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In-situ and *ex-situ* conservation of *Spiraea crenata* (*Rosaceae*) in Bulgaria*

Abstract

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This study presents the results of the conducted *in-situ* and *ex-situ* activities targeting the effective protection of the populations of *Spiraea crenata*, one of the rarest plant species in Bulgaria, currently known from two very restricted localities, near Kaspichan town (Northeastern Bulgaria) and near Stefanovo village (Western Bulgaria). A long-term monitoring of the two populations for identification of the main threatening factors, as well as the trends in the population state has been conducted. It is estimated that the potential for conservation *in-situ* actions exists in the locality near Kaspichan (the other locality is very small, situated in private lands and surrounded entirely by cultivated fields). The main activities conducted are the clearing of the habitat of branches of competitive trees and shrubs, as well as support of breeding abilities by *in vitro* propagation and return of acclimated plants back to the locality. Two *ex-situ* live collections of *in vitro* propagated plants were created and stored at the Institute of Biodiversity and Ecosystem research and Shumen Agricultural Institute.

Key words: breeding support, endangered plants, in vitro propagation.

Introduction

Spiraea crenata L. (Scalloped spirea) is one of the rarest species of the Bulgarian flora, known from two small localities: near Kaspichan town, Shoumen district (Northeastern Bulgaria) and near Stefanovo village, Pernik district (Western Bulgaria) (Fig. 1). Its geographic range includes Eastern and Northeastern Europe, the Balkan Peninsula and Northwestern Siberia, as the Bulgarian populations represent the southernmost point of its distribution area.

In Bulgaria this species is protected, included in Annex 3 of the Biological Diversity Law (2002, 2007), the Red List of Bulgarian vascular plants (Gussev 2009), as well as in

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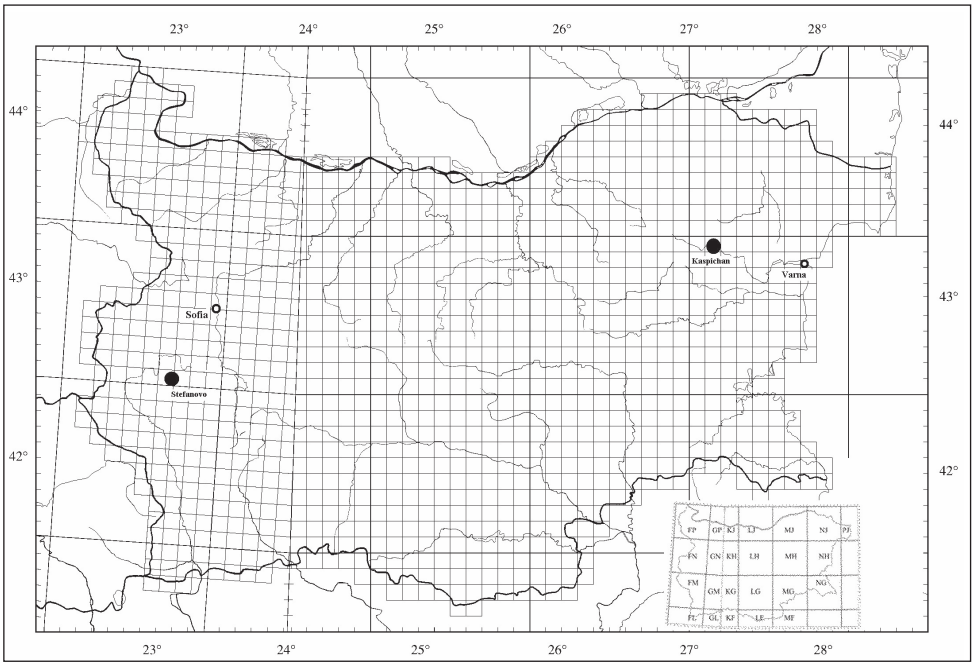

Fig. 1. Distribution map of *Spiraea crenata* L. in Bulgaria.

the Red Data Book of Bulgaria with as "Critically Endangered" (Gussev 2015). For its conservation a Protected Site "Kanyona" with an area of 17.58 ha was declared by the Minister of Environment and Water (State Gazette No 44 of 2012). Scalloped spirea belongs to the category of species for which restoration and maintenance activities are foreseen under the National Biodiversity Conservation Plan 2005-2010 (2010).

The biological characteristics and specific ecological requirements of the species, together with the existing threats to the populations and their habitats, determine the need for prompt protection measures.

This survey was conducted between 2010 and 2018 and aims to provide effective protection for populations of *S. crenata* by applying appropriate *in-situ* and *ex-situ* activities. The following tasks are included: 1. Study of the status of the species populations in Bulgaria and identification of the threatening factors; 2. Development of *in vitro* multiplication technique; 3. Implementation of *in-situ* activities for strengthening of the population of the species in the locality of Kaspichan; 4. Carry out long-term monitoring to track the effect of the activities carried out.

Material and methods

The present study focuses on the two populations of the critically endangered species *S. crenata* in Bulgaria and was implemented in the period 2010 – 2018.

Experimental work for obtaining authentic material for *in vitro* reproduction of *S. crenata* L. has been conducted at the Tissue Culture Laboratory of the Shumen Agricultural Institute. Stem cuttings of *S. crenata* were collected in 2010 from the locality near Kaspichan town. Apical meristems of plants are sterilized by plunging into a solution of 0.04% $HgCl_2$ and 0.1% Tween for 120 minutes and, subsequently by washing them 3 times with sterile distillated water (each of the tips for 30 minutes). In a sterile box under binocular are separated the inner parts of the apical meristems and put into a media for induction in thermostat for 15 days at 32-34˚C. The meristems were sub-cultivated six times on different induction media. The most intensive development of clonal regenerates from explants and whole plants are registered on medium, following Nanova (2007), which is a modification of the LePoivre (LP) nutrient medium (Quorin & al. 1977). In the phase of *in vitro* rooting a rooting media supplemented with macro salts (NH_4NO_3, KNO_3, $MgSO_4$, etc.), microelements ($ZnSO_4$, H_3BO_3, $MnSO_4$, $CuSO_4$, etc.), vitamins (C, B8), etc, was used (Slavova 1998). The *in vitro* rooted plants are taken out to controlled conditions room with temperatures of 26-28˚C and light regime with 16 hours daylight and 8 hours darkness.

The monitoring and assessment of the population state of the species were implemented in accordance with the Methodology for Monitoring of wascular plants and the Methodology for assessment of the state of vascular plants of the National system for Monitoring of the Biodiversity of Bulgaria (Executive Environment Agency-EEA) (http://eea.government.bg/en).

Results and discussion

Morphological description

S. crenata is a deciduous bush, up to 1.40 m high. Branches somewhat angulate, greyish, woody. Leaves of non-flowering shoots 2.5–3.5 cm long, about 2 cm wide, lanceolate to lanceolate-obovate, entire or crenate-serrate towards the apex, with 3 conspicuous veins running on both sides of the mid-vein from leaf base to leaf apex. Leaves of the flowering shoots smaller, oblong-obovate, and entire. Inflorescence umbellate, 10–20-flowered. Flowers small, 6–7 mm in diameter, white to yellowish-white. Follicles small, dehiscent along the ventral suture when ripe; seeds usually two in each follicle (Markova 1973). Flowering V, fruiting VI–VII. Reproduces by seeds and suckering.

The species inhabits gravelly and stony, dry, calcareous places. It is a element of the Pontic-Sarmatian shrub steppe communities, which refer to the priority habitat 40C0 Ponto-Sarmatian deciduous shrubs according to Annex I of the Directive 92/43/ EEC.

Study of the state of the populations of S. crenata *in Bulgaria*

The first population is located 2 km west of Kaspichan station, just near the railroad Kaspichan-Rousse, on the right bank of the river Kamenica (area “Canyon”) of 126 m. above sea level (Fig. 2). The population occupies an area of about 2.5 acres (Bancheva & Delcheva 2014). The species participates in the composition of mixed shrub communities dominated by Oriental Hornbeam. Projective cover of tree species is 10% with the participation of *Acer monspessulanum* L., *Carpinus orientalis* Mill., *Ulmus minor* Mill.

Fig. 2. The locality of *Spiraea crenata* near Kaspichan town, Shoumen district (Northeastern Bulgaria).

Projective cover of the bushes is 30% including *Corylus avellana* L., *Ligustrum vulgare* L., *Spiraea crenata*, etc. Projective cover of herbaceous species is 40%. As a result of the present study, the following threatening factors of anthropogenic origin have been identified: damage and/ or destruction of *S. crenata* in the cleaning of the railway's easement (high degree) and soil contamination with herbicides and fertilizers (medium degree). From the non-manageable factors key role in limited distribution of the species play a low regeneration ability of the species.

The second population is situated on the southern slopes of Golo Bardo Mountain, near the Stefanovo village, Pernik district, among cultivated fields, at 775-850 m above sea level (Fig. 3). It consists of 3 subpopulations of about 1 km distance from each other, as real the occupied area is 0.211 ha (Bancheva &Delcheva 2014). Projective cover of the bushes is 50% including *Cornus sanguinea* L.*, Crataegus monogyna* Jacq.*, Prunus spinosa* L.*, Rosa* sp.*, Spiraea crenata, Ulmus minor.* Projective cover of herbaceous species is 48% among which there are also agricultural crops. The main threatening factor in this locality is the destruction of the population of Scalloped spirea in the plowing of the surrounding fields and the passing of the dirt roads.

The study of both Bulgarian populations of *S. crenata* showed that conservation measures are urgently needed to strengthen the state of the species at national level. It is estimated that the potential for *in-situ* activities exists in the locality near Kaspichan (the other

Fig. 3. The locality of *Spiraea crenata* near Stefanovo village, Pernik district (Western Bulgaria).

locality is very small, situated in private lands and surrounded entirely by cultivated fields). The main activities that would help to strengthen the population are the clearing of the habitat of branches of competitive trees and shrubs, as well as support of breeding abilities by *in vitro* propagation and return of acclimated plants back to the locality.

Development of a technique for in vitro *propagation*

In vitro culture introduction

It was registered a weak development of the meristems, and that's why they were subcultivated again and again on different induction media. The best development was registered for the medium described by Nanova (2007).

In vitro cloning of the developed plants

For a 3 months period the developed plants were sub-cultivated repeatedly on different micro-propagation media. The most intensive development of clonal regenerates from explants and whole plants are registered on the medium, described by Nanova (2007). It should be underlined the comparatively low regeneration potential of the studied materials on the tested media. Nevertheless, enough plants for the subsequent phases of stabilization of the regenerated plants were obtained.

In vitro rooting

The most difficult appeared to be the choice of a media for *in vitro* rooting of the developed plants. After repeated sub-cultivations a rooting media supplemented with macro salts (NH_4NO_3, KNO_3, $MgSO_4$, etc.), microelements ($ZnSO_4$, H_3BO_3, $MnSO_4$, $CuSO_4$, etc.), vitamins (C, B8), etc., was chosen (Slavova 1998). The induced roots were simple and weak, which makes difficult their further development (Fig. 4).

Adaptation and acclimatization

The *in vitro* rooted plants are taken out to controlled conditions room with temperatures of 26-28˚C and light regime with 16 hours daylight and 8 hours darkness. After the careful separation from the nutrition media the plants are planted in PVC pots with 20 cm diameter in a soil substrate of peat, sand and perlite in 2:1:1 ratio. A humid camera is created via covering with a nylon bag for 1-2 weeks, which is regularly removed, so that there is no risk for over moistening and moulding of the substrate. A month later the plants form new roots, and then they are taken out consequently in a greenhouse for 2 months, and then in external conditions. The collection of *in vitro* plants number 50 individuals.

In-situ *activities for reinforcement of the population of Kaspichan locality*

In order to help strengthen the Kaspichan population, 15 of the *in vitro* propagated plants were planted in the spring of 2012 (Fig. 5). During the monitoring carried out in the

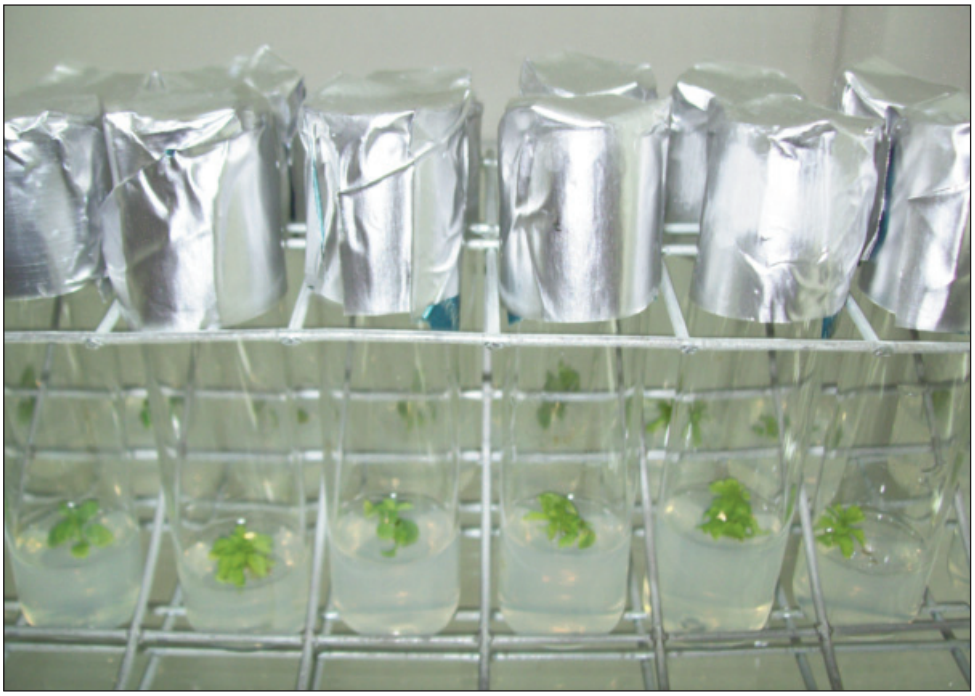

Fig. 4. *In-vitro* plantlets of *Spiraea. crenata.*

Fig. 5. Planting the *in-vitro* plants in the Kaspichan population.

autumn of the same year, 6 plants survived, while one year later, in the autumn of 2013, the number of the survived ones decreased to 3. Their number remained the same in the subsequent monitoring carried out in 2014, 2016 and 2018.

Another *in-situ* activity with a favourable effect on the state of the population of *S. crenata* was the clearing of branches of competitive trees and shrubs organized by the employees of the Bulgarian Railways serving the railway line passing through the locality of the species.

Ex-situ *activities*

For *ex-situ* conservation of the species, a live collection of 15 *in vitro* propagated individuals was created in the Greenhouse of Institute of Biodiversity and Ecosystem Research. Such a collection is also grown in the Shumen Agricultural Institute. If necessary, plants from these collections can be used to strengthen the existing populations of *S. srenata*, as well as to reintroduce the species in the locality of Kabiushka mogila (Shumen district) from where the species is considered extinct in the last decades.

Long-term monitoring of the populations' state and the effect of the activities carried out in

The monitoring of the species is based on long-term observation of selected parameters for the population status, the threats and the consequences thereof, and aims at identifying the trends in the development of the populations. Generally, carrying out systematic monitoring leads to the timely identification of negative population trends and serves as the

basis for adequate management measures. The monitoring of both species populations takes into account all parameters provided in the EEA Monitoring Methodology.

The reporting entity is a "group of bushes", because the biology of the species is such that visually cannot differentiate separate individuals.

Kaspichan's population

In 2010, 48 "groups of bushes" of the observed species were found, mostly concentrated along the edge of the canyon of the river Kamenica. The main threatening factors are damaging and/ or destroying of *S. crenata* in the cleaning of the railway's easement and soil contamination with herbicides and fertilizers. The most important factor, however, is the low regeneration ability of the species.

In the spring of 2012, two *in-situ* activities aimed at improving the state of the population were carried out: clearing the habitat from branches of trees and bushes of competing species and planting 15 *in vitro* propagated individuals (marked with permanent markers buried in the soil). In the autumn of 2013, the number of "groups of bushes" was 79, as 3 of them are the surviving *in vitro* plants. In the following observations in 2014, 2016 and 2018 the number is kept the same, which leads to the conclusion that the activities are in the right direction and the state of the population near Kaspichan can be defined as "Favorable".

Stefanovo's population

It is fragmented into 3 subpopulations about 1 km apart, surrounded by cultivated fields. The plants in each fragment are so densely positioned that individuals or "groups of bushes" can not be distinguished. The monitoring takes into account the area occupied by these fragments. In 2013 the total occupied area is 0.211 ha. In the next monitoring survey carried out in 2014, this area remains unchanged. The main threatening factor in this locality is destroying the population in plowing the surrounding fields and pave the roads. The state of the population near the village of Stefanovo is defined as "Unfavorable-bad".

Conclusions

As a result of the present study and the *in-situ* activities, the state of the Kaspichan population of the endangered species *S. crenata*, was improved. An effective method for its *in vitro* propagation has been developed. The state of the Kaspichan's population is "Favorable", while the Stefanovo's population is in "Unfavorable" state. Two *ex-situ* live collections of *in vitro* propagated plants were created and stored at the Institute of Biodiversity and Ecosystem research and Agricultural Institute in Shumen.

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