Annuaire de l'Université de Sofia "St. Kliment Ohridski"
Faculte de Biologie
First National Conference of
Reintroduction of Conservation-reliant Species, Sofia 2015
University Press 2016, pp. 63-70

PRESERVATION OF THE BULGARIAN ENDEMIC VERBASCUM DAVIDOFFII (*SCROPHULARIACEAE*) BY MEANS OF *IN VITRO* PROPAGATION

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Keywords: plant biotechnology, seed germination, endangered plants, reintroduction

Abstract: *Verbascum davidoffii* Murb. (*Scrophulariaceae*) is one of the rarest plant species in the Bulgarian flora. It is a Bulgarian endemic, protected by the national Biodiversity Act (2002), included in the Red List of vascular plants in Bulgaria and in the Red Data Book of Bulgaria with conservation status "Critically Endangered". The populations of this biennial herbaceous plant consist of sparse individuals and are severely fragmented; all known localities are within Pirin National Park and in a site of the European ecological network Natura 2000 in Bulgaria, between the valleys of Banderitsa River and Razlozhki Suhodol, at an altitude of 1800 up to 2300 m a.s.l. The distribution of *V. davidoffii* is limited due to its peculiar biology related to specific ecological requirements and low reproductive capability, as well as to the anthropogenic pressure: forest felling, destruction and pollution of the habitats because of tourism and infrastructure development in the region.

In order to preserve this species of conservation value, *ex situ* and *in situ* activities have been designed concerning elaboration of a specific protocol for *in vitro* propagation, followed by establishment of an *ex situ* collection, and strengthening of the wild populations. As a first step, seed germination has been studied. Seeds were gathered in August 2015 from the locality close to the "Banderitsa" rest-house and were successfully disinfected through a standard laboratory procedure. Seed germination was poor on the basal MS medium: only 1 seedling was obtained from 100 seeds for a period of 6 weeks; and no seed germinated on MS medium supplemented with 1 mg/l Kin. The stimulation of the process by seeds soaking in 0,35 % solution of gibberellic acid for 22 hours increased the germination rate up to 61 % and 18 % for the two media, respectively. The effect of the gibberellic acid was strong even if applied for only 2 h, and the concentration of kinetin was better when supplemented in 10-fold less concentration. Seed stratification with low temperature at 6°C for a month prior to cultivation had additional effect on germination which depended on the presence of kinetin in the medium. *In vitro* seedlings with several leaves and roots were potted in soil substrate of soil mixture, sand and coconut fiber (2:1:1), and easily *ex vitro* adapted into the ambience of the laboratory phytotron, under controlled temperature, light, and humidity variations.

INTRODUCTION

Verbascum davidoffii Murb. (Scrophulariaceae) is a biennial herbaceous plant with erect unbranched stem, densely haired leaves, calyx covered with long glandular black hairs, and with golden yellow flowers (Stefanova-Gateva, 1995). It is a Bulgarian endemic (Petrova, 2006), protected by the National Biodiversity Act (2002), included in the Red List of vascular plants in Bulgaria (Assyov & Denchev, 2009) and in the Red Data Book of the Republic of Bulgaria (Assyov & Denchev, 2015) classified as "Critically Endangered" acording to the IUCN criteria. All known localities are within Pirin National Park, some of them in Bayuvi Dupki-Dzhindzhiritsa Strict Nature Reserve, and in a protected site of the European ecological network Natura 2000 in Bulgaria.

The population is represented by several subpopulations, located between the Valley of Banderitsa River and Razlozhki Suhodol peak, at an altitude of 1800 to 2300 m alt. Habitats are calcareous, stony and grassy places in subalpine belts and in open forests of *Pinus heldreichii.* The distribution of *V. davidoffii* is limited due to its peculiar biology (reproduction by seeds), related to specific ecological requirements and low reproductive capability, as well as to the anthropogenic pressure: forest felling, destruction and pollution of the habitats because of tourism and infrastructure development in the region. According to the monitoring of the population in 2014, the species is currently in unfavorable-bad condition. This is due to the small number of individuals, established on an area of 30 hectares.

In order to preserve this species of conservation value, *ex situ* and *in situ* activities have been designed concerning elaboration of a specific protocol for *in vitro* propagation, followed by establishment of *ex situ* collection, and strengthening of the wild populations.

MATERIALS AND METHODS

The monitoring of *V. davidoffii* was held in July 2015 according to the approved monitoring methodology, developed for the needs of NSEM (National System for Environmental Monitoring).

Seeds were gathered in August 2015 from the locality close to the "Banderitsa" rest-house. Their germination capability was tested immediately as well as after one month of stratification with low temperature at 6°C. The two sets consisted of 400 seeds each, divided in four groups, to test additional stimulation with both gibberellic acid (GA_3) and / or Kinetin (Kin). Seeds were soaked in 0,35 % solution of GA_3 for 22 h before sterilizing. Then, they were disinfected through standard laboratory procedure (consecutive soaking in 70 % ethanol for 1 min, and in commercial bleach, chlorine < 5 %, for 10 min, followed by thrice rinses in sterile distilled water, 10 min each). The plant growth regulator Kin was added to the nutrient medium in 2 concentrations: 1 mg/l or 0,1 mg/l (media MS-K1 and

MS-K01). Basal MS medium (Murashige and Skoog, 1962) and seed soaking in tap water instead of gibberellic acid, was used as a control variant. Additionally, the effect of GA_3 was studied for shorter time: 2 h of seed treatment, only for MS basal medium.

The germination rate was calculated as an average percentage of germinated seeds on the basis of two repetitions of 50 seeds per variant, for a period of 4 weeks.

In order to compare the germination under different laboratory conditions, seeds were treated with GA_3 as described above (or water in the control variant) and placed either in petri dishes supplied bilaterally with wet filter paper, 25 seeds per petri dish, 3 replications per variant, or in a special terrine of 96 small pots with soil substrate, covered by polyethylene folio to maintain suitable air humidity.

The bigger 35 seedlings were *ex vitro* adapted in soil substrate consisting of soil mixture, sand, and coconut fibers in proportion 2:1:1, two plantlets per pot with diameter of 9 cm. The first step of the adaptation was conducted in a growth camera (POL-EKO Aparatura) for 4 weeks, under strict control of the important ambient conditions, simulating their natural daily dynamic: 10 h "day" under 2070 lx white light at 23°C, 8 h "night" in a dark, at 18°C, and two intermediate periods of 3 h each, under 1500 lx at 20°C. Plants are currently in the second step of the *ex vitro* adaptation, on the shelves of a room phytotron with a window, with less strict control of the temperature (22 ± 4°C), air humidity (between 35 and 60 %), and light (mixed: daylight and artificial light provided by warm white LED bands 16 h per day).

All the other seedlings were sub-cultured on fresh media supplemented with Kin or 6-benzylaminopurine (BAP) in combination with α-naphthaleneacetate (NAA) or Indole-3-butyric acid (IBA) to stimulate further shoot multiplication.

RESULTS AND DISCUSSION

The results of the monitoring, conducted in July 2015, showed that in an area of about 5 ha, including the transect route Vihren hut-Banderitsa hut-Malkiya Kazan locality, *V. davidoffii* was represented with 52 adult and 16 vegetative individuals. In comparison, the data of NSEM concerning the species in 2014 reported 30 flowering and 15 juvenile plants counted between the huts of Banderitsa and Vihren (Karakiev, unpublished). In view of the size of the inspected area which was about twice larger in 2015, the numbers of the *V. davidoffii* population was similar. The variable correlation between flowering and new individuals along the years depended on seed propagation success of this biannual plant species. Less successful seed germination resulted in less new individuals next year and superiority of the adult flowering plants over the new ones.

Because of the low competitiveness of the species, plants were noticed mainly along the asphalt road between the huts of Banderitsa and Vihren or on other open sites such as stony and erosive terrains on the path connecting the Banderitsa and the Kazan localities.

Non-stimulated seed germination under laboratory conditions was poor: only one seedling was obtained on the control MS medium from 100 seeds for a period of 4 weeks. The stimulation of the process by seeds soaking in 0,35 % solution of gibberellic acid for 22 hours increased the germination rate to 61 % on the basal MS medium (Fig. 1). The effect of the gibberellic acid when applied for 2 h was significant but less strong, in this case 28 % of the seeds germinated (Fig. 2). The stratification slightly enhanced the stimulation effect of GA_3, and seed germination reached 66,1 % on the basal MS medium. In comparison, the seed stratification applied alone was useless for *V. davidoffii* as only 3 % of the seeds soaked in water germinated on MS medium.

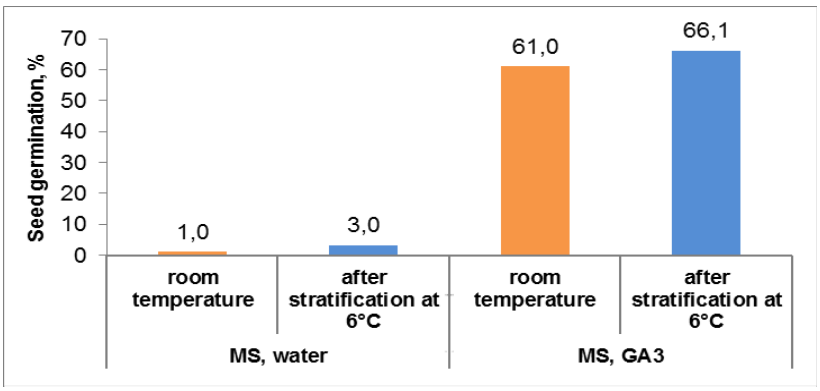

Fig. 1. Germination of *V. davidoffii* seeds on basal MS medium, with or without stratification and pretreatment with GA_3.

Fig. 2. Germination of *V. davidoffii* seeds on basal MS medium after soaking in: **A**) water, for 22 h; **B**) 0,35 % GA_3 for 22 h; **C**) 0,35 % GA_3 for 2 h.

No seed germinated on medium MS-K1 supplemented with 1 mg/l Kin (Fig. 3). Furthermore, the presence of kinetin in the medium inhibited the process as it caused both delayed germination and decreased effect of GA_3, and only 18 % of the seeds germinated on medium MS-K1. To decrease the negative influence of kinetin, the medium supplemented with this plant growth regulator was modified, its concentration being reduced to 0,1 mg/l. The use of Kin in a 10-fold lower concentration in the medium had a significant stimulation effect. Thus, 29,2 % of the seeds soaked in water germinated on medium MS-K01 which was about ten times more compared to that of the basal MS medium (Fig. 3 & Fig. 1). It is possible that the stratification had an additive influence. The combined effect of GA_3 and 0,1 mg/l Kin resulted in additional increase of the germination, up to 63,2 %. The highest germination rate of 66,1 % was however observed in the variant without Kin, when seeds were pretreated with both one-month low temperature at 6°C and soaked in 0,35 % GA_3 for 22 h.

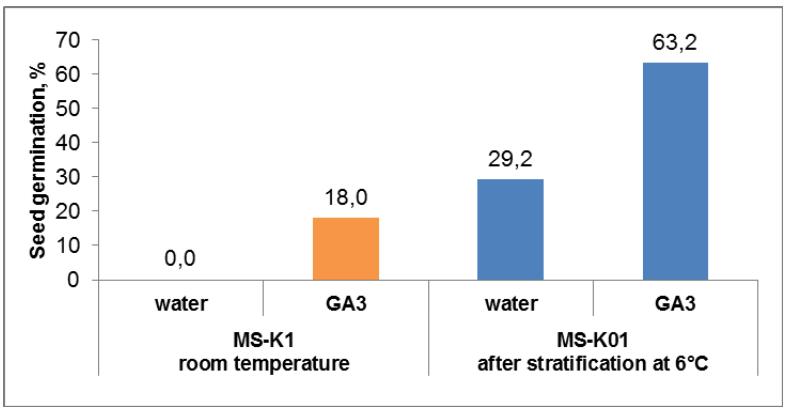

Fig. 3. Germination of *V. davidoffii* seeds on media supplemented with different concentrations of Kin, with or without stratification and pretreatment with GA_3.

It is worth to mention that a part of the seedlings had etiolated and deformed leaves due to the long seed treatment with GA_3 and finally 8,2 % of them were lost. This effect was not observed in the case of the short seeds soaking. In all variants on basal MS medium seed germination began in the first week when stimulated with GA_3 pretreatment and in the second week without its usage. The end of the germination was noticed after 3 weeks. In the variant of medium MS-K1 the beginning and the end of the process were delayed with 2 weeks. The stratification with low temperature enhanced the germination time with about one week in all tested variants.

Seeds placed in the terrine with soil substrate had similar germination rates to those of the *in vitro* germinated seeds. No seed germinated during the first

4 weeks when soaked in water, while 60 % of the seeds pretreated with GA_3 germinated. The highest seed germination rates were noticed in the petri dishes: 72,7 % when pretreated with GA_3 for 22 h and 9,4 % when soaked in water. However, these seeds were tiny, and their survival was problematic.

Low seed germination is a common problem for many endangered species and endemics. There are different approaches for increasing the germination rates or for the seed dormancy break: treatment with plant growth regulators, stratification with high or low temperature, application of red light or different light regime, etc.

The effect of some of these factors was tested on two *Verbascum* species endemic for Turkey: *V. bithynicum* and *V. wiedemannianum* (Senel et al., 2007). Contrariwise to the authors expectations, GA_3 prevented their seed germination even if applied in very low concentration, between 20 and 200 mg/l. To our opinion this was due to the application way: seeds were incubated in petri dishes with the GA_3 solution. We also noticed the inhibitory effect of the gibberellic acid on seed germination of another Bulgarian endemic of this genus, *Verbascum tzar-borisii*, when added in the nutrient medium (unpublished data).

Gibberellic acid is frequently used to pretreat seeds of many species in order to stimulate their germination, by seed soaking for several hours prior to cultivation (Kabar, 1990; Srivastava et al., 2011; Roychowdhury et al., 2012). It was successfully applied in a large concentration range for different time. The usual way of kinetin application is also as solution for seed pretreatment (Kabar, 1990; Roychowdhury et al., 2012).

The results of our study showed that the gibberellic acid was the most important stimulator of the germination of *V. davidoffii* seeds. The effect of Kin was less expressed and ambiguous. The reason for this difference could however be due to the way of the application of the two plant growth regulators: we tested GA_3 only as pretreating solution while Kin was added in the nutrient medium.

Different plant growth regulators: NAA, IAA, GA_3, as well as some simple combinations like KNO_3, HNO_3, HCl, H_2SO_4 were found to have various stimulating effect on seed germination when applied as solutions for seed pretreatment (Srivastava et al., 2011). Their effect depended on the concentration, the time of seed soaking, and the temperature. The seed response differed also from one plant species to the other.

Other authors also tested combinations of GA_3 and Kin on seed germination. The effect depended on the species as well as on the way of application. Thus, the germination of the lettuce seeds was enhanced by the combination of kinetin and suboptimal concentration of gibberellic acid (Ikuma and Thimann, 1963) while the same combination applied for 72 h caused a slight inhibitory effect on dark-grown *Lepidium* seeds (Evans and Fratianne, 1977). Different effects of these two plant growth regulators were noticed when applied to several dicots and monocots under saline conditions. Kinetin was more effective for seed germination

enhancement of most tested dicots, and the combination of GA_3 and Kin did not exceed its effect (Kabar, 1990). Even seeds with relatively high germination rate were influenced by solutions of GA_3, Kin, and IAA (Roychowdhury et al., 2012). Authors found out the optimal concentration of each of these stimulants and revealed their inhibitory effect in higher and lower concentrations.

Seedlings in all the variants were not uniform because seeds needed different time to germinate. The faster growing *in vitro* seedlings with several leaves and roots each were ready to be *ex vitro* adapted 8 weeks after the beginning of the experiment. They were easily adapted to the used soil mixture in the growth chamber with a strict control of temperature, humidity and light regime. The gradual decrease of the air humidity is of crucial importance for most of the *in vitro* obtained plantlets. The drastic difference of the survival rate due to the air humidity was reported in our previous work on other species (Stanilova et al., 2013). In the case of *V. davidoffii* 31 plants of the first 35 survived, strengthened and grew enough to be transferred to the shelves of the room phytotron after 4 weeks (Fig. 4). All of them are growing well and will be further used for establishment of an *ex situ* collection and for reintroduction in their population of origin.

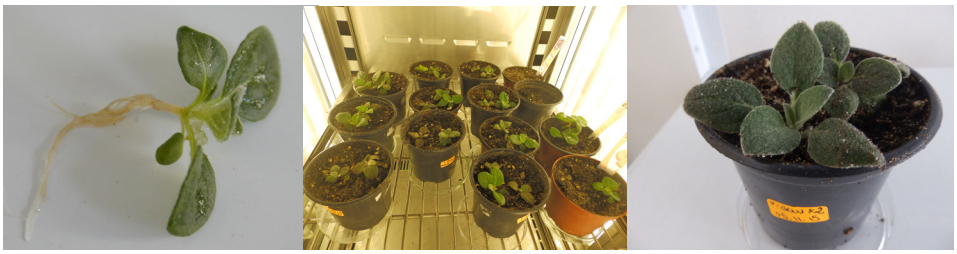

Fig. 4. *Ex vitro* adaptation of *V. davidoffii*: **A**) In vitro obtained 8-week aged seedling; **B**) Potted in vitro plants, in the growth chamber; **C**) Plants on the shelves of the room phytotron

CONCLUSIONS

In vitro cultures of *Verbascum davidoffii* have been successfully initiated. The seed germination rate reached its maximum of 66,1 % when seeds were placed on basal MS medium after being pretreated with both: one month stratification at 6°C, and 22 h soaking in 0,35 % solution of gibberellic acid. The first 31 *ex vitro* adapted plants are currently growing well in the phytotron. They will be used for establishment of an *ex situ* collection and for reintroduction in their population of origin.

The study will continue with sub-cultivation of the seedlings on fresh media with different compositions, supplemented with Kin or BAP in combination with NAA or IBA, to test their effect on shoot multiplication. The reproductive peculiarities of *Verbascum davidoffii* will be clarified, as well as the genetic diversity of the populations and the genetic fidelity of the *in vitro* multiplied plants.

Acknowledgment: This work was supported by grant DFNI-BO2/18 of the Bulgarian National Science Fund.

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