Scientific Papers. Series B, Horticulture. Vol. LXIV, No. 1, 2020
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

POSSIBILITIES FOR *EX SITU* CONSERVATION OF BULGARIAN ENDEMIC *BETONICA BULGARICA* DEGEN. & NEIČ.

Neli GROZEVA[1*], Galya PANAYOTOVA[2], Mariya GERDZHIKOVA[2], Mima TODOROVA[2]

[1]Trakia University, Faculty of Agriculture, Department of Biology and Aquaculture, BG-6000, Stara Zagora, Bulgaria
[2]Trakia University, Faculty of Agriculture, Department of Plant Growing, BG-6000, Stara Zagora, Bulgaria

*Corresponding author email: grozeva@uni-sz.bg

Abstract

The Bulgarian endemic Betonica bulgarica Degen. & Neič. is a protected species included in the Red Data Book of the Republik of Bulgaria vol.1. Plants and fungi. On the territory of "Sinite Kamani" Natural Park it forms populations in Ablanovo area, Slancheva polyana area, Upper lift station area and Karandila area. Main threats to the populations in the Park are the anthropogenic impact, difficult seed reproduction, soil erosion processes and the spread of eagle fern (Pteridium aquilinum) in the border areas of the population in Slancheva polyana area. According to evaluations, both in-situ, and ex situ conservation measures should be included for the protection and stabilization of populations of Betonica bulgarica. The aim of this study was to develop a technology for growing species from mature seeds in laboratory conditions. In order to realize the objective, a vast amount of literature was studied and biennial field research of the populations in the "Sinite Kamani" Natural Park was conducted. The developed technology is successful and fully complies with the environmental conditions of the natural habitat of the species in the Park. Using it, the species has been successfully propagated in the scientific laboratories of the Faculty of Agriculture at Trakia University from mature seeds to 6-8 leaves (phenophase) for replenishment of the populations. The technology can be used to replenish other natural populations of Betonica bulgarica in Bulgaria. In order to determine the effectiveness of the application of this measure for ex-situ conservation is necessary to continue observations after introduction of the plants grown in laboratory conditions, to follow their adaptation and further development, and if necessary to protect them and stabilize their condition.

Key words*: Betonica bulgarica Degen. & Neič., "Sinite Kamani" Natural Park, ex situ conservation.*

INTRODUCTION

The Bulgarian endemic *Betonica bulgarica* Degen. & Neič. is a species protected by the Biological Diversity Act (2002), included in the Red Data Book of the Republic of Bulgaria vol. 1. Plants and fungi in the category "endangered" (Genova, 2011). Main threats for the populations are trampling, grazing or collection of the aboveground and underground parts of the plant for medicinal purposes (Koeva, 1984; 1989; Genova, 2011). Genova (2011) reported that the conservation of the species *Betonica bulgarica* in the country would require studying the biology and ecology of the species, determining the numbers and area of the populations and the possibilities for cultivation, as well as collecting seeds for the National Seed Genebank in Sadovo.

The present study is part of project No 5103020-15-658 "Restoration of the habitats and conservation of the biological diversity in "Sinite Kamani" Natural Park. One of the aims of the project is *ex situ* conservation of protected and endemic plant species, located on the territory of "Sinite Kamani" Natural Park, in order to stabilize their populations.

According to preliminarily conducted biennial field research one of the species, whose conservation in the Park requires the use of *ex-situ* measures for conservation, is *Betonica bulgarica.* On the territory of "Sinite Kamani" Natural Park, the Bulgarian endemic was first described by Grozeva et al. (2004) with one population in Ablanovo area. Later three more populations were registered - in Slancheva polyana area, near Upper lift station and east of Microyazovir in Karandila area (Grozeva et al., 2014). The assessment of their condition showed that main threats for the species on the territory of the Park were: anthropogenic impact, difficult seed reproduction, the spread of eagle fern in the border areas of the

population in Slancheva polyana area, and erosion processes (Grozeva et al., 2014).
The aim of this study was to develop a technology for growing *Betonica bulgarica* from mature seeds in laboratory conditions in order to replenish and stabilize the populations of the species in "Sinite Kamani" Natural Park.

MATERIALS AND METHODS

For the development of the technology for growing *Betonica bulgarica* were used various literary sources (Hayek, 1929; Yankulov, 1964; 2000; Medicinal plants 2001; Grozeva et al., 2004; Panayotova et al., 2014) and data from conducted observations and performed analyses related to the realization of project No 5103020-15-658 "Restoration of the habitats and conservation of the biological diversity in "Sinite Kamani" Natural Park.
The morphological characteristic of the species corresponds with the one indicated in the Flora of the Republic of Bulgaria (Koeva, 1989) and the Red Data Book of the Republic of Bulgaria vol.1 (Genova, 2011).
The laboratory analyses were conducted in the scientific laboratories of the Faculty of Agriculture, Trakia University - Stara Zagora.
The mature seeds were taken from a population of the species in Ablanovo area after receiving a permit from the Minister of Environment and Water. In accordance with the permit conditions, 250 seeds were given for storage in the National Seed Genebank in Institute of Plant Genetic Resources "Konstantin Malkov" - Sadovo.
To prepare the soil for planting were used the data from the soil analyses of each population (Grozeva et al., 2014).
All actions related to the collecting of mature seeds, growing them in laboratory conditions and returning the grown plants in the natural populations of the species in "Sinite Kamani" Natural Park are in compliance with the Protected Areas Act (PAA, 1998), the Biological Diversity Act (BDA, 2002) and Ordinance No 8.

RESULTS AND DISCUSSIONS

Morphology and biology of the species

Betonica bulgarica is a perennial plant from the family *Lamiaceae* (Figure 1). It has a horizontal rhizome. The stem is erect, four-edged, with no branches, covered with bristles facing down.
The leaves are opposite each other. Their form is oblong ovate, heart-shaped at base, coarsely crenate to crenate-dentate at the edge, on both sides pubescent. The leaves at the base have stalks longer than the lamina, the 2-3 pairs of stem leaves have short stalks and the upper leaves are sessile. The inflorescence has concise and densely spiked raceme, rarely the lowest is separate. Bracts are of equal length to the calyx, lanceolate at the top, barbed at the edge and the veins - fibrous. The calyx is 8-9 mm long, almost bare or bare only at the base, from the middle upward - densely fibrous with long, white, hard bristles; the tube is 5 mm long; the teeth - narrowly triangular, 3-4 mm long, hairy at base, bare upward with a long apical spine.

Figure 1. *Betonica bulgarica* - general view (photo N. Grozeva)

The petal is pink-purple, layered with white bristles; the tube is narrow, slightly curved, 9-10 mm long, the upper lip flat or slightly bulging, entire or furcate at the apex, 4 mm long, the lower lip trilobite, 6 mm long, the middle part is repand and large at the edge, the lateral parts are small and ovate. There are 4 stamens with fibrous handles.

The fruit is coenobium, thereof named tetraeremum based on nutlets number. The nutlets are brown, triangular, elongated, 4 mm long, 2 mm wide, the outside almost flat, the edges with narrow wings, which at the top edge go into irregularly toothed membranous appendage.
Flowering in July-August, fruiting in August-September. The pollination is entomophilous. The species reproduces by seeds and vegetatively.

Natural habitat
The species inhabits Central and East Balkan range and Thracian lowlands on open grass lands in the oak and beech forest belts at an altitude from 540 to 1500 m (Genova, 2011; Asyov et al., 2012; Grozeva et al., 2014). It grows on Chromic Luvisols, Eutric Cambisols and Rendzinas, moderate structured soils with crumb structural aggregates and on soil texture from sandy clay loam to silty clay loam. It grows successfully both in acidic soil reaction with pH values (H_2O) = 4.23, and in slightly acidic to neutral reaction with pH values (H_2O) of 6.01 to 7.04. It dwells on soils with low mineral nitrogen content, poorly to well stocked with available forms of phosphorus and with high available potassium content. The presence of carbonates in the soil does not affect negatively the development of the species.

Environmental requirements
Betonica bulgarica is a sun-loving plant but can endure some shade. It prefers open sunlit meadows near forests. It is a cold-hardy plant. It can endure low temperatures during the winter-spring period. The species isn't particularly demanding regarding soil moisture - it's drought-tolerant but it can develop well in more humid places. It has no specific requirements for the soils, as long as they have good drainage. It can grow on Chromic Luvisols, Eutric Cambisols and Rendzinas, moderate structured soils with crumb structural aggregates and on soil texture from sandy clay loam to slity clay loam, as well as stony and sandy ground. It prefers soils with neutral or lightly acidic reaction, however our studies (Grozeva et al., 2014) show that it can grow successfully in acidic soil reaction.

Actions for growing *Betonica bulgarica*
The species propagates by seeds and vegetatively by dividing the root.
In laboratory condition it is propagated by seeds. The seeds are collected the previous autumn from the natural habitats of the plant. Isolation bags are placed on the racemes after the flowers wilt (Figure 2 A). The isolators are collected after the seeds mature, when the stems have fully dried and the fruit separate by themselves (Figure 2 B). The racemes are shed from the isolators (Figure 2 C) and the seeds checked for mechanical and biological mixes (Figure 2 D).
The seeds are cleaned manually.
To determine the seed sowing qualities a mean sample is taken according to the method of split middle diagonal sample.
The appearance of the batch of seeds is inspected for colour, smell, shine, etc. The health status of the seeds regarding diseases and pests is determined through the use of microscopic technology (Figure 2 E). The seeds collected from *Betonica bulgarica* should be stored in tightly closed, if possible sealed containers labeled with the necessary information for the population, date and year of acquiring the seeds (Figure 2 F), in order to ease their future use. Additionally, the net/gross weight or number of seeds can be indicated, as well as the name and address of the person responsible for the labeling and storing.
The moisture of the seeds is of vital importance. They will retain good sowing qualities if they are well dried and stored in appropriate places and low positive temperature from 1 to 5 °C and air humidity at around 50-55% is maintained. The seed qualities will worsen if a significant increase or drop in the air temperature is allowed. In order to remove the excess moisture, immediately before storing the seeds, they must be spread on a sheet of paper and sun dried for 1-2 hours on dry and sunny days with temperature of the air in the shade around 20°C. At this temperature they are not at risk to lose germination. It is best to store them in cloth or paper bags. If the seeds are well dried, they can also be stored in well closed, fully dry on the inside glass containers (jars, bottles), filled 2/3 of their volume or in well-closed box. Beads or an entire packet of Silica gel can be placed in the containers, in

order to suck up the moisture if any occurs. This way the valuable seeds can be preserved for longer without disturbing their sowing qualities. It's preferable that during the period of storage the containers should be opened only if necessary, for example to check the health status of the seeds. Frequent opening or change of containers can lower the sowing qualities and germination characteristics of the seeds. If there is the faint odour of mold or a weird smell, when opening the containers, this is a sign for a disease or unfitness of the seeds. For longer term storage the seeds can be stored in a fridge, sealed well, at temperature between 2-5°C.

The soil for sowing the seeds is prepared according to the data from the soil samples, taken from the natural habitats of the species. Before placing it pots or plastic containers, it should be made friable and sieved to remove rocks and large lumps Figure 2 G).

Betonica bulgarica has no special requirements regarding the soil fertility and can be grown with no fertilizer but for better development of the plants it is best to use organic or mineral fertilizers. From the organic ones decomposed manure can be used which should be mixed with the soil before planting of the seeds, ratio soil: fertilizer = 2: 1. From the industrial fertilizers - superphosphate 60-80 kg/ha, potassium sulfate 40-60 kg/ha and others can be used, added to the soil before planting, and a nitrogen fertilizer in amount of 100-120 kg/ha like ammonium nitrate or a different fertilizer is applied in the soil during the preparation of the soil and like water solution when watering the pots several times after planting the seeds. Complex foliar fertilizers containing the necessary macroelements also have a good influence on the growth and development. The exact amount of fertilizer can be determined according to the results of the soil sample analyses and the condition of the plants.

The seeds are sown from the middle of January to the beginning of March. Due to the prolonged slower germination period and low germination (35%), there are two possibilities for sowing - sowing the mature seeds at 1-1.5 cm in the soil (Figure 2 H) or sowing germinated in Petri dishes seeds (Figure 3 A, B). Panayotova et al. (2014) found that *B. bulgarica* was characterized by a prolonged period of germination and low rate of germination - 35.0%.

Figure 2. A - placing of isolators; B-C - collecting of isolators and shelling of seeds mature; D - seeds free of mechanical pulp; E - checking the health status of the seed; F - packaging and labeling; G - soil preparation for sowing; H - sowing the seeds matur

Sowing the germinated seeds reduces the costs for preparing the fertilizer-soil mix and the care until sprouting, including the use of nitrogen fertilizer. After planting the seeds must be watered with water or water solution of nitrogen fertilizer. The differences in the development of the plants in both types of sowing have not been discovered as the sown germinated seeds go through a period of adaptation, and the directly sown seeds sprout during that time (Figure 3 C).

The care during vegetation is related to ensuring optimum conditions for the development of the plants. The soil is made arable and periodically watered. Plant nutrition can be done with combined fertilizers two or three times, during the cotyledon phase (Figure 3 D), the formation of four (Figure 3 E), and 6-8 true leaves (Figure 3 F). Any weeds which appear should be removed manually. Economically important diseases and pests

were not discovered but if needed it can be used authorized plant protection products.
The plants are grown in laboratory conditions until they form a rosette of 6-8 leaves (Figure 3 G). The plants should be returned to their natural habitats during autumn and/or spring (Figure 3 H). The planted young plants should be observed and watered, if they are dug out by wild boars - they should be pressed into the soil again until their condition stabilizes.

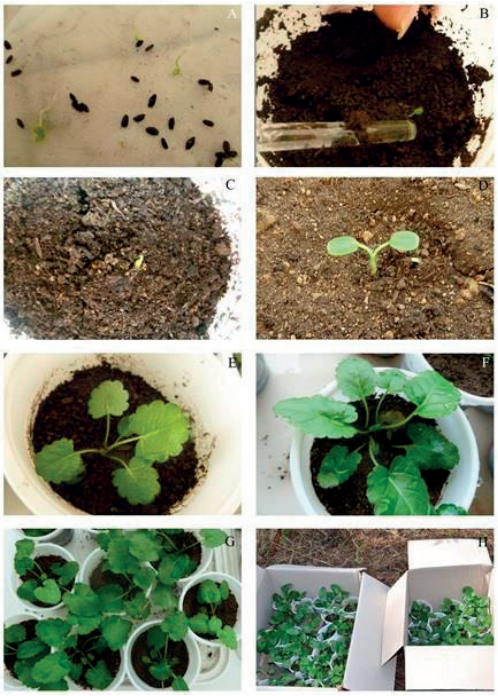

Figure 3. A - germination of seeds in Petri plates; B - planting seeds germinated; C - germination of seeds sown; D - stage cotyledons; E - 4-5 leaves stage; F-G - 6-8 leavesstage; H - plant prepared to return to their natural habitats

CONCLUSIONS

The technology developed for growing the Bulgarian endemic *Betonica bulgarica* from mature seeds to 6-8 leaves phenophase in laboratory conditions was successful and can be used to replenish the natural populations of the species in Bulgaria. In order to determine the effectiveness of the application of this measure for *ex*-situ conservation is necessary to continue observations after introduction of the plants grown in laboratory conditions to the Natural park, to follow their adaptation and further development, and if necessary to protect them and stabilize their condition.

ACKNOWLEDGEMENTS

This research was funded by Agreement OPOS 2-36/24.07.2014 between Faculty of Agriculture, Trakia University - Stara Zagora and Directorate of Natural Park "Sinite Kamani" - Sliven, Operational program "Actions for finding, restoring, maintaining of local plant species on the territory of «Sinite Kamani» Natural Park - Sliven", for project of "Sinite Kamani" Natural Park - Sliven, No 5103020-15-658 "Restoration of the habitats and conservation of the biological diversity in «Sinite Kamani» Natural Park", funded by Agreement 5103020-C-002 on Priority axis 3 Preservation and restoration of biodiversity of OPERATIONAL PROGRAMME "ENVIRONMENT 2007-2013"

REFERENCES

Assyov, B., A. Petrova, D. Dimitrov., Vassilev, R. (2012). Conspectus of the Bulgarian vascular flora. Distribution maps and floristic elements Fourth edition. Bulgarian *Biodiversity Foundation, Sofia. Edited by B. Assyov & A. Petrova* .

Biological Diversity Act, Republic of Bulgaria (2002). State Gazette No. 77/9.08.2002 http://lex.bg/en/laws/ldoc/ 2135456926.

Genova, E. (2011). *Betonica bulgarica* Deg. et Neic. – In: Peev D (ed.), Red *Data Book of the Republic of Bulgaria,* vol. 1. Plants & Fungi, http://e-ecodb.bas.bg/rdb/en/vol1/Betbulga.html.

Grozeva, N., M. Georgiewa., Vulkova, M. (2004). Flowering plants and ferns. In: Stoeva, M. (ed.), Biological diversity of Sinite Kamani Nature Park. Kontrast - Bogomilovo, Stara Zagora, pp 9-112.

Grozeva, N., M. Todorova, M. Gerdzhikova, G. Panayotova, N. Getov., Dohchev, D**.** (2014). New data for Bulgarian endemic *Betonica bulgarica* Deg. et Neič. of Sinite Kamani Natural Park Sliven. J. *BioSci. Biotech*. SE/ONLINE: 205-210.

Hayek, A. (1929). Prodromus Fl. Penins. Balc. II: 281.

Koeva, Y. **(**1984). *Stachys bulgarica* (Deg. & Neič.) Hayek (322), *Stachys balcanica* Ball (323), *Stachys scardica* (Griseb.) Hayek (322), *Stachys serbica* Pancic (325) - In: Velchev, V. (ed.), *Red Book of PR Bulgaria. vol. 1. Plants*. Publishing House of BAS, Sofia.

Koeva, Y. (1989). Genus *Betonica*. In: Jordanov, D. (ed.), *Flora Reipublicae Bulgaricae, vol 9, Editio Acad. "Prof. Marin Drinov"*, Serdicae, 412–416 .

Medicinal plants (2001). *Bioselena Foundation,* 2-20.

Ordinance No 8 by the Ministry of Environment and Water and Ministry of Agriculture and Forestry, State Gazette No 4, 16 January 2004.
Protected Areas Act. State Gazette No. 133/1998. http://lex.bg/laws/ldoc/2134445060.
Panayotova, G., N. Grozeva, D. Pavlov, M. Todorova., Gerdzhikova, M. (2014). Seed Germination, Growth and Morphological Parameters of *Betonica bulgarica* Deg. et Neič. Cultivated under Different Conditions. *Turkish Journal of Agricultural and Natural Science*, 2: 2006-2013.
Yankulov, J. (1964). Cultivation of essential oil and medical plants. CKS "Bilkoop".
Yankulov, J. (2000). Main aromatic plants, 19 modern technologies for cultivation. Sofia. 225.