The auxins indole-3-butyric acid (IBA), NAA or IAA at 0.5 mg/l were used to study their effect on root formation, and a half-strength MS medium free of PGRs and containing 20 g/l sucrose was used as a control. The micropropagated shoots (1.5 – 2.0 cm) were transferred to the four tested media, 20 shoots per medium variant, in two replications (origin AMG). The percentage of rooted shoots after four weeks of culture, the number of roots per explant, and the root length, were used as criteria for media comparison.

All *in vitro* cultures were maintained in a culture room, at a temperature of 22±2 °C, and 16 h photoperiod, under illumination of 40 μmol $m^{-2}s^{-1}$ provided by Philips 36 W cool white fluorescent tubes.

Long-term sub-cultivation

To assess the potential for long-term *in vitro* clonal multiplication of *A. montana*, a 5-fold consecutive sub-cultivation was done with shoots of AMG origin on the best medium (1 mg/l

BAP and 0.1 mg/l IAA), using as explants 40 newly obtained shoots per sub-cultivation, for a period of 4 weeks for each sub-cultivation. The consecutive propagation coefficients were calculated as average of newly obtained shoots per explant, and theoretical PC was calculated for the whole period of 20 weeks, as number of shoots obtained per initial explant.

Ex vitro adaptation and acclimatization

In vitro obtained plants with well-developed roots were removed from the culture vessels and washed free of agar. A total of 134 *in vitro* rooted plants were transferred to small pots containing peat, perlite and coconut sawdust (Noramix Group, Sri Lanka) in proportion 2:1:1 (v/v/v), one or two per pot. To maintain high humidity, the pots were covered with transparent polyethylene. After two weeks, polyethylene covers were removed in order to adapt the plants to *ex vitro* conditions. After an adaptation period of 6 weeks, the plants were transferred to the greenhouse for further strengthening. Next spring they were planted at two experimental plots of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences: "Beglika" locality (Western Rhodope Mts.) at 1500 m a. s. l. and "Zlatni mostove" locality (Vitosha Mt.) at 1400 m a. s. l. The success of the acclimatization in the mountain regions and the flowering were evaluated over the next three consecutive years.

Prolonged *in vitro* Storage

Nutrient media for prolonged in vitro storage

Retarding the growth of *in vitro* cloned plants was optimized by testing of 8 medium variants, all of them with half strength MS medium (½ MS), reduced sucrose concentration (2%), and addition of osmotic regulator (sorbitol or mannitol) at concentrations (1, 2, 3, and 4%). The medium without mannitol or sorbitol was used as a control. Cultures were maintained under low light intensity (20 μmol $m^{-2}s^{-1}$ illumination). The percentage of survival plants was evaluated after 1, 3, or 6 months of cultivation. The survival of the prolonged storaged *in vitro* cultures was determined by the presence of green plants with healthy growing tips without necrosis, and the average height of the shoots.

Viability restoration of long-term storaged shoots

The recovery of plants' quality after prolonged cultivation on medium with sorbitol or mannitol was initiated by culturing under optimal conditions for growth and development. Plants were restored on optimum propagation medium (full strength MS medium supplemented with 1 mg/l BAP and 0.1 mg/l IAA), at a temperature of 22 ± 2 °C and light intensity of 40 $\mu Mm^{-2}s^{-1}$.

Statistical Analysis

Data were subjected to one-way ANOVA analysis of variance for comparison of means, and significant differences were calculated according to Fisher's least significance difference (LSD) test at the 5% significance level using a statistical software package (Statgraphics Plus, version 5.1 for Windows). Data were presented as means ± standard error.

RESULTS AND DISCUSSION

Influence of PGRs on Shoot Multiplication of *A. montana*

The stem segments cultured on control MS medium free of PGRs, and containing full strength of salts and vitamins did not induce new shoots during the tested culture period, however they grew in height and 30% of them rooted. The effect of cytokinins supplemented alone in MS medium on the shoot induction of *A. montana* was studied previously, and PC reached 3.12±0.25 shoots per explant in the best variant, containing 1 mg/l BAP (Petrova et al. 2012). To enhance the multiplication effectiveness, nutrient media supplemented with both cytokinins and auxins were elaborated. Initial explants expressed different multiplication potential depending on PGRs type and concentration (Table 1, Fig. 1).

Table 1. Effect of the PGRs on the clonal propagation of *A. montana* (origin AMG)

PGRs [mg/l]	Organogenesis frequency [%]	PC [shoots/explant]	Shoots height [cm]	*In vitro* rooting [%]
BAP + NAA				
0.5 + 0.1	62.5	2.72 ±0.29^{a}	2.84 ±0.12^{b}	0
1.0 + 0.1	75.0	3.82±0.33^{bc}	2.32 ±0.10^{a}	0
BAP+ IAA				
0.5 + 0.1	77.5	2.97±0.28^{ab}	2.63±0.11^{b}	0
1.0 + 0.1	95.0	4.25±0.35^{c}	2.25 ±0.05^{a}	0
Zea + NAA				
0.5 + 0.1	45.0	1.45±0.11^{a}	2.91±0.08^{a}	8
1.0 + 0.1	72.5	2.82±0.25^{b}	3.23±0.14^{b}	10
Zea +IAA				
0.5 + 0.1	82.5	3.07 ±0.26^{bc}	2.94 ±0.10^{a}	0
1.0 + 0.1	87.5	3.52±0.29^{c}	2.78 ±0.07^{a}	0
2-iP + NAA				
0.5 + 0.1	5.0	1.05±0.03^{a}	3.12 ±0.15^{b}	45
1.0 + 0.1	12.5	1.12±0.05^{ab}	2.74±0.11^{a}	50
2-iP + IAA				
0.5 + 0.1	7.5	1.07±0.04^{ab}	3.03±0.14^{ab}	50
1.0 + 0.1	17.5	1.20±0.07^{b}	2.85±0.10^{ab}	55

The data are presented as means of 40 shoots per medium variant ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing one way ANOVA analysis

Figure 1. Multiplied Shoots Developed on MS Medium with Different Composition: a) MS + 1.0 mg/l BAP + 0.1 mg/l IAA, b) MS + 1.0 mg/l Zea + 0.1 mg/l IAA and c) MS + 1.0 mg/l 2-iP + 0.1 mg/l IAA

In all PGR combinations, the organogenesis frequency and the propagation coefficient of the cultures increased with the increase of cytokinin concentration from 0.5 to 1.0 mg/l. Further increase of the cytokinin level from 1.0 to 2.0 mg/l did not improve any of the studied parameters and reduced proliferation of shoots (data not shown). It was established that the most effective for rapid and mass micropropagation of *A. montana* was MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l IAA (Table 1), which yielded 4.25 shoots per explant with mean height 2.25 cm. This PRG combination provoked the highest organogenesis frequency (95%). The addition of BAP and NAA (1.0 mg/l + 0.1 mg/l), and combinations of Zea (1.0 or 0.5 mg/l) and IAA also promoted the multiplication and the mean number of shoots per explants in these variants exceeded 3. In the medium variants supplemented with 2-iP alone (Petrova et al., 2012) or applied in combination with NAA or IAA (Table 1), a small part of explants produced shoots, and shoot proliferation was lower. However, the presence of 2-iP stimulated the rhizogenesis of about 50% of the shoots, while those grown on medium variants with the highest PC did not root (Table 1).

The data obtained showed that the most effective for rapid multiplication of *A. montana* was the combination of 1.0 mg/l BAP and 0.1 mg/l IAA added to full strength MS medium, followed by the combination of 1.0 mg/l Zea and 0.1 mg/l IAA. Other authors working with *A. montana* reported a maximum of 7.7 shoots per explants for 6 weeks on MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA (Conchou et al., 1992), while Malarz et al. (1993) obtained a high number of shoots per explant on MS medium containing 2.5 mg/l kinetin and 0.5 mg/l NAA within 8 weeks of cultivation. Surmacz-Magdziak and Sugier (2012) reported that 0.5 mg/l BAP in the medium had more beneficial effect on micropropagation and morphology of multiplied shoots than the higher concentration of BAP (1 or 2 mg/l). The difference in the culture response to the growth regulators may be due to differences in their absorption by the cells and the mechanism of action of cytokinins and auxins (Sujatha and Reddy, 1998; Kim et al., 2001; Sujatha and Kumari, 2007).

Repeated sub-cultivation is an effective way to obtain new plants. *In vitro* cultures (cultivar 'Arbo') were maintained and propagated on MS medium supplemented with BAP and IAA for a long period of time without loss of morphogenetic potential (Fig. 2). Newly obtained plants from *A. montana* were sub-cultured five-fold to produce a large number of vegetative shoots. The average number of shoots per explants increased to 7.46±0.38 in the second subculture, and some explants formed up to 16 shoots. The propagation coefficient increased significantly with each sub-cultivation, and reached 12.32±0.82 in the fifth subculture. Thus, a large number of cloned plants were obtained. The theoretical PC for the whole period of sub-cultivation (20 weeks) was assessed to over 42 thousands of *in vitro* shoots obtained per initial explant. Other authors starting with seedlings or zygotic embryos as initial material reported different results in other species: sub-cultivation significantly increased multiple shoots induction and direct organogenesis in *Boscia senegalensis* (Khalafalla et al., 2011), while decrease of multiplication rate in subsequent subcultures were noticed for *Carlina acaulis* and *Inula germanica* (Trejgell et al., 2010, 2018).

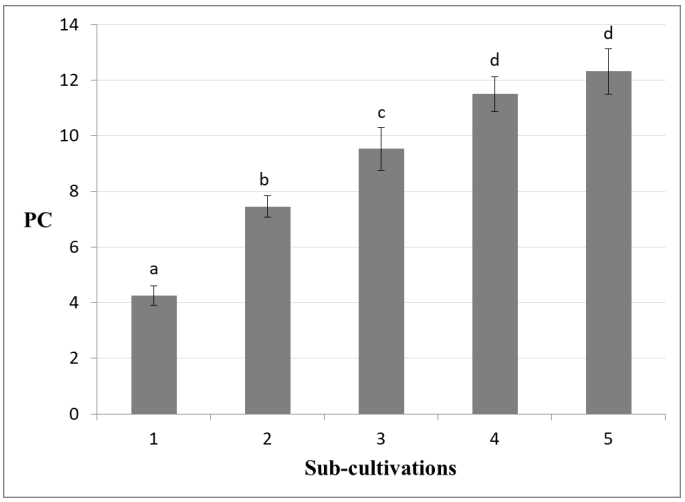


Figure 2. *In vitro* multiplication of *A. montana* (cultivar 'Arbo') during 5 successive subcultivations The data are presented as means of 40 shoots per subcultivation ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing one-way ANOVA analysis

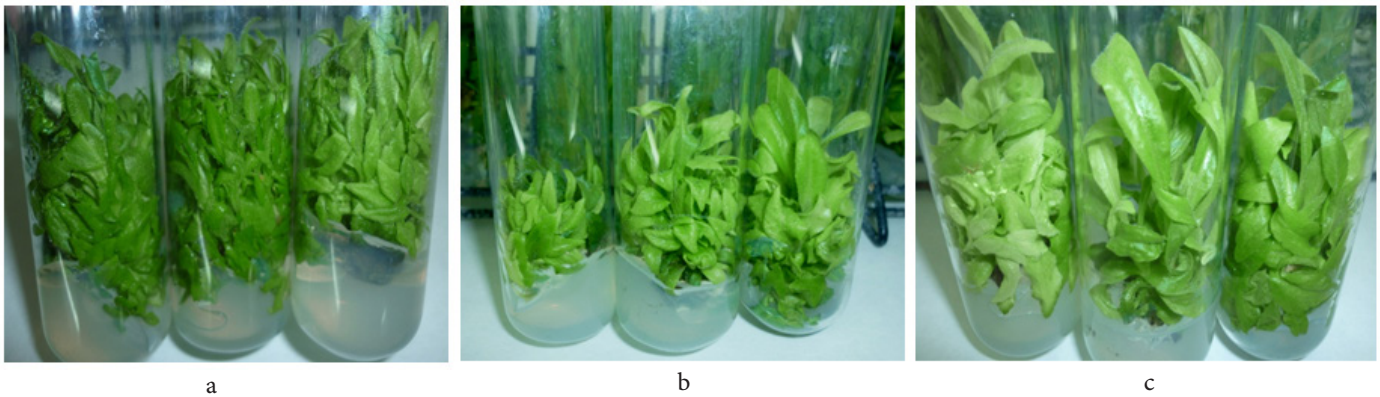
Effect of *A. montana* Origin on Multiplication Effectiveness

Plants derived from different origins showed different potential for *in vitro* organogenesis frequency and propagation rate. On the optimum medium (1.0 mg/l BAP and 0.1 mg/l IAA) the mean number of shoots per explant was significantly higher for cultures from origins AMG and AMU, compared to that from AMA (Table 2). Shoots were numerous and difficult to count. Besides the well-formed shoots, many small buds were observed, as well (Fig. 3). They needed one or two sub-cultivations to grow and stabilize. The influence of the combination 1.0 mg/l Zea and 0.1 mg/l IAA was most pronounced for cultures from AMG origin, but all the three origins differed significantly from one another. The impact of the genotype was reported as crucial for the success of the *in vitro* clonal propagation in some species (Bogdanova et al. 2009). Regardless of the different multiplication efficiency in cultures of different origin, the established protocol for clonal propagation could be successfully applied to other genotypes. The medium variant containing 1.0 mg/l BAP and 0.1 mg/l IAA has been chosen as the best one for all tested cultures, ensuring the highest propagation rate and formation of multiple small buds.

In vitro Rooting and Acclimatization of *in vitro* Obtained Plants

A higher frequency of *in vitro* rooting (50%) was recorded on MS medium with half strength of micro and macro elements and 2% sucrose, compared with full strength MS medium containing 3% sucrose (30% rooted shoots). For this reason ½ MS containing 2% sucrose was used in the next experiments. Reducing the amount of salts and sucrose in the nutrient medium usually improves rhizogenesis in many other plant species (Joshi and Dhar, 2003; Birmeta and Welander, 2004; Baskaran and Jayabalan, 2005; Trejgell et al., 2009). The rooting process has been studied on MS medium without auxins in *A. montana* (Malarz et al., 1993; Weremczuk-Jeżyna and Wysokińska, 2000) but the authors did not mention the percentage of the rooted plants. Induction of roots in basal medium may be due to the presence of endogenous auxin in the regenerated shoots. However, the addition of auxin in the medium proved necessary for the process of rhizogenesis of a number of plant species.

In the present study root formation in the ½ MS medium free of PGRs was poor with the lowest percentage of rooted shoots and the lowest mean number of roots per plant compared to the auxin-containing tested media (Table 3). Rooting of the shoots started 7-12 days after their transfer to ½ MS media for *in vitro* rhizogenesis, first on that containing IBA, at the end of the first week. In the fourth week of culture shoots had well developed root system suitable for *ex vitro* adaptation (Fig 4a). All the three tested auxins had a positive effect on root induction; however, IBA was the most effective at triggering new roots, ensuring not only 100% of rooted shoots, but also the highest number of roots per plant (Table 3). Other authors have also found that IBA was more effective for root induction than NAA and IAA (Butiuc-Keul et al., 2002; Baskaran and Jayabalan, 2005). IBA stimulated root formation in a number of species from Asteraceae family: *Centaurea rupestris* (Perica, 2003), *Wedelia chinensis* (Kameri et al., 2005), *Emilia zeylanica* (Robinson et al., 2009), *Achillea millefolium* (Turker et al., 2009; Shatnawi, 2013). Kousalya and Bai (2016) suggest that IBA was more effective than the other auxins on root formation and development because of its easier uptake/transport, and successive gene activation. No difference was observed regarding *in vitro* rooting of shoots derived from the three tested origins.



a b c

Figure 3. Multiplied shoots from different origins a) AMG; b) AMU; c) AMA, cultivated on MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l IAA

Table 2. Effect of plant origin and PRGs on shoot multiplication of *A. montana*

Origin	Plant growth regulators					
	1.0 mg/l BAP + 0.1 mg/l IAA			1.0 mg/l Zea + 0.1 mg/l IAA		
	OF [%]	PC [shoots per explant]	Shoot height [cm]	OF [%]	PC [shoots per explant]	Shoot height [cm]
1. AMG	95.0	4.25±0.35^{b}	2.25±0.13^{b}	87.5	3.52±0.29^{c}	2.78±0.12^{a}
2. AMU	87.5	3.87±0.25^{b}	2.02±0.08^{a}	80.0	2.70±0.20^{b}	2.63±0.14^{a}
3. AMA	85.0	2.85±0.17^{a}	2.31±0.09^{b}	70.0	2.07±0.14^{a}	2.74±0.14^{a}

OF – Organogenesis frequency; PC – Propagation coefficient. Data are presented as means of 40 shoots per treatment ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing one-way ANOVA analysis

Table 3. Effect of auxins on in vitro rooting of *A. montana* (origin AMG)

Type of auxin	Auxin concentration[mg/l]	Rooted plants [%]	Number of roots/plant	Root length [cm]
Control	0	50	1.22±0.06^{a}	1.34±0.12^{b}
IBA	0.5	100	5.0±0.16^{d}	1.51±0.11^{b}
NAA	0.5	100	4.12±0.28^{c}	1.84±0.15^{c}
IAA	0.5	85	2.52±0.19^{b}	1.02±0.13^{a}

The data are presented as means of 20 shoots per medium variant ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing one-way ANOVA analysis

Rooted *in vitro* plants were sucesfully adapted to substrate mixture consisting of peat, perlite and coconut sawdust in proportion 2:1:1, in small plastic pots (Fig. 4b). It was found suitable for the plants' hardening (survival rate of 89.6%). All plants survived during their adaptation in the greenhouse, and formed several offspring rosettes. No phenotypic variations were observed in the regenerated adapted plants. Most of the plants were sucessfully acclimatized on the experimental *ex situ* collections in "Beglika" locality (Western Rhodope Mts.) at 1500 m a. s. l. and "Zlatni mostove" locality (Vitosha Mt.) at 1400 m a. s. l. (Fig. 4c). The formation of offspring rosettes continued over the next three years of observation. About 70% of a total of 140 plants started to bloom in the second year. In the third year, all plants developed flower stems and bloomed, forming several flower heads per plant (Fig. 4d). Detailed information about growth, development and flower heads yield of *in vitro* acclimatized plants was described by Vitkova and Balabanova (2018). Regarding our previous study, *in vitro* propagated two-year plants in full flowering stage cultured at the experimental plots accumulated higher amount of lactones in comparison with *in vivo* propagated three-year plants (Todorova et al., 2016).

a b c d

Figure 4. *In vitro* rooting and acclimatization of *A. montana*: a) *In vitro* rooting of *A. montana* on ½ MS with 0.5 mg/l IBA; b) *Ex vitro* adapted plants; c) Plants cultured in the experimental field "Beglika"; d) Three-year old plants during flowering stage

In vitro Storage by Slow Growth

The valuable *in vitro* cultures of *A. montana* could be stored through clonal propagation of plants, but this process would need plant sub-cultivation on fresh medium every month. *In vitro* conservation by retarding the growth allows sub-cultivation at significantly longer periods (several months to a year), which is economically more profitable (Moges et al., 2003). In our study, the control plants cultured on nutrient medium ½ MS containing 2% sucrose without osmotic agent grew faster and had to be transferred on fresh media after one month of culture due to exhaustion of nutrients. The increasing of mannitol and sorbitol concentration in nutrient media led to retarded growth and decrease of plant height (Table 4, Fig. 5). The addition of low level of mannitol (1 or 2%) was effective to extend the subculture interval up to three and six months, respectively. These concentrations of mannitol established conditions of osmotic stress, which reduced the growth rate. The stems were shortened; the leaves and roots were of small size. Further increasing of mannitol concentration to 3% led to yellowing of leaves and necrosis of plant tips. The plants died after the third month. The content of 4% mannitol in the medium blocked the growth and development of plants. The survival rate on this medium was low (20%) and plants died after the first month of cultivation. The optimum nutrient medium for prolonged *in vitro* storage was ½ MS containing 2% sucrose and 3% sorbitol where culture growth was slow but plants remained viable (Fig. 5d). Their survival rate was 70% after six months of cultivation. The average height of these plants was 1.84 cm after six-month storage and the difference with that of the control plants (2.8 cm after on month) was well expressed. In a medium containing 2% sorbitol plants needed sub-culturing after the third month due to nutrient depletion. The content of 4% sorbitol in the medium blocked the growth and development of plants, and the survival rate was low (30%) after one-month storage. The influence of sorbitol on the conservation of medicinal plants under slow growth has been reported by other authors (Lata et al., 2010; Razavizadeh and Adabavazeh, 2017). Osmotic agents such as sorbitol and mannitol have established conditions of osmotic stress in plants that lead to reduction in plant water uptake (Shibli et al., 2006; Sharaf et al., 2012) and a drop in turgor pressure below normal required for cell division, thus inhibiting growth (Moges et al., 2003). The three tested *A. montana* origins did not show significant differences in their survival for a period of six months of storage suggesting genotype independence in viability under selected slow growth conditions.

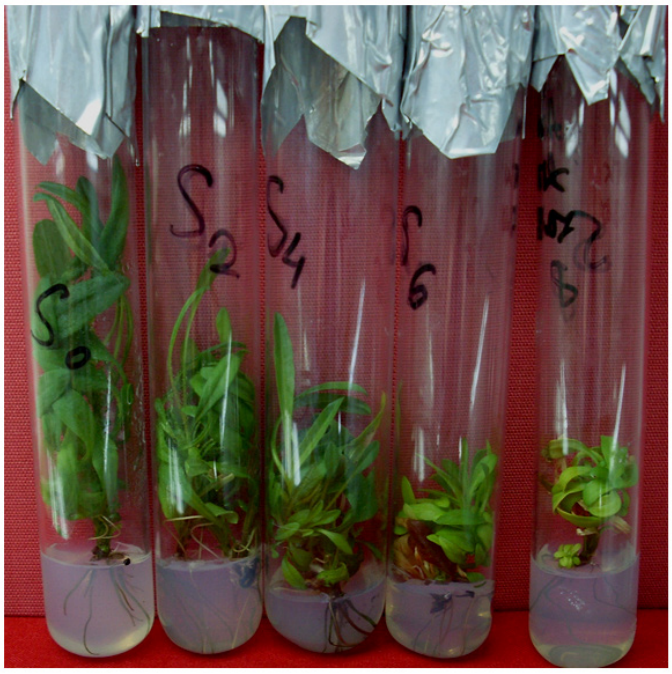


Figure 5. Plants of *A. montana*, grown on nutrient media with sorbitol after one-month storage: a) Control plants; b) 1% sorbitol; c) 2% sorbitol d) 3% sorbitol; e) 4% sorbitol

Table 4. Effect of osmotic agents on growth and survival rate of *A. montana* plants (origin AMG)

Nutrient media	Osmotic agent concentration [%]	Shoot height [cm]	Survival [%] after		
			1 month	3 months	6 months
Control	0	2.80±0.13^{e}	100	Transf.	Transf.
Mannitol	1	1.71±0.11^{c}	90	90	Transf.
	2	1.22±0.12^{b}	65	65	60
	3	0.75±0.07^{a}	55	40	Transf.
	4	-	20	Transf.	Transf.
Sorbitol	1	2.64±0.06^{e}	95	90	Transf.
	2	2.18±0.04^{d}	85	80	Transf.
	3	1.84±0.05^{c}	80	75	70
	4	1.42±0.09^{b}	30	Transf.	Transf.

Transf. – culture transferred on optimum medium for restoration of the growth

The data are presented as means of 20 plants per medium variant ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing one-way ANOVA analysis

Multiplication of *A. montana* Plants after Prolonged *in vitro* Conservation

After the storage experiments all the survived *in vitro* cultures were transferred to MS medium free of PGRs and osmotic agents in order to return towards normal growth and development. The plants were subsequently micropropagated on the optimum medium after three and six months of storage. The shoots that had been incubated for 6 months multiplied slower in comparison with those stored for 3 months. Furthermore, they produced only 2 or 3 shoots within 5 weeks of culture recovery period, while plants which had been kept 3 months under slow growth conditions produced 4-5 new shoots. The culture morphology remained the same like that of the control plants, in terms of growth pattern, leaf shape, and height of plants. All viable plants retaining green color after *in vitro* storage period, showed good regeneration capacity.

CONCLUSION

The present study provides successfully elaborated *in vitro* protocols that can be further used for rapid clonal multiplication and slow-growth storage of selected valuable genotypes of the medicinal plant *A. montana*. *In vitro* obtained plants showed high survival efficiency during the *ex vitro* adaptation and were successfully acclimatized to their approprate environmental conditions in the experimental field plots "Beglika" (Western Rhodope Mts.) and "Zlatni mostove" (Vitosha Mt.), Bulgaria. The developed *in vitro* protocols could be of great value for commercial propagation and sustainable conservation of this threatened medicinal plant.

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