Ex situ and in situ conservation of Centaurea pseudaxillaris (Asteraceae) by means of plant biotechnology

ABSTRACT
Centaurea pseudaxillaris Stef. & T. Georgiev (Asteraceae) is a Bulgarian endemic plant species with limited area comprising two populations located in the floristic regions Thracian Lowland and Toundzha Hilly Country. It is critically endangered, protected by the Biodiversity Act. To preserve the species both ex situ and in situ measures have been applied. Seeds germinated in vitro after 70 days of incubation on basal MS medium. Leaf explants isolated from the seedlings formed callus and leaf rosettes on MS medium supplemented with 1 mg/l BAP. The rosettes rooted spontaneously on the basal medium. Callus formed also on root segments and was fast growing on medium containing 0.5 mg/l BAP and 0.2 mg/l 2,4-D. Root-derived calli sub-cultured on MS free of plant growth regulators developed only roots while those transferred to media with 0.5 mg/l BAP or 1 mg/l 2iP regenerated whole plantlets. They were potted in soil substrate and ex vitro adapted using phytotron. The well-developed roots and the gradual humidity decrease were of crucial importance for the success of this step. Plants were further adapted to greenhouse and finally acclimatized to the field plot. The ex situ collection consisted of 14 plants which bloomed and gave seeds during their first year. After the wintering they grew bigger and two years later, with the help of volunteers, 9 of them were transferred to their population of origin, in order to strengthen it. Two months later the monitoring confirmed the survival of the plants which were all in very good condition.

Key words: Centaurea, in vitro propagation, ex situ collection, population strengthening, biodiversity conservation

Abbreviations: BAP – 6-benzylaminopurine; 2,4-D – 2,4-Dichlorophenoxyacetic acid; 2iP – 6-(γ,γ-dimethylallylamino) purine; PGRs – plant growth regulations

Introduction
Centaurea pseudaxillaris Stef. & T. Georgiev (Asteraceae) is a Bulgarian endemic plant species with a few very restricted populations located in the floristic regions Thracian Lowland and Toundzha Hilly Country (Bancheva & Raimondo, 2003; Bancheva, 2015). In 2014 a new population of the species, near to Dobrich village, Haskovo district was found (Bancheva & al. 2013). The area of the population and the number of the individuals are limited, consisting of several hundred individuals distributed in groups. Seed propagation is problematic due to the low seed germination and the insect attacks. The species is protected by the Bulgarian Biodiversity Act (2002) and it is included in the Red Book of Bulgaria (Bancheva, 2015) and the Red List of the vascular plants in Bulgaria (Bancheva, 2009), as critically endangered after IUCN criteria.

One of the major factors for the plant diversity loss is due to the destruction and fragmentation of the habitats (Holsinger & Gottlieb, 1991; Falk, 1992). C. pseudaxillaris is
The seeds and the primary plant explants were put in plastic containers (Duchefa, NL) with basal MS medium (Murashige & Skoog, 1962) in a culture room at 23±2°C and 16 h light daily of 2000 lx. The secondary explants excised from the leaves and the roots of the in vitro obtained plantlets were cultivated on media supplemented with plant growth regulators (PGRs) as follows: medium B1 (1 mg/l BAP), medium C2 (0.5 mg/l BAP), medium IP1 (1 mg/l 2iP), medium CA3 (0.5 mg/l BAP and 0.2 mg/l 2,4-D), while the shoots were sub-cultured on media free of PGRs: MS or ½ MS (with half-strength macrosalts) in smaller containers (Magenta, USA) or in glass test tubes.

The regeneration capacity (RC) of the explants was calculated as percentage of explants with organogenesis. Leaf and root segments were tested in four repetitions 16 explants each while calli were in repetitions of 9 pieces. The propagation rate was presented by the propagation coefficient (PC) which indicated the number of obtained adventitious shoots per initial shoot. The criteria for medium assessment were RC, PC, and the quality of the regenerated in vitro plants.

Ex vitro adaptation and acclimatization

In autumn 2012 and in summer 2013, in vitro regenerated plantlets were taken out of the containers, their roots were washed from the agar medium, then rinsed in diluted solution of KMnO₄ and transferred to autoclaved mixture of soil, sand and coconut fiber (2:1:1) one per plastic pot with diameter 9 cm. They were gradually adapted ex vitro, the first four weeks in a growth chamber with strict control of the temperature, light and humidity, set in four time segments in order to simulate the diurnal atmospheric conditions: 3 hours “daybreak” at 20°C and 2070 lx, followed by 10 h “day” at 23°C and 3300 lx, 3 h “dusk” at 20°C and 2070 lx, and 8 h “night” at 18°C in a dark. The ventilation was permanent while the humidity was gradually decreased every few days starting from 90 % in the “night”, 80 % in the “day” and 85 % during the intermediate time segments “daybreak” and “dusk”, and reaching finally 65 %, 55 %, and 60 % in the same segments, respectively. Plantlets spent two months on the shelves of the room phytotron with less control on these parameters: temperature between 16 and 28°C, humidity varying daily from 25 to 60 %, and mixed illumination of daylight and artificial warm-white LED light added 16 hours per day. Strengthened plantlets were transferred to larger pots

Material and Methods

Monitoring

For the monitoring and assessment of the status of the populations “Methodology for monitoring of vascular plants” and “Methodology for assessment of the status of vascular plants” elaborated for the National System for monitoring of the biodiversity of the Executive Environment Agency are used (http://eea.government.bg/bg/nsmos).

In vitro propagation

Mature seeds and capitula with unripe seeds were gathered from both populations of C. pseudaxillaris, as well as vegetative organs: flower stems and leaves. They were washed with tap water for one hour and then disinfected by consecutive soaking in KMnO₄ solution, 70% ethanol for a minute and commercial bleach (chlorine < 5%) for 10 min, followed by thrice rinse with distilled sterile water. For stimulation of the seed germination, stratification (4 weeks in a fridge at 6°C) or treatment with gibberellic acid (soaking in 0.35% GA₃ for 20 h) were used.

In autumn 2012 and in summer 2013, in vitro regenerated plantlets were taken out of the containers, their roots were washed from the agar medium, then rinsed in diluted solution of KMnO₄ and transferred to autoclaved mixture of soil, sand and coconut fiber (2:1:1) one per plastic pot with diameter 9 cm. They were gradually adapted ex vitro, the first four weeks in a growth chamber with strict control of the temperature, light and humidity, set in four time segments in order to simulate the diurnal atmospheric conditions: 3 hours “daybreak” at 20°C and 2070 lx, followed by 10 h “day” at 23°C and 3300 lx, 3 h “dusk” at 20°C and 2070 lx, and 8 h “night” at 18°C in a dark. The ventilation was permanent while the humidity was gradually decreased every few days starting from 90 % in the “night”, 80 % in the “day” and 85 % during the intermediate time segments “daybreak” and “dusk”, and reaching finally 65 %, 55 %, and 60 % in the same segments, respectively. Plantlets spent two months on the shelves of the room phytotron with less control on these parameters: temperature between 16 and 28°C, humidity varying daily from 25 to 60 %, and mixed illumination of daylight and artificial warm-white LED light added 16 hours per day. Strengthened plantlets were transferred to larger pots
with 11 cm of diameter in a mixture of peat enriched with mineral salts, and sand (2:1), and acclimated to greenhouse ambience. Finally, 14 of the plants were planted on the experimental field plots of the Institute of biodiversity and ecosystem research (IBER) in Sofia; at altitude of 500 m. During the last two years they were watered and weeded regularly from spring to autumn, and spent the winter under the snow.

**Strengthening of the population**

In the early spring 2015, nine *C. pseudaxillaris* plants of the *ex situ* collection of IBER were uncovered from the snow, uprooted and put in large pots of 18 cm diameter. After a week, they were planted into their population of origin near the village of Dobrich, with the help of volunteers. Plants were marked with red-tinted bars and their GPS points were noted as well. In the same time a monitoring of the population was done to observe the status of the native plants of the species. Two months later, in the end of May 2015, a second monitoring was realized to check the survival of the *in vitro* regenerated plants and their status in comparison with that of the native plants of the population.

**Results**

**Monitoring**

The monitoring conducted in 2015 confirmed that the only known populations of the species had a very limited area and number of individuals – several hundred individuals with group distribution (Figure 1). The main threats are: low reproductive and migration potential; weak competitiveness of the species; destruction of its specific habitat – xerothermic oak forests; fires (Bessaparski ridove locality), etc.

**In vitro propagation**

*In vitro* culture was initiated from seeds despite their heavy microbial contamination and the extremely low germination rate: 0.7 % of 615 seeds germinated after 70 days of incubation on MS medium. Neither the seed stratification at 6°C for one month, nor the treatment with 0.35% GA3 stimulated the germination. A part of the contaminated seeds were transferred to soil mixture but no one of them germinated.

In *vitro* plants obtained on the different media seemed similarly: all of them had a good leaf turgor and deep green color. The upper part of the leaves faded with the time but new leaves appeared. Although all plantlets rooted spontaneously on medium free of plant growth regulators, the number and the quality of their roots and leaves differed which seemed to depend on the presence of cytokinins and...
auxins in the previous media (Table 1). Thus, the best previous media concerning the in vitro rooting were medium C2 which contained twice less cytokinin compared to C2 and IP1, and CA3 which contained auxin in addition. Plants which began their regeneration on medium CA3 developed long and ramified roots on the PGR-free media, while those obtained on media IP1 and B1 formed shorter and less numerous roots for the same period on the basal medium. Unexpectedly, on medium ½ MS used for rooting, new shoots regenerated by direct organogenesis at the base of some of the shoots. The propagation rate on ½ MS medium for a period of 2 months also depended on the previously tested media and was highest after cultivation on medium IP1 (1.7 shoots per initial shoot). Obviously, BAP had less long effect on the formation of adventitious shoots compared to 2iP. The prolonged cultivation on ½ MS medium after medium C2 led to considerable increase of the propagation rate.

The disinfection of the vegetative parts of the plants gathered from the wild populations was not successful due to the very heavy microbial and fungi contamination. Similarly, the leaf explants taken from the ex vitro adapted plants in the greenhouse were lost because either of microbial contamination or of tissue necrosis in case of more strong sterilization.

**Figure 2.** In vitro cultivation of *C. pseudaxillaris*: A) Formation of callus and leaf rosettes on leaf explants grown on MS medium containing 1 mg/l BAP; B) Callussogenesis on root explants on MS medium supplemented with 0.5 mg/l BAP and 0.2 mg/l 2,4-D; C) Formation of leaf rosettes on root-derived callus, on medium containing 0.5 mg/l BAP; D) In vitro plant developed from leaf-derived leaf rosettes on B1 medium, rooting on ½ MS; E) In vitro plant developed from root-derived leaf rosettes on CA3 medium, rooted on ½ MS; F) In vitro plants developed from root-derived leaf rosettes on C2 medium, rooted on ½ MS.
Table 1. Morphological features of in vitro plants regenerated on media with different PGR composition and grown on the half-strength basal medium ½ MS.

<table>
<thead>
<tr>
<th>Initial medium</th>
<th>Time on ½ MS [months]</th>
<th>PC</th>
<th>Rooting %</th>
<th>Root features</th>
<th>Number of leaves per shoot</th>
<th>Number of roots per shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA3</td>
<td>8 weeks</td>
<td>1.0 ± 0.0</td>
<td>100</td>
<td>Long, ramified</td>
<td>11.3 ± 3.0</td>
<td>4.8 ± 2.5</td>
</tr>
<tr>
<td>IP1</td>
<td>6 weeks</td>
<td>1.2 ± 0.4</td>
<td>100</td>
<td>Short</td>
<td>9.0 ± 3.3</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>IP1</td>
<td>8 weeks</td>
<td>1.7 ± 1.2</td>
<td>100</td>
<td>Medium</td>
<td>11.0 ± 3.0</td>
<td>3.7 ± 3.8</td>
</tr>
<tr>
<td>B1</td>
<td>9 weeks</td>
<td>1.3 ± 0.5</td>
<td>100</td>
<td>Short</td>
<td>5.8 ± 2.1</td>
<td>3.2 ± 2.2</td>
</tr>
<tr>
<td>C2</td>
<td>13 weeks</td>
<td>2.6 ± 1.8</td>
<td>100</td>
<td>Long, ramified</td>
<td>17.2 ± 6.7</td>
<td>9.0 ± 3.7</td>
</tr>
</tbody>
</table>

**Ex vitro adaptation and acclimatization**

The mixture of soil, sand and coconut fiber (2:1:1) proved to be suitable for ex vitro adaptation of the in vitro regenerated C. pseudaxillaris plants. The well-developed roots and the gradual humidity decrease were of crucial importance for the success of this step. Thus, most of the plants with weak roots obtained on medium B1 stopped their growth and dropped out. All plants higher than 5 cm and possessing abundant roots survived easily in the growth chamber, and the room phytotron (Figure 3-A). Moreover, the plants were resistant to the usual trips and insects occurring in phytotrons and greenhouses. They supported well the low temperature in the unheated greenhouse and bloomed in summer (Figure 3-B). Plants acclimated easily to the open-air field plot, as well. A month later they developed new leaves and grew considerably (Figure 3-C). All of them formed buds, bloomed and produced seeds the first season. The aboveground part of the plants withered after the hot summer months but in autumn new leaves appeared and a second flowering was observed. After the wintering on the field plot, the plants developed multiple additional shoots and formed multiple flowerets (Figure 3-D).

**Ex situ collection**

The ex situ collection of C. pseudaxillaris has been established in spring 2013, and completed in autumn the same year. It consisted of 14 plants which continued to grow. The largest ones developed several dozens of shoots while the others were smaller (Figure 4). Currently, 5 plants are remaining on the field plot after the in situ restoration actions, and there are several more in the greenhouse. The in vitro propagation continue with new genotypes aiming at conservation of the genetic diversity of the species.

**In situ activities on population restoration**

In the early spring of 2015, nine of acclimatized plants on the field plot were transferred to their population of origin, near the village of Dobrich, Haskovo district, in order to strengthen it. When uprooting from the frozen soil of the field plot the main root were cut at a depth of about 20 cm but there were still many smaller adventitious roots. The leaves under the snow were green even they looked pressed (Figure 5-A).
They straightened up quickly during the week in the unheated greenhouse and the plants were nice and vital at the moment of planting into the population (Figure 5-B). Several volunteers participated in this activity, enthusiastic to contribute to the conservation of the Bulgarian endemic species. The campaigns that we organized to make popular the conservation measures related to the biodiversity loss had a wholesome effect on the people ecological awareness. Special brochures and calendars about several plant endemics were dealt to the citizens in the library club. It is worth to mention that the native *C. pseudaxillaris* plants were very small at this time even if the climate in the region is softer compared to the Sofia country.

Two months later the monitoring confirmed the survival of the introduced plants into the population. All of them were in very good condition; however, some differences between them and the native individuals were noticed. The native plants were in blossoming stage (Figure 5-C) while the introduced ones had already formed seeds. Besides, the in vitro multiplied plants were larger, consisting of numerous shoots with many florets each while the native plants had a few stems which were higher, with a single flower at the top (Figure 5-D, E).

**Figure 4.** *Ex situ collection of C. pseudaxillaris in the Institute of Biodiversity and Ecosystem Research, Sofia.*

**Figure 5.** *Strengthening of the population of C. pseudaxillaris near the village Dobritch, Haskovo district: A) Acclimatized in vitro regenerated plant from the ex situ collection in mid-March, designed to be returned to the wild population; B) Plant transferring to the population on 29th of March 2015; C) General view of the wild population in bloom, May; D) 2015In vitro regenerated plant 2 months after the transfer to the population, on 25th of May 2015; E) Native plant from the wild population, on 25th of May 2015.*
Discussion

The genus Centaurea is the richest one with species of conservation important in the Bulgarian flora. C. pseudaxillaris is a cross pollinating species but its propagation is hindered because of the low seed germination. The relations between the Centaurea species and the insects are complex as the latter are reliable for the pollination, but they also destroy the seeds for their high nutritive value (Clement & al., 1988; Story & al., 1995). Our observations proved that the insects infested C. pseudaxillaris capitula with unripe seeds in the populations as well as in the ex situ collection. Thus, in 2015 only six mature seeds were available among several hundred capitula formed by the plants in the living collection and none of them germinated. In this context, the in vitro multiplication of the species is appropriate for overcoming the reproduction difficulties. The seeds are more suitable for initiation of in vitro culture of C. pseudaxillaris for both preserving the genetic diversity of the regenerated plants and because they are easier to disinfect compared with the vegetative organs grown in the nature. However, the in vitro germination rate was very low regardless the conditions of seed keeping: in paper-bags at room temperature as recommended by Kurt & Erdağ (2009) for Centaurea zeybekii or in a fridge at 6°C for a month to simulate the winter season by stratification. The soaking in solution of gibberellic acid primary to culture initiation which is a common practice for enhancement of seed germination in many species, was useless as well. These were the reasons to multiply the plants by clonal propagation starting from a few seedlings.

The most frequently used nutrient medium for in vitro cultivation formulated by Murashige & Skoog (1962) was suitable also for C. pseudaxillaris. It was reported to be appropriate for many other Centaurea species (Alaiwi & al. 2012, Cuenca & al. 1999, Cuenca & Amo-Marco 2000, Kurt & Erdağ 2009, Mallón & al. 2010). The basal MS medium supplemented usually with BAP in concentration between 0.5 mg/l and 1.0 mg/l have been used for in vitro cultivation of all species from Asteraceae family included in the Red Book of Spain (González-Benito & Martín 2010). Benzylaminopurine was reported as more effective for in vitro cultivation of Centaurea species compared with the other commonly used cytokinin, kinetin (Emek & Erdag 2013). Our experiments showed that BAP was better than 2iP tested in the same concentrations. Cytokinins were indispensable for the formation of the vegetative bud from the callus induced by the root segments. After the dedifferentiation of the root tissues under the influence of BAP and 2,4-D (medium CA3) the callus cells were able to induce only rhysogenesis on basal MS medium. This fact suggested that on PGR-free medium the callus cells had the tendency to reproduce the tissues they had derived from. Once regenerated from callus in presence of cytokinin, either BAP or 2iP, the in vitro plantlets grew successfully on medium without PGRs. Another feature of the C. pseudaxillaris callus was its color. Regardless of the medium composition, the leaf-derived callus was green while the root-derived callus was greyish. This particularity indicated that in spite of the cell dedifferentiation the callus cells kept their chloroplasts.

The formation of vigor in vitro roots was proved to be one of the two crucial prerequisites for the successful ex vitro adaptation, the other being the gradual decrease of the air humidity during the first weeks after the in vitro stage of the regenerated plantlets (Stanilova & al. 2013). According to the literature data most of the Centaurea species needed the addition of auxins in the culture medium to develop roots. Cuenca & al. (1999) reported difficult in vitro rooting of different Centaurea species and necessity of special medium composition. Authors’ best result was 40% rooting for one month on medium supplemented with 2 mg/l IAA in combination with 2 mg/l IBA; 70% of the rooted plantlets were successfully ex vitro adapted. In contrast, C. pseudaxillaris formed easily roots on the basal media MS and ½ MS ensuring 100 % in vitro rooting which facilitated a lot the stage of ex vitro adaptation. Our data showed also the prolonged influence of the PGRs present in the previous medium on the organogenic potential of the in vitro cultures. Thus, the in vitro rooting on ½ MS medium was better in case the previous medium contained auxin or at least the cytokinin was in low concentration. To ensure better in vitro rooting it would be useful to consider the effect of the cytokinins to inhibit the root formation even regarding the media before the last one.

Finally, a protocol for in vitro regeneration of C. pseudaxillaris was elaborated starting from seed germination and including indirect organogenesis. The full cycle of in vitro cultivation comprises all the steps of the method from...
the seed disinfection to the ex vitro adaptation and acclimatization to open-air field plot and to the nature population conditions. Plants obtained in vitro via indirect organogenesis are normally developing, they are fertile and seemed to adapt well into the wild population. The genetic stability of the in vitro plants of genus Centaurea has been studied by Radić & al. (2005). Authors found out that the chromosome and mitotic anomalies occurred in similar degrees in C. ragusina in vitro cultivated plants on ½ MS medium containing 0.1 mg/l BAP and 0.7 mg/l GA3 and in native plants growing in the locality of their seed origin as well as in another locality. The frequencies of the mitotic anomalies were 12% for the in vitro cultured plants and 10% and 9% for the two localities, respectively. It is worth to mention that the polyploidy and the aneuploidy did not concern the whole plants but only single cells, and neither the PGRs nor the age of the culture affected the cytogenetic stability of the plants.

The monitoring will last two years to study the development of the introduced C. pseudaxillaris individuals into the population and to check the possible changes in their features related to the new ambience without human care. The height of the plants could increase, for example, because of the high grasses in the population, the lack of weeding and the need of competition for sun light. The natural water regime is also expected to affect the plant growth. Plant material will be gathered for further investigations. The assessment of the population status of C. pseudaxillaris will continue, with respect to the impact of the in vitro obtained plants which have been introduced into one of the two populations of the species. The investigation will be further extended to clarify the reproductive peculiarities and the genetic diversity of C. pseudaxillaris and of other unstudied endemic species of the genus.

Acknowledgements

This research was supported by the Bulgarian National Science Fund, Bulgarian Ministry of Education and Science (project DFNI-BO2/18).

References


Emek Y, Erdag B. 2013. In vitro propagation of critically endangered endemic rhaponticoides mykalea (hub.-mor.) by axillary shoot proliferation, Current Progress in Biological Research, Dr. Marina Silva-Oppe (ed.).


Reasearch Article


