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In vitro cultivation of some endemic and rare *Alchemilla* species in Bulgaria

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Abstract

Alchemilla achtarowii Pawł., *Alchemilla jumrukczalica* Pawł. and *A. bundericensis* Pawł. (Rosaceae) are medicinal plants, Bulgarian high-mountain endemics. *Alchemilla* species have a broad medicinal application and are being collected from their wild habitats, which threatens their populations. Along with their limited distribution, the ability of their populations for natural reproduction is suppressed by natural factors and human impact. The ex situ conservation measures taken include the maintenance of a living collection, as well as in vitro cultivation in order to enhance propagation. In the present work, the germination of seeds of the species was tested in vitro and was stimulated by treatment with gibberellic acid (GA_3). This treatment significantly increased the germination rate. The germination without using the growth regulator was lower than 50%, *A. mollis* having the highest and *A. bundericensis* – the lowest rate. The obtained seedlings were propagated by subcultivation on a MS-based medium containing 5,37 μM α-naphthaleneacetic acid (NAA) and 1,33 μM benzylaminopurine (BAP). The species differed in their propagation coefficients, *A. mollis* having the highest one. They also formed clusters of stems with varying length. The grown plants were subsequently adapted ex vitro and all surviving plants were successfully transferred to a mountain environment.

Key words: medicinal plants, *in vitro* germination, gibberellic acid, propagation coefficient

Introduction

Alchemilla achtarowii Pawł., *A. jumrukczalica* Pawł and *A. bundericensis* Pawł. (Rosaceae) are medicinal plants, Bulgarian high-mountain endemics, the former two occurring in the Central Balkan range and the latter one – in Pirin mountain. *A. mollis* (Buser) Rothm. is a medicinal plant, critically endangered in Bulgaria. The concentration of flavonoids and tannins in *A. mollis and A. jumrukczalica* is one of the highest among members of the genus (VITKOVA [1]). Due to these substances, *Alchemilla* species have a broad application in hematology and gynecology, for the treatment of myoma and chronic inflammation and kidney disease, for post-operation treatment as well as in veterinary medicine for the treatment of foot-and-mouth disease. Therefore the plants are being collected from their wild habitats, which threatens their populations. All four species are included in the Red data book of Bulgaria, the Red list of Bulgarian vascular plants, the Biodiversity act and the Medicinal plants act of Bulgaria. Most of their habitats are included in Natura 2000 sites, national and nature parks and reserves. Along with their limited distribution in Bulgaria, the ability of their populations for natural reproduction is limited by low pollen fertility (less than 10% in *A. achtarowii* (pers. comm.), low seed germination (unpublished data), slow growth and negative human impact (VITKOVA & al. [2]).

The complex studies on these species are a way towards their sustainable use and conservation by providing an alternative source of plant material to replace the collection from wild populations. Some *ex situ* conservation measures have been taken within the project "Complex study on high-mountain protected, endemic and rare medicinal plants from the Bulgarian flora – conservation and sustainable use" (DTK 02/38) supported by the National science fund. They include the maintenance of a living collection at the Institute of Biodiversity and Ecosystem Research (BAS), the establishment of *ex situ* collection on Vitosha Mt. and Rhodopes Mts., and *in vitro* cultivation as a tool for rapid plant propagation.

In the present work, the *in vitro* micropropagation of the studied *Alchemilla* species was tested, aiming at enhancement of the plants' multiplication and their *ex vitro* adaptation. Differences in seed germination and subcultivation efficiency were observed between species, and attempts to stimulate these processes were undertaken.

Material and methods

Seeds from the Bulgarian endemics *Alchemilla achtarowii, A. jumrukczalica,* and *A. bundericensis*, and the critically endangered for Bulgaria *A. mollis*, were collected in July 2010 from plants grown in the living collection of the Institute of biodiversity and ecosystem research in Sofia. Two hundred seeds from each of the tested species were used as plant material for *in vitro* cultures initiation.

Seeds were surface sterilized by consecutive treatment with 70% ethanol for 1 min and commercial bleach (chlorine <5%) for 10 min, followed by 30 min rinsing with sterile distilled water.

The germination of seeds was stimulated with both: stratification (4 weeks in refrigerator at 9°C), and soaking in 10 mM solution of gibberellic acid (GA_3) for 26 hours prior to the seed disinfection, in combination or applying them separately.

The seeds were germinated on MS-based (MURASHIGE & SKOOG [3]) agar-solidified medium with 6,5 g l^{-1} Plant agar (Duchefa®, Haarlem, The Netherlands) and 30 g l^{-1} sugar, in plastic containers with grids (Duchefa®), 50 seeds per container. The seedlings were then subcultured on the basic medium supplemented with 1,33 μM benzylaminopurine (BAP) and 5,37 μM α-naphthaleneacetic acid (NAA). This medium was used during all the period of *in vitro* plant multiplication. Successive subcultivations were applied every three months. The cultivation conditions were constant: 23±2°C and 16 h light daily, with intensity of 2000 lux.

The propagation efficiency was assessed by propagation coefficient (PC), calculated as number of obtained shoots per initial explant at every subcultivation.

Rooted plantlets were *ex vitro* adapted in mixture of soil, sand and coconut fibres in proportion 2:1:1, first at room conditions, covered with polyethylene foil progressively adapted to normal room humidity, than transferred to greenhouse, and finally planted in Vitosha Mt. near Sofia.

The germinating energy of the seeds from the different species, tested under equal conditions, was compared by a paired T-test; the differences concerning their germination efficiency and propagation coefficient were assessed by ANOVA single factor analysis based on three repetitions of 50 seeds or shoots each, respectively.

Results and discussions

***In vitro* seed germination**

According to our first *in vitro* tests with fresh seeds, the species differed in their seed germination rate, *A. mollis* having the highest rate, *A. achtarowii* an intermediate one, and *A. bundericensis* – the lowest one: 45%, 25%, and 16% of the seeds respectively, when stratification was applied alone. The combined application of low temperature and gibberellic acid had better effect on seed germination, resulting in 51%, 60%, and 28% germinated seeds of these species.

Interestingly, the best germination rate was reached when GA_3 was used the only stimulator without stratification, although the seeds were one year old. In this case the percentages of germinated seeds for the same duration were 87% for *A. achtarowii*, 77% for *A. bundericensis*, and 63% for *A. jumrukczalica*. Negative effect of the seed storage at low temperature on the germination potency was observed for *A. achtarowii* and *A. mollis* under non-sterile laboratory conditions, as well (unpublished data). Possibly, in the nature germination occurs long before the winter cold.

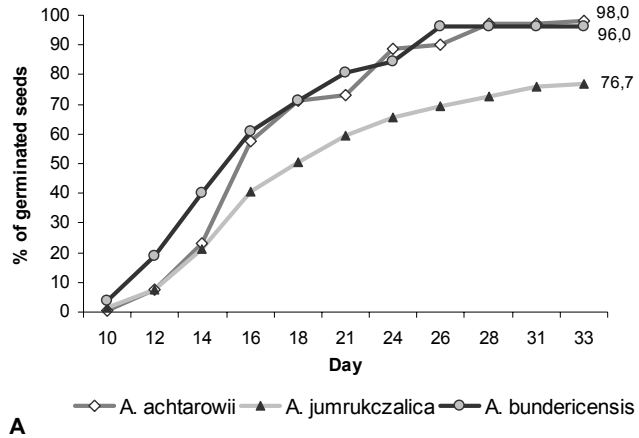
At sterilization, most of the soaked seeds sunk to the bottom of the vial, but near ¼ of them were floating on the solution surface despite the repeatedly stirring up. The floating seeds were supposed to be empty or damaged therefore they were germinated separately. Indeed, the difference between the percentages of the germinated seeds in the two groups was obvious (Table 1). Almost all sunken seeds of *A. achtarowii* and *A. bundericensis* germinated (Fig. 1-A). These two species had similar germinating efficiency while *A. jumrukczalica* significantly differed from them ($P \leq 0{,}001$, single-factor ANOVA).

The germination energy of the three species was similar (T-test paired); the very first germinating seeds were observed on the tenth day, the great mass of seeds germinated between the 14th and the 18th days, and less than 10% of all germinated seeds started the process after the 26th day (Fig. 1-B). Some seeds stopped their development at the cotyledon stage; however, their number was relatively low.

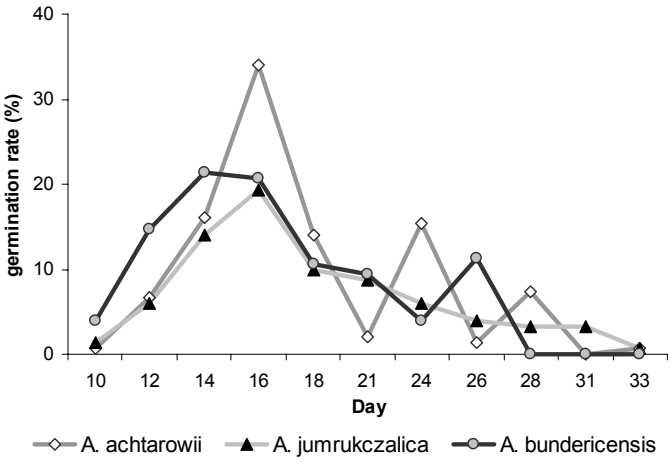
Table 1. Germinated seeds and seedlings with cotyledons and leaves of four *Alchemilla* species, obtained for one month, after stimulation with 10 mM gibberellic acid.

Species	Seeds			% of germinated seeds*	% of seedlings with leaves
	Total number	Features	% of all seeds		
A. achtarowii	200	sunken	76,9	98,0 a	94,0
		floating	23,1	48,9	48,9
A. jumrukczalica	200	sunken	81,0	76,7 b	72,7
		floating	19,0	19,1	8,5
A. bundericensis	200	sunken	75,4	96,0 a	82,7
		floating	24,6	18,4	12,2
A. mollis	200	sunken	77,7	N/A**	N/A**
		floating	22,3	41,9	32,6

* Significant differences assessed with ANOVA single factor are marked with different letters; ** This variant was discarded because of a bacterial contamination.



A



B

Fig. 1. *In vitro* seed germination of 3 endemic for Bulgaria *Alchemilla* species, after stimulation with 0.35% of geberellic acid: A) Percentage of germinated seeds; B) Germination energy.

In vitro plant multiplication

In vitro seedlings at stage three to five leaves were subcultured to upper and lower stem segments on fresh medium supplemented with BAP and NAA after removing of their roots. New shoots appeared at the base of the stem cuttings (direct organogenesis), grew, and were used as explants for further shoot multiplication. The optimal duration between two passages with subcultivation was found to be three months. At the end of this period shoots rooted spontaneously and their size allowed their separation from one another.

Cluster non-uniformity was typical for *Alchemilla in vitro* cultures. Even after germination, before any treatment, 23% of the seedlings grew an adventitious stem. Usually, the shoot explant was growing faster than the few new shoots originated from it; however, some shoots were withering, giving rise to numerous thin shoots which needed more time to gain strength (Fig. 2). The biggest shoots, with stem diameter about 2 mm and stem length up to 2 cm, were cut to stem segments in order to obtain more explants while the smaller ones were used whole. The number of the shoots per shoot-cluster depended on the explant type, the lower stem segments originating many new shoots in consequence of the apical dominance breakage, the top stem segments giving rise to only one or few shoots (STANILOVA & al. [4]). The number of leaves per *in vitro* plant varied as well (Fig. 3). Once rooted, regenerated plantlets grew faster, especially their leaf blades, and obtained dark green color.

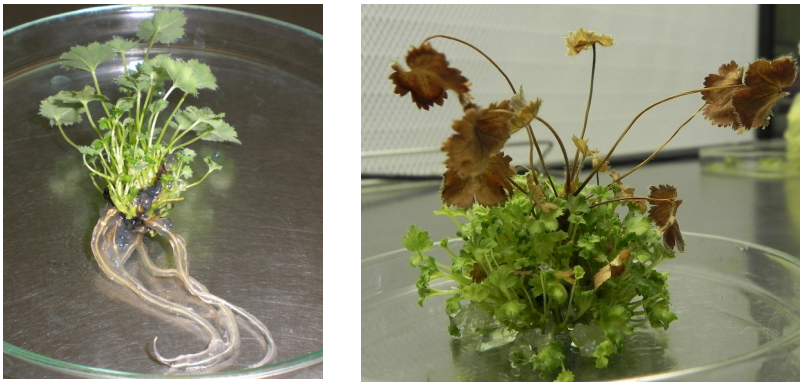


Fig. 2. A cluster of stems of different length (left); a large shoot cluster with a dead main stem and numerous new small stems.

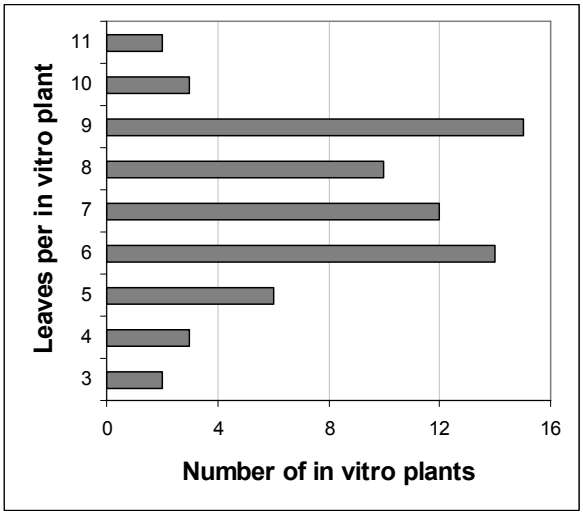


Fig. 3. Non-uniformity of *in vitro* culture of *Alchemilla achtarowii*, consisting of plants with different number of leaves and different stem length, after 9 months of cultivation.

The species differed in their propagation coefficients, *A. mollis* having the highest one. The PC was different depending on how the plants were subcultivated: if the stems were not only separated from each other, but were also cut in smaller segments, the coefficient increased (up to 3,3 compared

to 2,5 for the separated stems). However, these segments required longer time to grow than the separated stems and some of them did not survive, so the separation of stems alone proved to be more efficient. Also, the PC increased with every subcultivation, but the differences between the species were preserved (Table 2). At the third subcultivation (after 9 months), the differences, assessed by the single-factor ANOVA, were very significant (P = 0,001). For that period, 32 plants were obtained from a single seeds of *A. mollis*. The number of plants increases exponentially with every subcultivation. The work on *Alchemilla jumrukczalica* started at a later stage, so the data is still not available.

Table 2. Propagation coefficient of the three species at the third subcultivation (after 9 months).

Species / PC	*A. achtarowii*	*A. bundericensis*	*A. mollis*
Repetition 1	4,3	2,3	7,0
Repetition 2	6,0	3,0	7,1
Repetition 3	6,2	2,8	6,2
Average PC	**5,4±1,0**	**2,7±0,4**	**6,7±0,5**

Ex vitro adaptation

For the adaptation, single plants with average stem length of 1,4 ± 0,3 cm and root length of 5,9 ± 0,7 cm, or plant clusters with common roots were used. The number of surviving plants was still low, due to low-humidity stress during the first weeks of the *ex vitro* adaptation, and fungal contamination. Once adapted to ambient conditions in pots, plants were easily transferred to the Vitosha Mt. with a 100% rate of survival (currently 85 *A. mollis* and 11 *A. achtarowii* plants – Fig. 4).

Fig. 4. Plants acclimated to ambient conditions in Vitosha mountain (1450 m a.s.l.)

Conclusions

Successful *in vitro* germination in all four species was achieved at a high rate (98% in *A. achtarowii*) by stimulation with gibberellic acid. The plants formed non-uniform clusters of stems with varying length. The optimal duration between two subcultivations was found to be three months. The propagation coefficient proved to be species-dependent and was as high as 6,7±0,5 (*A. mollis*, for a period of 3 months). For nine months, 32 plants were obtained from a single seed of that species. The plants rooted and were adapted to *ex vitro* conditions, where the adjustment of air humidity was of great importance for their survival. Currently, 96 *A. mollis* and *A. achtarowii* plants altogether are acclimated to ambient conditions in Vitosha mountain.

Acknowledgement

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