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Original Scientific Paper

Propagation of *Campanula bayerniana* (Campanulaceae) by seeds as a reproduction model for the conservation of threatened small-seed plant species

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ABSTRACT:
The *in vitro* seed propagation of small seeded *Campanula* species often incurs complications in the manual handling of the tiny seed material from seed sterilisation to seedling transplantation in soil. The lack of a simple and effective protocol which minimises manual operations makes the propagation of such species both challenging and inefficient. This study aimed to develop a protocol for the seed reproduction of threatened *Campanula* and other threatened small-seed plant species. The method was successfully tested on *Campanula bayerniana* subsp. *bayerniana.* The saplings were cultivated over a 5-month period through seed germination under *in vitro* conditions followed by their transfer to containers with a soil mix. The protocol employs small filter paper discs on which the seeds germinate and continue their development in soil substrate until the full biodegradation of the discs. The method involves surface treatment of the seeds with potassium permanganate solution, thus avoiding additional washing phases and direct contact with the seeds and seedlings, thereby preventing mechanical damage. This also facilitates the handling of each seed separately, minimising seed loss particularly when working with a small number of seeds. This study is also the first known example of the *ex situ* propagation of *Campanula bayerniana.*

Keywords:
Campanula bayerniana, *ex situ* conservation, seed reproduction, small-seeded plant species

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INTRODUCTION

The genus *Campanula* L. (*Campanulaceae*) in the flora of Armenia comprises 21 species 5 of which: *Campanula caucasica* M.Bieb., *C. propinqua* Fisch. & C.A.Mey., *C. zangezura* (Lipsky) Kolak. et Serdjukova, *C. minsteriana* Grossh. and *C. massalskyi* Fomin are included in the Red Data Book of Armenia (Tamanyan *et al.* 2010). The last of these is also a globally threatened species (IUCN 2023). None of these species is available in the living collections of the botanical gardens of Armenia, and only a few examples of the cultivation of local *Campanula* species in these gardens have been documented. Several attempts have been made to transplant individual plants from the wild, but they did not survive over the years. There is only one documented success of *C. caucasica,* which was transplanted to the Flora and Vegetation of Armenia plot at the Yerevan Botanical Garden where it naturalised well and flowered for a period of time (Oganesian 1999). No other examples of the *ex situ* propagation of the *Campanula* species in the local botanical gardens are known either, except for a report on the successful propagation of *C. armena* Steven from seeds sown in pots with soil and further growth of the plants in a rock garden during the period 1995–2003 Alexanyan (2004).

Fig. 1. *Campanula bayerniana* subsp. *bayerniana* in its habitat.

In the aim of creating a living collection of threatened *Campanula* species in the Yerevan Botanical Garden, in 2023–2024 we tested a new approach to plant propagation using the seeds of a widespread chasmophyte *Campanula bayerniana* Rupr. in order to apply it to rare species with low seed availability.

Campanula bayerniana is a perennial plant, (5)10–35(40) cm tall, with more or less depressed stems, rosette and lower stem leaves, long-petiolate, round or broadly ovate, with a round or reniform base (Fig. 1). The flowers are grouped in a loose paniculate inflorescence. The corolla is (1)1.5–3(3.5) cm long, the seeds are 0.2–0.25 mm wide and 0.6–1 mm long, oblong-elliptical, more or less flat (Oganesian 1995). *Campanula bayerniana* occurs on rocks (rarely on church walls) at altitudes ranging from 1300 to 3000 m a.s.l. There are two subspecies *C. bayerniana* subsp. *bayerniana* and *C. bayerniana* subsp. *choziatowskyi* (Fomin) Oganesian in the flora of Armenia. The distribution area of *C. bayerniana* subsp. *bayerniana* includes the south of the Caucasus and northwest of Iran. *C. bayerniana* subsp. *choziatowskyi* is endemic to Armenia and differs from the other subspecies in terms of its bigger size and larger flowers, the absence of cilia along the edges of the wider calyx denticles and the marked constriction of the corolla (Oganesian 1995).

We succeeded for the first time in the propagation of *C. bayerniana* subsp. *bayerniana* using our specific methodology. We raised healthy seedlings over a 5-month period and transplanted them into an open-air biotope in the Yerevan Botanical Garden where they all successfully overwintered.

MATERIALS AND METHODS

Plant material. One hundred seeds of *C. bayerniana* subsp. *bayerniana* from the seed bank of the Department of Conservation of Genetic Resources of Armenian Flora stored in a paper envelope at 20–25°C were used (Accession data: 1320, collected the 30 July 2018 in the vicinity of Jermuk, Vayots Dzor, Armenia, Leg. & Det. A. Nersesyan). The seeds were 100% viable according to the cut test result carried out prior to the experiment.

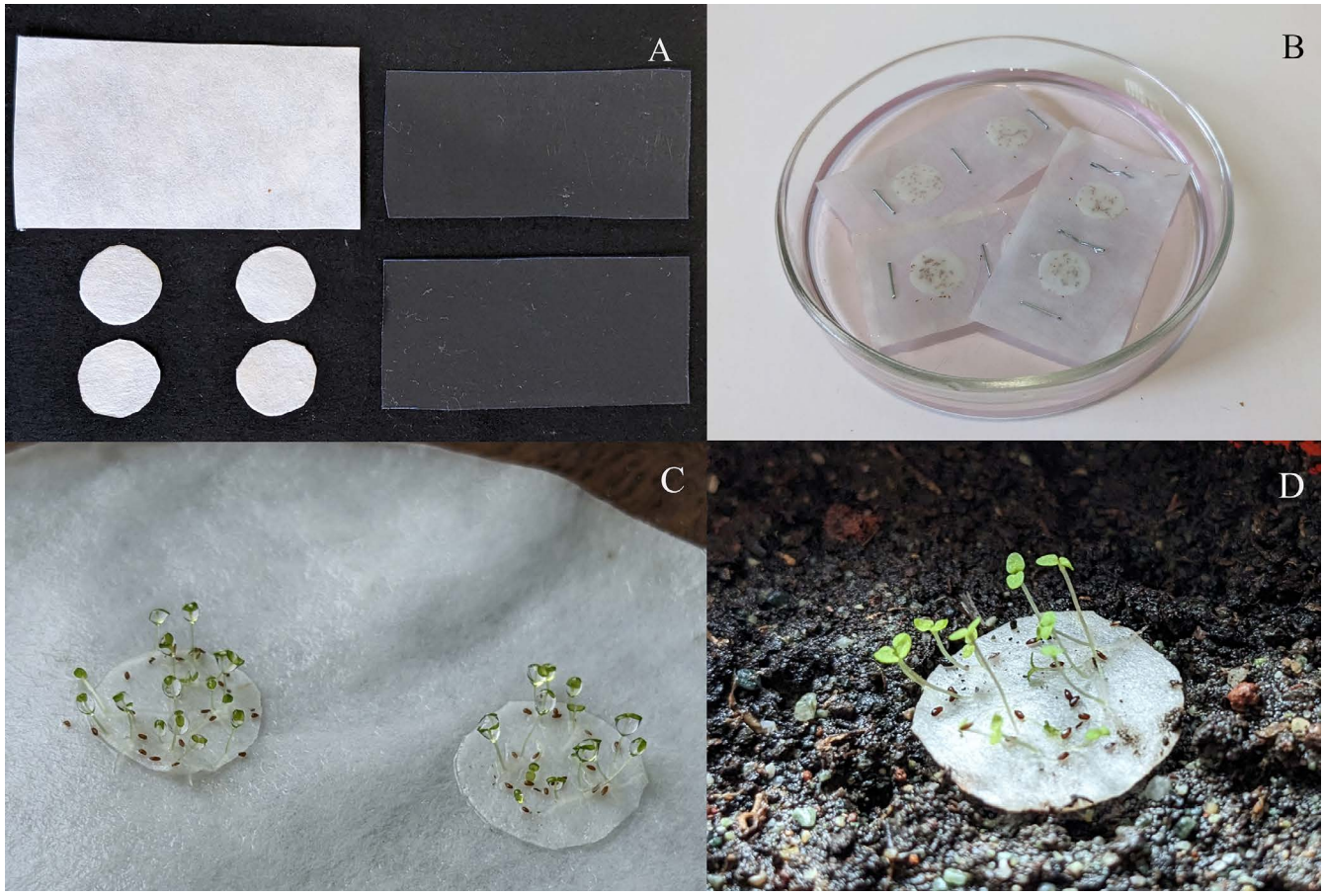

Fig. 2. A) Material used to form the envelopes for the sterilisation of small sized *Campanula* seeds; B) Envelopes soaked in $KMnO_4$ solution; C) *C. bayerniana* subsp. *bayerniana* seedlings during in vitro development on filter paper discs; D) Disc with seedlings after transfer to *in vivo* conditions.

Protocol for seed sterilisation in $KMnO_4$ with reduced manual intervention. The following materials were used (Fig. 2A): one sterile Petri dish with 2 layers of sterile filter paper, a 0.0005 mol/l solution of $KMnO_4$, 4 filter paper discs of 8–10 mm in diameter, 2 layers of 300 µ transparent plastic film of 5cm × 2.5 cm in size, a piece of filter paper (3 cm × 6 cm), a piece of double folded filter paper (approximately 10 cm × 10 cm), a small sized (N10) stapler, tweezers, a needle or any other tool for working with very small seeds, small scissors, and a dry Petri dish containing 100 seeds.

The sterilisation process comprised the following steps:

- The seeds were divided into 2 groups in a Petri dish. [When working with a small seed number, they can be grouped into portions of one to several]
- One piece of plastic film was covered with the 3 cm × 6 cm filter paper slightly soaked in the $KMnO_4$ solution. Subsequently, 2 paper discs were soaked in the $KMnO_4$ solution and covered by seeds by pressing each disc slightly onto the seeds in the dry Petri dish. The discs with seeds on the top were placed on the top of the double-layered base. The two seed-containing discs were covered with two moistened discs. The whole construction was covered with another piece of transparent film and secured with a stapler (Fig. 2B).
- The envelope was soaked in the $KMnO_4$ solution for 10 min. The solution was able to permeate between the plastic layers due to the filter paper layer. After soaking, the envelope removed from the solution and placed in the folded filter paper to remove any excess moisture. The envelope was then carefully cut open and the disc layers were separated so as to distribute the seeds onto each disc. Where necessary, a needle can be used to adjust the distribution of the seeds on the discs.

In vitro seed germination. The discs covered with seeds were placed in Petri dishes with double layered filter paper, moistened with $KMnO_4$ solution and maintained in a growth chamber under 25.5/18°C with a constant photoperiod of 16-h light/8-h dark.

In vivo development of the seedlings. After reaching the maximum germination rate, the paper discs were transferred to a 100 ml plastic pot filled with fine sifted and well moisturized 4:1:1 ratio soil, peat and river sand.

Fig. 3. A) Seedlings after 1.5 months of development; B) Root of a seedling after 5 months of development; C) Young plant of *Campanula bayerniana* subsp. *bayerniana* a few weeks after planting in the Alpine Corner of the Ecoepicenter at the Yerevan Botanical Garden; D) *C. bayerniana* subsp. *bayerniana* after overwintering.

The soil around the discs was slightly watered using a pipette to avoid damaging the seedlings while allowing the paper to absorb the water. The pot was covered with a transparent lid (the pots can also be placed in plastic bags with zip locks) to maintain the necessary humidity level and at the same time to allow some ventilation. The pot was then placed by a window in a room at 17–20°C. Further actions were aimed at maintaining the necessary humidity level and gradually increasing the ventilation by periodically keeping the lid (zip locker) open during the day. After an adaptation period of a month the lid was removed. At the four-leaf stage, the surviving seedlings were transplanted into individual 100 ml containers filled with the same type of substrate and kept in room conditions at about 20–23°C.

RESULTS AND DISCUSSION

On 24 March 2023, 100 seeds of *C. bayerniana* were surface-sterilised in a $KMnO_4$ solution and sewn on filter paper discs as described above. The seeds started to germinate within a period of two weeks, reaching a maximum

germination rate of 60% on 11 April 2023 (Fig. 2C). At this point the paper discs were transferred from *in vitro* to *in vivo* conditions. Within a few days after the transfer, the seedlings started to develop actively (Fig. 2D). After one month the first leaves appeared (29. April 2023) and 21 seedlings had survived by this date. The filter paper discs were fully biodegraded 30–40 days after the beginning of *in vivo* development and the seedlings grew in size at a greater distance from each other (Fig. 3A).

On 20 June 2023, at the four-leaf stage, the surviving seedlings (total number 14) were transplanted into individual 100 ml containers. Intensive development of the root was observed during the following weeks with the root becoming thick and firm, especially in its upper part (Fig. 3B). The leaves grew in size and number. On 25August 23, the young plantlets, having 5–6 well developed leaves, were transplanted to open-air conditions in the Alpine Corner of the Ecoepicenter at the Yerevan Botanical Garden.

The plantlets were planted between rocks filled with the substrate on which they had grown and covered with dry grass to protect them from excessive insolation. They were watered at least twice a day by spraying water all over them and the surrounding rocks. After a week the grass was removed. The plants naturalised well, did not exhibit any signs of stress and began to develop new leaves a week after transplantation (Fig. 3C). All the plants developed 3–4 new leaves over a 2-month period. These plants were watered just twice in winter – in dry and warm periods. On 8 February 2024 little rosettes of new leaves were observed on all the 14 plants, indicating their successful wintering and survival (Fig. 3D).

Species specific propagation protocols through *in vivo* seed germination have previously been developed for several rare *Campanula* species (Paunescu 2010; Stamenković *et* al. 2012; Grigoriadou *et al*. 2014; Villa *et al*. 2021; Konstantinov *et al*. 2022; Sarropoulou *et al*. 2022; Anestis *et al.* 2023; Yücel & Erken 2023) and these are useful when large quantities of seeds are available.

Additionally, there is a report (Melia & Barblishvili 2018) on the successful growth of *Campanula kachetica* Kantsch in *ex situ* conditions, from seeds sown in Petri dishes with agar and filter paper, and in pots with soil resulting in the further development of adult plants. The report does not provide any information on any seed pretreatment or on the handling of the seedlings in *in vivo* conditions, but the photographs show that a large quantity of seeds (hundreds) were sown in a single spot in a Petri dish, forming a compact bunch of seedlings with intertwined roots. This made the subsequent division of individual plants almost impossible because of the size and fragility of the seedlings during all phases of *in vitro* development. Although effective, this method cannot be used when working with a small number of seeds.

Micropropagation methods are applied for the propagation of different endemic and threatened *Campanula* species and are considered the starting point for *ex situ* conservation or for mass propagation, resulting in the rapid production of numerous new plantlets (Airo *et al.* 2010; Paunescu 2010; Seglie *et al.* 2012; Stamenković *et al.* 2012; Grigoriadou *et al.* 2014; Gabedava 2015; Pirhan *et al.* 2022; Anestis *et al.* 2023). However, the restoration of populations of threatened species using plants grown from seeds is preferable as it supports the conservation of intraspecific genetic diversity and thus increases the viability of the population. For the successful multiplication of endangered *Campanula* species with a small number of seeds, we would recommend a combination of the above-described method (to maintain genetic diversity) and micropropagation (to increase plant numbers and subsequently seed production outcomes).

CONCLUSIONS

The above-described method, while simple, allows sterilising and propagating *Campanula* seeds with the following benefits:

- The use of envelopes for sterilisation and a diluted $KMnO_4$ solution for surface sterilisation helps to avoid a number of complicated manual steps, which often pose a significant challenge in the sterilisation process (Stamenković *et al.* 2012).
- Seeds sown on paper discs are transferable and in cases of the contamination of the basal filter paper the discs with seeds can be moved to a new sterile Petri dish and treated by dropping a solution of $KMnO_4$ over them. If the discs themselves become contaminated, they can be removed individually with the remaining seed material treated with a solution of $KMnO_4$.
- Seeds fixed to the discs and subsequently the seedlings are protected from mechanical damage during the transfer from *in vitro* to *in vivo* conditions. Just sprouted, the seedlings are very fragile and can be easily damaged even by a water drop.
- The discs provide mechanical support for the roots, which grow through and on them, finding their way down to the substrate, thus allowing the seedlings to maintain a straight position and preventing them from falling over.
- The filter paper discs absorb water from the soil, which minimises stress during the first weeks of *in vivo* development of the seedlings: the roots are provided with the necessary amount of water and the air humidity around the seedlings is sufficient.
- The filter paper is fully biodegraded by the time the roots have developed deeper into the soil and the seedlings developed the first leaves. This is the most sensitive and slow growth period in the seedlings'

development. After this stage, the growth intensifies and at the 4-6 leaf stage the seedlings become viable and ready to be planted in open-air conditions. For better adaptation, they can be kept a week or two in pots outside, in the shade prior to planting.

- When working with a small number of seeds, each paper disc may contain only one to a few seeds, making management more effective. The paper discs can be transferred to in-vivo conditions during the entire period of germination as the seeds germinate thus minimising seed loss. This is especially important when handling the seeds of rare species.

This study is the first to present a successful propagation protocol for *Campanula bayerniana*. The sterilisation and propagation method using seeds presented in this paper may serve as a model for the development of propagation protocols for other threatened small-seeded plant species.

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REZIME

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Propagacija *Campanula bayerniana* (Campanulaceae) semenom kao model reprodukcije za očuvanje ugroženih vrsta sitnog semena

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In vitro razmnožavanje vrsta *Campanula* sa malim semenom često nosi teret komplikacija u ručnom upravljanju sitnim semenskim materijalom koji se kreće od sterilizacije semena do transplantacije sadnica u zemljištu. Nedostatak jednostavnog i efikasnog protokola sa smanjenjem manuelnog rukovanja čini razmnožavanje takvih vrsta izazovnim i neefikasnim. Ova studija je imala za cilj da razvije protokol za reprodukciju semena ugroženih vrsta roda *Campanula* i drugih ugroženih biljnih vrsta sitnog semena. Metoda je uspešno testirana na *Campanula bayerniana* subsp. *bayerniana*. Mladice su gajene u periodu od 5 meseci klijanjem semena u *in vitro* uslovima i njihovim daljim rukovanjem u posudama sa mešavinom zemljišta. Protokol koristi male filter papirne diskove na kojima seme klija i nastavlja svoj razvoj u zemljišnom supstratu do potpune biorazgradnje diskova. Metoda se zasniva na površinskoj obradi semena rastvorom kalijum permanganata – što omogućava da se izbegnu dodatne faze pranja i da se isključi direktan kontakt sa semenom i sadnicama kako bi se sprečila mehanička oštećenja. Takođe pomaže u rukovanju svakim semenom posebno u slučaju malog broja semena čime se minimizira gubitak semena. Ova studija je takođe prvi poznati primer *ex situ* razmnožavanja *Campanula bayerniana*.

Ključne reči: *Campanula bayerniana*, *ex situ* konzervacija, reprodukcija semenom, vrste malog semena

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