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In vitro micropropagation of *Astragalus physocalyx* (Fabaceae) and reintroduction of plants in its "*Locus Classicus*" on Mladezhki Hill in Plovdiv

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Abstract

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Astragalus physocalyx Fisch. is a tertiary relict, critically endangered worldwide according to the IUCN criteria, included in the Bern convention and protected by the Biodiversity Act of Bulgaria. It was discovered in 1834, on Mladezhki Hill in Plovdiv, but this locality was destroyed during the development of a stone quarry. Later, another population was discovered in Bulgaria with only 20 individuals and one each in North Macedonia, Greece and Turkiye. The objectives of this study were to develop a protocol for rapid *in vitro* propagation of *A. physocalyx* and to reintroduce the species into its natural habitats. Almost two-thirds of all collected calyxes were empty and *in vitro* seed germination was most successful after mechanical disruption of the hard seed coat, or after repeated seed treatment with boiling and ice water. Plants were multiplied by direct organogenesis on MS medium supplemented with 1.0 mg/L BAP + 0.2 mg/L NAA or with 0.3 mg/L GA_3 resulting in 16.0 and 48.4 new explants per *in vitro* tuft, respectively, for a period of 2 months. *In vitro* rooted plants were easily adapted to soil mixture but needed several years in the greenhouse to reach size suitable for transfer into the wild. In April, 2018, thirty-six plants were planted outdoors: 18 in the only Bulgarian population near Kulata village and 18 on Mladezhki Hill in Plovdiv; the latter survived, branched, and in 2021, the largest bloomed. The "*Locus Classicus*" of the species can be considered restored as all plants are currently in good condition.

Keywords: Leguminosae; endangered species; tertiary relict; seed germination; *in vitro* plant multiplication; restoration of plant population

Introduction

Astragalus physocalyx Fisch. is a tertiary relict, critically endangered worldwide according to the IUCN criteria (IUCN, 2012), included in the Bern convention (1979) and in the Red Data Book of Bulgaria (Stanev, 2015), and protected by the Biological Diversity Act of Bulgaria (2002). The species was discovered in 1834, on the Dzhendem Tepe (currently known as Mladezhki Hill in Plovdiv) by the Hungarian botanist Frivaldszky's assistants Hinke and Manolesko. It was initially misidentified as *Astragalus utriger* Pall. by Frivaldszky and subsequently described by the Russian botanist Fischer (1837). At the beginning of the 20th century, the type locality of Dzhendem Tepe was destroyed due to

the development of a stone quarry. In 1959, the Bulgarian botanists Velchev and Bondev discovered a second locality of the species in the southern Struma Valley, above the village of Kulata, Petrich district (Velčev & Bondev, 1961), which was also destroyed, and for nearly 50 years the species was considered extinct from the Bulgarian flora. In May, 2006, the species was rediscovered on the Kartaletsa hill near the village of Kulata, consisting of about 20 individuals (Stoyanov et al., 2006). The locality was designated as an "Important Plant Areas" (in fulfillment of the objectives of the Convention on Biological Diversity) and was declared a protected site "Kartaletsa". Until recently, *A. physocalyx* was considered a Balkan endemic, since apart from Bulgaria it was found in isolated localities in North Macedonia and in Greece, situated relatively close to each other, in close proximity to the state borders, but in 2002, it was reported to be found in Turkiye (Anatolia), which expanded the range of the species beyond the Balkan Peninsula (Nydegger-Hügli, 2002). In 2014, an Action Plan for the conservation of the species in Bulgaria through *in situ* and *ex situ* measures was developed and officially approved (Stoyanov et al., 2014).

The flowering of *A. physocalyx* usually starts in early April, and lasts about 3 weeks. The growth process of the calyx begins already in the first week and at the end of the flowering it acquires its usual bubble shape (Fig. 1-A) and dimensions (length about 20 mm and diameter about 15 mm). After the death of the corolla, the calyx teeth fuse and completely close the pod (Fig. 1-B), which is only 5–6 mm long and contains one to five seeds about 3 mm in diameter. Although already in July, the blistered calyx is completely dry, its mechanical strength prevents the release of the pod and the seeds, which is the main obstacle to the effective seed propagation of the species.

Information on *in vitro* cultivation of *Astragalus* species is scarce and mainly concerns callogenesis and somatic embryogenesis or plant regeneration through indirect organogenesis (Hou & Jia, 2004; Park & Choi, 2015). *In vitro* plant multiplication by direct organogenesis starting with *in vitro* germinated seeds was applied to *A. nitidiflorus*, another endemic and endangered species (Cano-Castillo et al., 2009). This method of propagation is best suited to preserve the genetic diversity of plants intended to be used for species conservation. The objectives of the present study were to develop a protocol for rapid *in vitro* propagation of *A. physocalyx* using seeds as starting material and to reintroduce the species into its natural habitats.

Fig. 1. *A. physocalyx* in its natural habitat near the village of Kulata: *A*) Flowering stage; *B*) Fruiting stage

Material and Methods

Plant material

In May, 2006, and April, 2007, plant material of *A. physocalyx* was gathered from the population on the Kartaletsa hill above the village of Kulata, Petrich district: 30 ripe seeds from previous years and several flowers. The collected specimens were deposited in the herbarium of the Institute of Biodiversity and Ecosystem Research (under the following numbers: SOM 162681, SOM 162682 and SOM 163917). In addition, 390 calyxes were gathered in August, 2011, from 6 of the larger plant individuals with abundant fruiting, intended for *in vitro* rapid multiplication. The last hundred ripe seeds (50 fresh and 50 one-year old) were collected in August 2017 to complete the study.

Pollen fertility assessment

Pollen fertility was assessed in 15 flowers (3 flowers per each of 5 randomly selected plants) based on 100 pollen grains per microscope slide, by standard method using Alexander dye (Alexander, 1969), which results in intense staining of the fertile pollen grains, while the sterile ones remain colorless, transparent.

Seed formation and germination

The percentage of calyxes with pods was calculated for each of the selected donor plants, as were the number of seeds per calyx and the percentage of small underdeveloped and withered seeds. Attempts to germinate seeds were carried out three times: in 2009 (with seeds stored for 3 years at sub-zero temperatures, at 25±2 °C or 15 °C, both in the light and in the dark), with freshly harvested seeds in 2011, and with fresh and one-year old seeds in 2017, at 23±2 °C and 16 h of light daily. Different methods were applied to stimulate seed germination: soaking in 0.35% gibberellic acid (GA_3) for 24 h (in 2009); treatment with boiling water for 10 seconds, alone, or in combination with one of the following: pre-stratification for 3 months at 8 °C, soaking in 0.35% GA_3 for 24 h, sounding with birdsong for 1 h per day after seeding *in vitro* (in 2011); 10-fold consecutive treatment with boiling and ice water for 10 seconds each, with or without mechanic

scarification (in 2017). Seeds were disinfected by soaking for 1 min in 70% ethanol followed by 10 min in sodium hypochlorite (chlorine < 5%) and repeated washings in sterile distilled water. Seeds were put on basal MS nutrient medium (Murashige & Skoog, 1962) supplemented with 30 g/L sugar and solidified with 6.5 g/L Plant agar (Duchefa, NL) (medium MS), in plastic containers. Non-germinated seeds were scarified by breaking their hard seed coat under sterile conditions and returned to the culture medium. To assess differences between plant individuals concerning formation of well-developed seeds, Excel ANOVA single factor was used.

In vitro propagation and ex vitro adaptation

Seedlings and cotyledons obtained on MS medium were transferred to media supplemented with different plant growth regulators (PGRs): 1.0 mg/L BAP + 0.2 mg/L NAA for plant multiplication by direct organogenesis (medium BN), 0.3 mg/L GA_3 for stem elongation (medium MSG), or 0.3 mg/L BAP + 4 mg/L 2,4-D for callogenesis (medium BD). Plantlets were sub-cultured on the same media by separation of the newly formed shoots and cutting of the long stems to segments, at every 2 months, and propagation coefficient (PC) was evaluated as number of new shoots and stem segments per explant. Control plants were cultivated on MS medium free of PGRs. Plantlets were rooted on MS medium or MS supplemented with 0.5 mg/L NAA (medium MSN). *In vitro* cultivation was carried out at 23±2 °C and a photoperiod of 16 hours of light per day, with an intensity of 3000 lx. Rooted plantlets were potted in soil substrate and *ex vitro* adapted first in a growth camera with strict control of the temperature, light regime, and air humidity and then in a phytotron, as described in Gorgorov et al. (2015). Plants were further strengthened in an unheated greenhouse.

Reintroduction of the plants into their natural habitats

Only well-developed plants were used for reintroduction. A total of 29 plants were transferred to the Mladezhki Hill in Plovdiv, the first 6 in April, 2015, another 5 in May, 2017, and the remaining 18 in April, 2018, the latter being regularly watered. Another 18 plants were transferred to the only Bulgarian population of *A. physocalyx* in April, 2018. The plants were monitored in subsequent years.

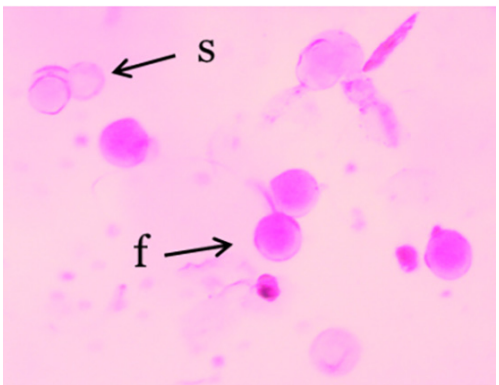

Fig. 2. Pollen grains: f – fertile; s – sterile

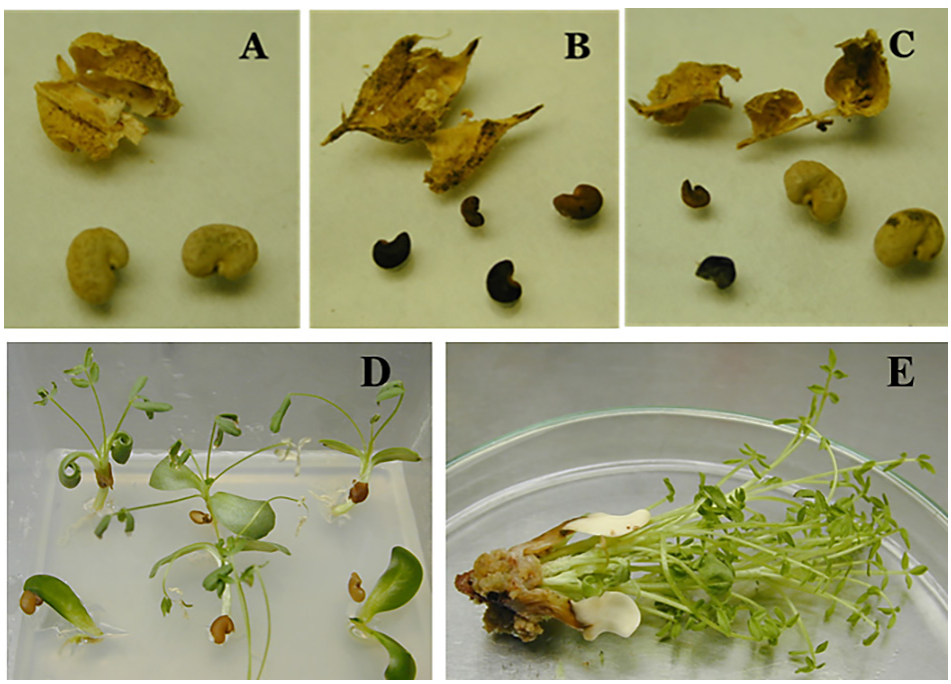

Fig. 3. Calyxes with seeds and *in vitro* seed germination: *A*) Well-developed seeds; *B*) Small underdeveloped and withered seeds; *C*) Both well-developed and small seeds; *D*) Normal seedlings and cotyledons grown from damaged embryos; *E*) Seed germination with formation of several stems and callus on medium containing GA_3

Results and Discussion

Pollen fertility and in vitro seed germination

Plants formed tufts of various sizes, with hundreds of flowers. Despite the high percentage of fertile pollen, which was in the range of 72.3% – 86.8% (Fig. 2), almost two-thirds of the 390 calyxes collected from 6 individuals were empty, and the others formed one to five seeds: 79.5% of these calyxes contained only well-developed and 6.2% only small withered seeds (Fig. 3, A-C), 87% of all seeds being well developed. Individual tufts differed in percentage of calyxes with pods, number of well-developed seeds per pod ($P < 0.001$, calculated for calyxes with pods), and percentage of small underdeveloped and withered seeds ($P < 0.01$) (Table 1). Similar data were reported for the critically endangered *Astragalus argaeus* (77% pollen viability on the day of full-bloom and high stigma receptivity), whose low reproductive success was associated with low seed germination due to impermeable and hard seed coat and small population size (Atasagun et al., 2021).

Our observations found that in March of the following year, there were still unreduced calyxes. Even well-ripened seeds have a chance to germinate only the following spring and possibly in the fall. It could be assumed that the mechanical obstacles, first of the calyx and then of the pod, provide a longer period for the seeds to mature and reach germination, as well as to protect them from destruction. The seeds stored for 3 years at sub-zero temperatures were successfully disinfected, but did not germinate either at 25±2 °C or at 15 °C, both in the light and in the dark. Soaking in 0.35% GA_3 for a day also proved useless. The problem was only resolved by breaking the seed coat with sterile instruments, although some embryos were injured. Seeds with intact embryos formed a radicle and large cotyledons, and well-formed unpaired leaves with several pairs of leaflets grew between them, as in nature, while those with damaged embryos formed only cotyledons and callus (Fig. 3-D). Surface sterilization of freshly harvested seeds in 2011 was less successful and most seeds were discarded, so a comparison between the germination stimulation variants tested was impossible. A total of 24.8 ± 6.7% of all seeds used germinated over a period of 100 days (in the first 3 weeks only 4.6% of seeds swelled and 1.8% germinated). Pretreatment with GA_3 led to formation of several shoots per seed and callus instead of radicle (Fig. 3-E). A more precise experiment with fresh and one-year old seeds collected in 2017 showed that the stratification by repeated treatment with boiling and ice water was very effective (Table 2). All scarified fresh seeds germinated but were more susceptible to infection than non-scarified, and this difference was also observed in one-year old seeds, but the percentage of contaminated and non-germinated seeds increased. Gibberellic acid was reported to be the most potent agent breaking seed dormancy in *A. cyclophyllon*, reaching 81% germinated seeds; however, the germination ability of this species was relatively high, as 55% of fresh untreated seeds also germinated (Keshtkar et al., 2008).

Table 1. Comparison between individuals of donor plants regarding seed formation

Plant individuals	Number of collected calyxes	Calyxes with pods: (number, %)	Number of seeds	Number of seeds per pod	Small withered seeds (%)
Tuft 1	50	22, 44.0	37	1.7	10.8
Tuft 2	130	22, 16.9	49	2.2	32.6
Tuft 3	80	30, 37.5	75	2.5	14.7
Tuft 4	30	12, 40.0	41	3.4	0.0
Tuft 5	50	25, 50.0	41	1.6	17.1
Tuft 6	50	35, 70.0	87	2.5	5.7

ANOVA Single factor: Well-developed seeds per pod, calculated for 6 individuals

Source of Variation	df	MS	F	P-value
Between Groups	5	9,959944	11,52114	2,5E-09
Within Groups	140	0,864493		
Total	145			

Table 2. Seed germination and plant development (seeds collected in 2017)

Seeds	Number		Contaminated [%]		Non-germinated [%]		Abnormal [%]		*Ex vitro* adapted [%]	
	Fresh	1-y old	Fresh	1-y old	Fresh	1-y old	Fresh	1-y old	Fresh	1-y old
Stratified & Scarified	26	24	26.9	41.7	0.0	16.7	7.7	20.8	65.4	20.8
Stratified, Non-scarified	24	23	8.3	30.4	25.0	43.5	8.3	8.7	58.3	17.4

In vitro propagation and ex vitro adaptation

Seedlings reaching 5–6 cm in height were transferred to MS or BN medium. Cutting off the roots stimulated the formation of new shoots at the base of the seedling stem, and the presence of PGRs enhanced shoots development (Fig. 4-A). Successive sub-cultivation with separation of the newly formed shoots from each other and cutting of shoots into segments led to an increase in the multiplication coefficient (Table 3). Cultivation on MSG medium containing GA_3 resulted in most elongation of the shoots, which allowed to cut them into segments according to the number of internodes, thus increasing the number of new explants per stem; however, 23.5% of them died and the survivors formed fewer shoots due to their smaller size. Exclusion of growth regulators from the medium resulted in stem shortening and plant appearance closer to that in nature (Fig. 4-B). *In vitro* rooting was long and difficult, occurring only on MS or MSN media, 47.9% and 34.5% rooted plants, respectively (Fig. 4-C). Similar results were obtained for *in vitro* rooting of *A. nitidiflorus* endemic to Cartagena: 46.7% rooted plants in the best variant using first MS medium supplemented with 0.5 mg/L IAA, and then half-strength MS with 0.9 g/L activated charcoal (Cano-Castillo et al., 2009). Many *in vitro* propagated shoots in 2011 were not able to root due to occurrence of endophytic microbial contamination, causing necrosis and death of plants (Fig. 4-D). Therefore, the normally developed *in vitro* seedlings obtained from fresh and one-year old seeds collected in 2017 (31 and 9 seedlings, respectively), were further grown without sub-cultivation (Table 2). Most of the *in vitro* seedlings and *in vitro* rooted plants were successfully *ex vitro* adapted in the phytotron (Fig. 4-E) and then acclimatized in the unheated greenhouse of IBER (Fig. 4-F). Callus formed on injured seeds and cotyledons, and around the shoot base of some plantlets, was very slow-growing even on medium BD and considered as unpromising.

Reintroduction of in vitro propagated plants into their natural habitats

Although plants were easily adapted to soil mixture, they needed several years in the greenhouse to reach size suitable for transfer into the wild: 10–15 cm height, rosette diameter about 20 cm, with about 20 leaves and well-developed roots, and many of them had one or more newly formed stems, which is a good sign of successful development (Fig. 5-A). This slow plant development is consistent with the monitoring of the population on the Kartaletsa hill, showing that only 5 new plants emerged over a 6-year period, of which 3 were flowering and fruiting and 2 were vegetative (Stoyanov et al., 2014).

Table 3. Propagation coefficient after 2 months *in vitro* cultivation on different media

Medium	PC		
	Shoots per stem	Segments per shoot	Explants per stem
MS, control	2.8	–	2.8
BN	4.2	3.8	16.0
MSG	3.8	12.7	48.4

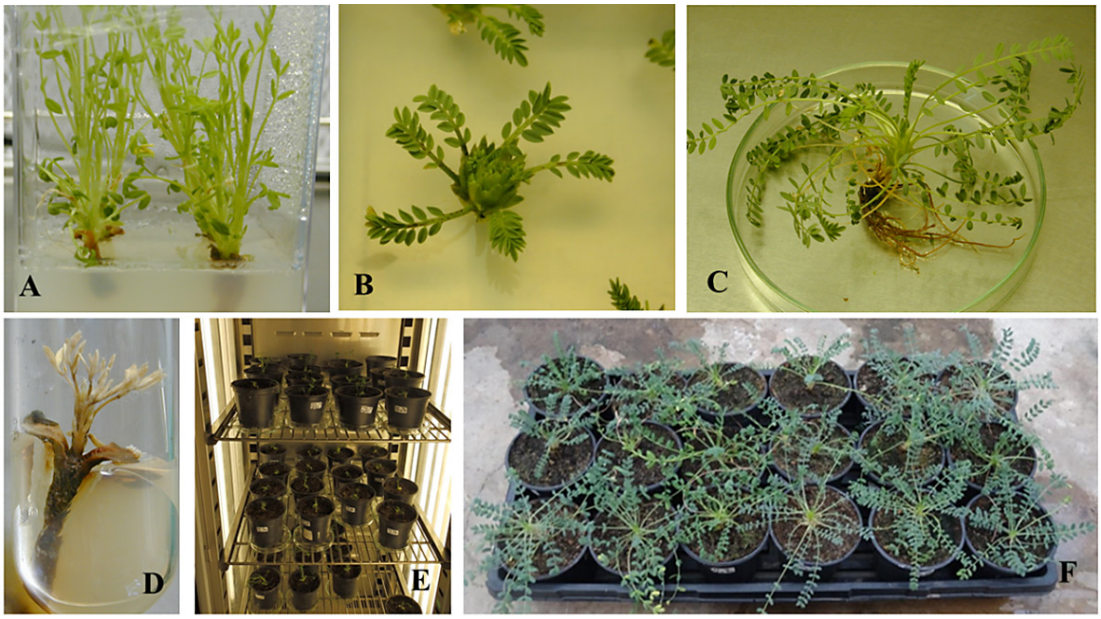

Fig. 4. *In vitro* propagation of *A. physocalyx*: *A*) Shoot multiplication on medium BN; *B*) *In vitro* plant transferred to medium MS; *C*) *In vitro* rooted plant; *D*) Failed *in vitro* rooting due to endophytic contamination; *E*) *Ex vitro* adaptation in a growth camera; *F*) Acclimated plants in the greenhouse

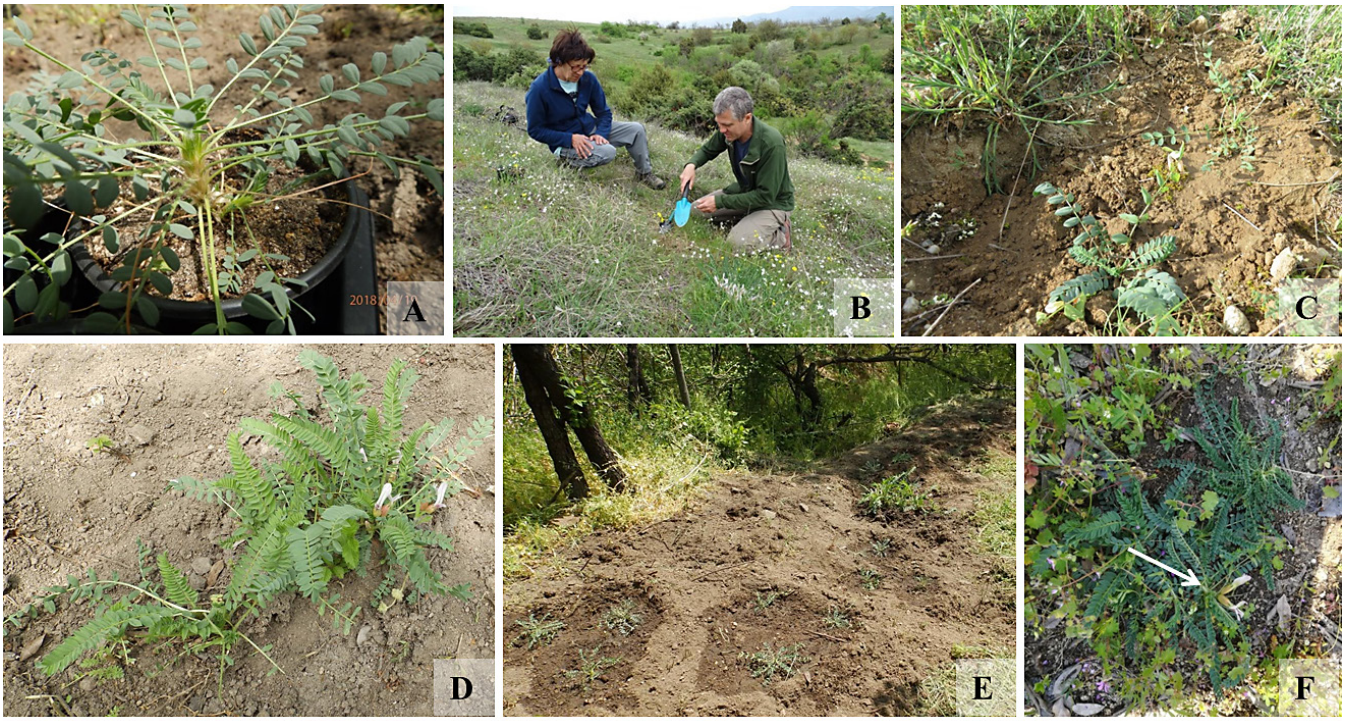

Fig. 5. Reintroduction of *in vitro* propagated plants:
***A*) Plant with a branched stem, 5 years after potting; *B*) & *C*) Plants transferring in the only Bulgarian population of *A. physocalyx* near Kulata village; *D*) The only surviving multi-year plant on Mladezhki Hill in Plovdiv; *E*) Plants transferred in 2018 into "*Locus Classicus*" of the species on Mladezhki Hill; *F*) First flowering of acclimatized plants on Mladezhki Hill, in 2021**

In April, 2015, the bigger 6 of the first *in vitro* propagated plants in 2009 were transferred to the Mladezhki Hill in Plovdiv and planted in a specially prepared place near the only surviving multi-year plant of the species that was flowering (Fig. 5-D). They didn't survive without follow-up care. Of the next 5 plants of the same set, transferred in April, 2017, to the same location, only one overwintered successfully. In April, 2018, thirty-six plants multiplied from the seeds collected in 2011, were planted outdoors: 18 in the only Bulgarian population of *A. physocalyx* near the village of Kulata, to strengthen it (Fig. 5-B,C) and 18 on Mladezhki Hill in Plovdiv to restore the "*Locus Classicus*" of the species (Fig. 5-E). The latter survived thanks to the care (watering) on the part of the Municipal Enterprise "Gardens and Parks" in Plovdiv, branched out, and in 2021, the largest ones bloomed (Fig. 5-F). All are currently in good state. The seedlings obtained in 2017 are still growing in the greenhouse of IBER.

Conclusions

Despite the high pollen viability (72.3–86.8%), the percentage of calyxes with pods was low (37 ± 17.3%). In addition, the germination of the seeds was hindered by their hard coat, which explains the low regeneration of the species in nature. A protocol for rapid *in vitro* micropropagation of *A. physocalyx* was established, allowing production of hundreds of well-developed plants in a relatively short time. Seed germination was improved by breaking the seed coat, multiplication of *in vitro* seedlings was achieved by sub-cultivation, and most of the *in vitro* rooted plantlets were successfully *ex vitro* adapted. *Astragalus physocalyx* was reintroduced in the only Bulgarian population of the species near the village of Kulata and on Mladezhki Hill in Plovdiv; its "*Locus Classicus*" can be considered restored, as all the plants transferred in 2018 to Mladezhki Hill have branched and since 2021 started to bloom.

Acknowledgements

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