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Trials on the introduction and cultivation of *Arnica montana* L. (Asteraceae) in Bulgaria

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***Abstract*:**

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The aim of the present study was to consider the possibilities for introduction and sustainable cultivation of *Arnica montana* in Bulgaria. The research was conducted on two experimental fields in different floristic regions of the country at 1 400-1 500 m altitude. The experiment was conducted with seeds of two origins: a natural population from the Carpathians (Ukraine) and the variety "Arbo" (Germany). Comparison between *in vivo* and *in vitro* propagated plants under field conditions was carried out. During four vegetation seasons the growth and development of the plants were monitored and the survival rate was evaluated. There were no significant morphological differences between the plants of both studied origins while *in vivo* and *in vitro* propagated plants had high parameters variation. The obtained results proved the possibility of sustainable cultivation of *A. montana* in the high-mountain regions of Bulgaria.

Key words: *Arnica montana*, Bulgaria, field cultivation, medicinal plant

***Apstrakt*:**

Vitkova, A., Balabanova, V.: ***Ispitivanja introdukcije i kultivacije vrste Arnica montana L. (Asteraceae) u Bugarskoj. Biologica Nyssana, 9 (1). Septembar, 2018: 21-29.***

Cilj ovog istraživanja bio je da se razmotre mogućnosti introdukcije i održive kultivacije vrste *Arnica montana* u Bugarskoj. Istraživanje je bilo izvedeno na dva ogledna polja koja su pripadala različitim floristićkim regionima na nadmorskoj visini od 1400-1500 m. Eksperiment je izveden sa semenjem čije je poreklo bilo iz dva različita izvora: prirodna populacija sa Karpata (Ukrajina) i varijetet „Arbo“ (Nemačka). Izvršeno je poređenje *in vivo* i *in vitro* propagiranih biljaka u terenskim uslovima. Tokom četiri vegetacione sezone, praćeni su rast i razvoj biljaka i procenjena je stopa preživljavanja. Nije bilo značajnih morfoloških razlika između biljaka različitog porekla, dok su *in vivo* i *in vitro* propagirane biljke imale visoke varijacije parametara.

Dobijeni rezultati dokazali su mogućnost održive kultivacije *A. montana* u visoko planinskim regionima Bugarske.

Ključne reči: *Arnica montana*, Bugarska, kultivacija na oglednim poljima, lekovita biljka

Introduction

Arnica montana L. (Asteraceae) is a valuable medicinal plant widely used in the phytotherapy in the form of numerous preparations (Merfort, 2002). The pharmaceutical industry uses the herbal drug Arnicae flos (arnica flower heads) (European Pharmacopoeia, 2011) that contains sesquiterpene lactones, flavonoids and phenolic acids (Lyss et al., 1998; Nikolova et al., 2013; Todorova et al., 2016) and it is applicable in the production of creams and lotions for muscles and bone injuries as well as in the treatment of hematomas (Klaas et al., 2002).

The studied species is native to Europe. It is distributed from Southern Norway and Latvia, Southward to Portugal, East across Europe to the North Apennines in Italy and South Carpathians in Romania (Ferguson, 1976; Smallfield & Douglas, 2008). Investigations have been carried out on the influence of environmental factors on plant material quality from natural populations of *A. montana* (Ucenic & Mastorakis, 2007), the effect of altitude on the phenolic content in arnica flower heads (Spitaler et al., 2008) and also on the presence of mycorrhiza under field conditions (Ryszka et al., 2010).

The results of the attempts to cultivate mountain arnica in some European countries could help to find appropriate environmental conditions for its sustainable cultivation. Such studies have already been made in Germany (Weyel, 1989; Bomme et al., 1995), Poland (Jurkiewicz et al., 2010; Sugier et al., 2013), Switzerland (Delabays & Mangel, 1991), Italy (Aiello et al., 2012), Finland (Galambosi, 2004) and Serbia (Plevljakušić et al., 2014). In Bulgaria, experiences on *in vivo* and *in vitro* propagation of *A. montana* have been conducted by Balabanova & Vitkova (2010), Evstatieva et al. (2012) and Balabanova et al. (2013).

A. montana is not native in the Bulgarian flora. However, more than 100 years ago, this species has been found in the subalpine zone in the Rila Mt. at 1 800 m a.s.l. and a specimen was deposited in the herbarium of Sofia University "St. Kliment Ohridski" (SO 86331).

The main purposes of the present study were: to establish an approach for cultivation of *Arnica montana* in Bulgaria; to find regions in the country with appropriate environmental conditions for its sustainable cultivation; to make a comparison of *in vivo* and *in vitro* propagated plants grown in different regions in Bulgaria on the basis of their biological features.

Material and methods

Our preliminary studies have been conducted on fields in the region Sofia (544 m a.s.l.) and the town of Pirdop (696 m a.s.l.). The obtained results showed that the climatic conditions at these altitudes are not suitable for successful cultivation of *A. montana* in Bulgaria (**Tab. 1**). After this conclusion we started to look for mountain areas with appropriate

Table 1. Experimental field environmental conditions

Ecological characteristic	Experimental fields			
	Vitosha Mt.	Sofia City	Western Rhodopes Mts.	Pirdop City
GPS coordinates	N 42º36´ E 23º14´	N 42º41´ E 23º20´	N 41º50´ E 24º07´	N 42°42´ E 24°11´
Altitude [m]	1 404	544	1 500	696
Exposition	SW	SW	NW	S
Slope [º]	8	1	3	1
Soil type	brown mountain-forest	alluvial-diluvial meadow	brown mountain-forest	cinnamon forest
Area [m^2]	30	10	30	20
N [%]	0.459	-	0.147	-
P_2O [mg/100 g]	4.10	-	19.83	-
K_2O [mg/100 g]	7.8	-	16.6	-
pH	6.1	-	6.5	-
Humus %	7.02	-	2.84	-

environmental conditions where the study could be realized.

The field experiments were carried out during the period 2009-2013 at two experimental fields of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Science: "Beglika" locality (Western Rhodope Mts.) at 1500 m a.s.l. and "Zlatni mostove" locality (Vitosha Mt.) at 1400 m a.s.l. The experimental fields are located in the European Continental climatic region of Bulgaria (Subev & Stanev, 1963).

The experimental fields' environmental characteristics are presented in **Tab. 1**. The soil analysis was carried out at the Laboratory of soil analysis at the University of Forestry, Sofia, Bulgaria. The soil type is determined according to Ninov (2002). The data for the average temperature and precipitation for the experimental fields were obtained by National Hydrometeorological Institute, Bulgarian Academy of Science, Sofia, Bulgaria.

The experiment was carried out with seeds (achenes) of two origins: a natural population from the Carpathians (Ukraine) and a variety "Arbo" (Germany).

Comparative study on *in vivo* and *in vitro* propagated plants under field conditions was carried out. The field observation on 1-, 2- and 3-year old plants was conducted through 2011-2013 growing seasons.

In vivo seedlings were produced from seeds of both origins under greenhouse conditions following the Smallfield and Douglas (2008) methodology. In December 2010, dark and mature achenes were sown in plastic plates with 50 jacks, each 5 cm in diameter, in commercial peat substrate "Tera Mix" and pine bark (1:1) (**Fig. 1**). Thereafter, the soil was slightly pressed in order to improve its close contact with the seeds. The seeds germinated after 10-12 days at 18-20 °C. When the plants formed six leaves, they were put into individual pots (d =12 cm) with the above - mentioned substrate.

We received the *in vitro* propagated plants bred by Assoc. Prof. Ely Zayova from the Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria. The seedlings were planted in coconut sawdust (Noramix Group, Sri Lanka) and their adaptation was conducted under greenhouse conditions. Two months later, the seedlings were planted into individual pots (d=12 cm) in peat substrate with natural zeolite (Tera Mix, Bulgaria) and pine bark (1:1). The *in vitro* plants were included in the study to determine the possibility of plants growing associated with a future selection based on biotechnological breeding approaches.

In May 2011, six-month-old *in vivo* and *in vitro* plants were transferred to the experimental fields of "Beglika" and "Zlatni mostove" localities. The seedlings were transplanted in 30 x 40 cm distance, thus ensuring a plant density of 10-12 plant/m^2 according to a previous research (Sugier et al., 2013). Further, the cultivation model should be adjusted for field plantation and the seedlings should be planted in 70 x 30 cm pattern due to the mechanical maintenance.

The experimental replication was made up of 46 plants. Experimental complete plot consisted of two and three replications for *in vitro* and *in vivo* propagated plants, respectively (each replication of 3.8 m^2). The areas were kept clean by hand hoeing and a regular irrigation was made.

Direct seed sowing on the field was conducted in the beginning of April and mid-September. The seeds were sown in shallow furrows in the soil which were at a distance of 30 cm in depth of 4-5 cm. The furrows were filled with a mixture of peat substrate with natural zeolite (Tera Mix, Bulgaria) and pine bark (1:1). After sowing, the soil was treated with a wooden roller and the areas were regularly moistened.

The phenological observations were carried out following Beydeman (1974). The plant development was monitored throughout 5 phenophases and the duration of each phase was recorded (**Tab. 2**).

Table 2. Duration of phenophases in *A. montana* development under field conditions

	Experimental fields	
Duration of phenophase (days)	Vitosha Mt.	Rhodopes Mts.
Vegetative	18	20
Flower buds formation	20	16
Full flower	30	24
Ripe fruit	19	28
End of vegetation	60	55
Total duration of vegetation	147	143

In order to establish the plant morphometric variability due to the seed origin, *in vivo* and *in vitro* seedlings production and the environmental conditions in the regions of plants cultivation, 8 parameters were examined on 3-year-old plants, as follows: rosette diameter stem height; affiliated rosettes number; leaves number per a rosette; length and width of rosette leaf; flower head diameter; flower heads number per plant. Morphometrical measurements were performed on 3-year-old plants during the full flowering stage. The obtained data were statistically calculated by MEDCALC Program,

Table 3. Morphometric characteristic of the plants at Vitosha Mt. and Rhodopes Mts.

parameter [M±sd] sample	rosette diameter	plant height	rosette number	leaves number per rosette	leaf length	leaf width	flower head diameter	flower heads number
AMVU1	28.11±4.19	46.33±5.51	3.56±0.92	7.78±1.67	11.80±1.56	3.60±0.76	6.77±0.59	7.94±4.29
AMVU2	24.25±6.27	42.94±7.09	5.75±3.20	8.25±1.28	9.60±2.16	3.44±1.10	5.41±0.48	16.63±12.32
AMVG1	25.36±5.50	40.90±4.53	3.64±1.69	8.18±1.89	10.62±1.98	3.57±0.79	6.28±0.64	8.09±5.57
AMVG2	22.30±2.05	38.50±4.21	4.60±0.89	8.00±1.41	9.20±1.15	3.45±0.72	5.36±0.31	12.40±8.36
AMRU1	31.87±5.64	33.91±4.12	8.83±2.23	7.65±0.98	11.72±2.51	2.82±0.51	6.57±0.79	23.00±11.87
AMRU2	29.50±4.04	30.33±3.62	7.67±2.42	7.67±0.82	11.68±1.93	3.77±0.59	6.12±0.80	20.60±10.53
AMRG1	33.41±4.10	32.23±5.95	9.71±2.46	8.23±0.89	12.77±2.70	2.61±0.41	6.56±0.80	24.02±17.93
AMRG2	25.80±1.92	28.40±6.54	6.00±1.58	7.60±0.89	10.70±1.35	3.10±0.55	5.88±0.25	13.80±3.49

Legend: AMVU1 - *A. montana*, Ukraine, *in vivo* plants, Vitosha Mt.; AMVU2 - *A. montana*, Ukraine, *in vitro* plants, Vitosha Mt.; AMVG1 - *A. montana*, Germany, *in vivo* plants, Vitosha Mt.; AMVG2 - *A. montana*, Germany, *in vitro* plants, Vitosha Mt.; AMRU1 - *A. montana*, Ukraine, *in vivo* plants, Rhodopes Mts.; AMRU2 - *A. montana*, Ukraine, *in vitro* plants, Rhodopes Mts.; AMRG1 - *A. montana*, Germany, *in vivo* plants, Rhodopes Mts.; AMRG2 - *A. montana*, Germany, *in vitro* plants, Rhodopes Mts.

expressed by average value (M) and standard deviation (sd), and the differences of the parameters variation were shown ($p<0.1\%$) (**Tab. 3**).

Results and discussion

Plants development in greenhouse and adaption to field conditions

In vivo propagated plants

The present study proved that the seeds from both origins - natural population (Ukraine) and variety ”Arbo” (Germany) germinated jointly 10 - 12 days after sowing under greenhouse conditions and the germination reached a rate up to 70 - 80%. During the first two months, the seedlings formed roots (5-6 cm) and 4 small leaves (**Fig.1**). Six-month-old plants had rosettes with diameter of 10.00 – 12.00 cm consisting of 8-10 leaves. Kating & Seidel (1967) reported that the nursery stage is critical for arnica cultivation, which prompted us to be careful during this period of plant growing.

In vitro propagated plants

Two-month-old *in vitro* plants were transferred from the coconut sawdust into a mixture of commercial (Tera Mix, Bulgaria) and pine bark (1:1) substrate. After the transplanting, some of the plants failed to adapt and 15-20% of them died. The surviving plants had a successful adaptation and developed well in the greenhouse conditions during the next 4 months. The 6-month-old plants with well-formed roots and a rosette of leaves with diameter of 10.00-12.00 cm were transferred to the fields.

Direct seed sowing on the fields

The seeds from the two investigated origins did not germinate on the experimental fields of "Beglika" and "Zlatni mostove" localities. The literary data on cultivated *A. montana* by direct seed sowing on the field indicated that the seeds germinated jointly, but the juvenile plants were vulnerable to the climate conditions (Sugier et al., 2013). Our results proved that this approach is not a reliable method for cultivation of mountain arnica in Bulgaria.

Plant adaptation after transferring to the fields

In the middle of May 2011, *in vivo* and *in vitro* plants were transferred from greenhouse to the mountain experimental fields. Plants have successfully adapted to the new conditions. At the end of the studied period

Fig.1. Two-month-old *in vivo* seedlings bred under greenhouse conditions

(2013) in the Rhodope Mts. ("Beglika" locality), 80% *in vivo* and 60% *in vitro* plants (variety "Arbo") and 65% *in vivo* and 60% *in vitro* plants (Ukraine) have adapted and completed their full development. Regarding Vitosha Mt. ("Zlatni mostove" locality), 75% *in vivo* and 33% *in vitro* plants (variety "Arbo") and 41% *in vivo*, 25% *in vitro* plants (Ukraine) have been adapted. These results showed that the percentage of successfully adapted *in vivo* plants at field conditions was higher than *in vitro* plants and the best results were established in "Beglika" locality.

Our research found a chlorosis in 5% of the plants at "Beglika" locality which had resulted on plant death. This is the evidence that the chlorosis correlates with high phosphorus content in the soil (Delabays & Mange, 1991). The soil analysis has proven that in the locality "Beglika" P_2O_5 content was 19.83 mg/100 g while on the locality "Zlatni mostove" it was significantly lower (4.10 mg/100 g). It was found that the plants are very sensitive to drought stress which was also reported by Weyel (1989). This conclusion shows that a regular irrigation of the experimental fields is needed, especially during the driest and warmest months July and August. A drought stress was observed in the plants grown on Vitosha Mt. This is mainly due to the southwest exposure of the experimental area, which leads to intense and prolonged sunshine during the summer months. To the causes of the drought stress on the plants, the soil drying due to the slope of the terrain (8o) can also be added as well as the inability to keep the precipitations falling.

Due to the meteorological survey, the average monthly air temperatures at "Zlatni mostove" locality during April 2009 - October 2013 were as follow: at the beginning of the period the temperatures were slightly above the monthly norms (perennial average). During the years 2010, 2011, 2012 and 2013, the temperatures were close to the monthly rates. According to the precipitation values for the above-mentioned period, a significant fluctuation in the amount was recorded. In the spring and late autumn, the rainfalls were generally in the norm, while in summer and early autumn, the received data were close to the minimum.

Regarding "Beglika" experimental field, the average monthly air temperatures during January 2010 - October 2013 were generally close to the above-mentioned perennial averages and the temperatures were considerably high in August - September. Concerning the precipitation values, the data were more variable compared to the perennial average. In the spring and early autumn, rainfalls were close to the average monthly rate, but were below the norm during the period August - October.

The data obtained in the study showed that the most suitable period for planting and establishing a mountain arnica field plantation in Bulgaria is at the end of May or in early June. In this period, the plants assimilate the spring humidity, temperature is appropriate and the plants adapt to the field conditions. If the planting is done in late autumn (November/December), some plants will fail to accommodate to a sharp drop in temperature, freezing of the soil and a large number of them will not survive. If the planting is done at the early October, the plants adaptation will be successful.

Vegetative propagation

It was found that at the beginning of their development, the plants of mountain arnica grown under greenhouse conditions formed offspring of rosettes. At the end of the first vegetation season, 100% of the plants produced up to 2-3 rosettes. Two, three and four-year-old arnica plants had about 3-5, 6 - 10 and up to 11-25 offspring rosettes per plant, respectively (**Fig. 2**). Through a division of offspring rosettes, seedlings which can be planted in the field were obtained. The observations showed that 100% of the plants survived and 40% of them blossomed in the same vegetation season.

Fig. 2. Affiliated rosettes of mountain arnica

Phenological observation of plants grown in different floristic regions

The phenological observation is important in the process of plant introduction and cultivation into a new geographical area. This study provides information on the duration of the vegetation season and different phenophases. In the presented research it was found that the growing season on "Beglika" and "Zlatni mostove" experimental fields lasted 143 and 147 days, respectively and that the plant development passed throughout five phenophases (**Tab. 2**).

Vegetative phase. The growing season of 2-, 3- and 4-year-old plants began in the second half of April in the two experimental fields. Regarding Vitosha Mt., the plants began to form new leaves in the middle of May and the vegetative phase lasted 18 days, while at the Rhodopes Mts. that happened 5 days later and the vegetative phase lasted 20-22 days.

Flower buds. This phase started at the end of May in the "Zlatni mostove"locality and in the first half of June in "Beglika" locality. This phase ended in the second half of June for the plants on both fields. Mountain arnica plants began to bloom in the second growing season as 70% of them formed flower buds. In the third growing season, all plants have flower buds and entered into a generative period. It was found that the plants formed single flower buds until the middle of August.

Full flowering. The plants in "Beglika" locality began to bloom two weeks after the flower buds formation and the phase lasted 14 days (**Fig. 3B**). During the period 1-5 July, all the plants were blossoming and unripe seeds in the flower heads were observed. On the same plant, single flower buds, heads in full flowering stage and blooming heads with unripe achenes were present. The full flowering of the plants at "Zlatni mostove" locality started in middle of June and ended in middle of July (**Fig. 3A**). These results showed a slightly longer flowering phase for the plants from this locality compared to "Beglika" locality. This is probably mainly due to the climatic features of these two plots. The number of flower stems and flower heads increased during the plant growth and development. Concerning 4-year-old plants, the flower stem and flower heads number for one plant was 12-20 (25) and 35-50, respectively (**Fig. 4**).

Ripe fruits. It was found that after the blossoming of the first flower heads, in the middle of July, the seed formation began at both experimental fields. In the beginning of this period, there were a small number of mature seeds in the flower heads. Ripe fruit formation was observed in the first half of August, when 80-90% of the seeds were mature.

End of vegetation. This phase began in early September when the stems were dying. Leaves that have appeared in the same year usually survived the winter. The dried stems fell off at the end of October.

Variation of plant morphometric characteristics

The data on the morphometrical research showed the following results: the rosette diameter was characterized by a very low to medium variability and a large scattering of the sign; stem height was

Fig. 3. Three-year-old plants in flowering phase at "Zlatni mostove" (A) and "Beglika" (B) experimental fields

Fig.4. Four-year-old plants in flowering phase at "Beglika" experimental field

characterized by low to medium volatility and a very large sign scattering; rosette number and inflorescence number per plant were characterized by high to very high variability and a large scattering of the parameters; leaves number per rosette was from low to high variability and relatively large sign dissipation; length/width of rosette leaf was characterized by medium to high volatility and a wide scattering of the signs; flower head diameter was characterized by low volatility and low parameter dissipation.

Plant morphometric variability according to their geographic origin

Our study found that statistically proven differences exist on plant height, leaves number in the rosette and flower head diameter ($p<0.1\%$) for the plants of both studied origins (**Tab. 3**). The plants from Ukraine, grown on both experimental fields, had higher values of the morphometrical characteristics compared to those of variety "Arbo" (Germany).

Morphometrical variability of in vitro and in vivo propagated plants

Statistically proven differences exist on all studied parameters, except stem height and leaves number per rosette ($p<0.1\%$) (**Tab. 3**).

Morphometrical variability of the plants depending on the region of cultivation

Significant differences for the plants grown at both experimental fields for the most parameters were proven except leaves number per rosette and leaf length ($p<0.1\%$) (**Tab. 3**). It was found that plants of the two origins grown at "Zlatni mostove" locality had higher stems and larger leaves number per a rosette. Higher values for number of the flower heads and flower head diameter were established for the plants at "Beglika" locality.

The least changing parameter was flower head diameter indicating that it is a conservative feature. The flower heads number per plant is an important characteristic for mountain arnica cultivation and its commercial use. The data proved that number of flower heads was significantly larger for the plants cultivated on "Beglika" experimental field.

Arnicae flos yield

Regarding our previous study, a higher yield was obtained for the plants originating in Ukraine. The highest yield was registered for *in vivo* propagated plants - up to 268 kg/ha (Balabanova & Vitkova, 2016). The harvest of the two studied origins was relatively similar in the experimental plot on Vitosha Mt. unlike those grown in Rhodope Mts. The highest quantity of herbal drug Arnicae flos was reported for 5-year-old mountain arnica.

Conclusion

A comparative study on the cultivation of *A. montana* in two floristic regions in Bulgaria was carried out for the first time. The obtained results proved good acclimatization of the seedlings to the experimental fields at altitude of 1 400 m (Vitosha Mt.) and 1 500 m (Rhodopes Mts.). The *in vivo* plants were better adapted than those propagated by *in vitro* methods. The most important factors for *A. montana* sustainable cultivation were the climate and soil conditions. For the establishment of field plantation should be preferred flat terrain with northern exposure or a northern component. The soil should be well structured and slightly acidic (pH 6.1-6.5). The plants propagated by seeds passed into generative phase during the second year, while those

obtained by dividing of offspring rosettes - during the first year. The total duration of mountain arnica growing season was 143-147 days and the plants passed throughout five phenological phases. There were no significant morphological differences between the plants of both studied origins while *in vivo* and *in vitro* propagated plants had high parameters variation. The most variable was the parameter flower heads number per plant. The present study proves that *A. montana* could be cultivated successfully in the high-mountainous regions of Bulgaria.

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