WINTER SURVIVAL AND FLOWERING PERFORMANCE OF IN VITRO PROPAGATED *DIANTHUS GIGANTEIFORMIS* SUBSP. *PONTEDERAE* (A. KERN.) SOO UNDER DIFFERENT OVERWINTERING CONDITIONS

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Abstract

Dianthus giganteiformis subsp. *pontederae* (A. Kern.) Soó is a protected perennial endemic to the Pannonian region whose fragmented populations require effective conservation measures. Although in vitro propagation has been successfully applied to this taxon, little information is available on the post-acclimatization performance of propagated plants under outdoor conditions. The aim of this study was to evaluate the overwintering survival, flowering, and post-winter performance of in vitro-propagated plants under outdoor and greenhouse overwintering conditions.

A total of 160 acclimatized plants representing 20 seed-derived genotypes (Seed IDs) were included in the experiment. Half of the plants were overwintered outdoors under a protective horticultural fleece, while the remainder were maintained in a moderately heated greenhouse (13–15 °C). Plant survival, flowering, flowering stem production, and plant vigor were monitored from November 2025 to June 2026. Treatment effects were evaluated using Pearson's chi-square test, Cramer's V, the paired Wilcoxon signed-rank test, and Spearman's rank correlation.

Outdoor overwintering resulted in higher survival (75.0% vs. 62.5%) and substantially higher flowering (75.0% vs. 30.0%) than greenhouse overwintering. Although the difference in survival was not statistically significant ($p = 0.088$), flowering differed significantly between treatments ($p < 0.001$). Genotype significantly influenced post-winter performance, with considerable variation among Seed IDs. The Final Performance Score was significantly higher following outdoor overwintering ($p < 0.001$), while genotype performance remained positively correlated between the two overwintering treatments (Spearman's $\rho = 0.659$, $p = 0.002$).

These results demonstrate that in vitro-propagated *D. giganteiformis* subsp. *pontederae* can successfully establish, survive winter, and flower under outdoor conditions. The observed genotypic variation highlights the importance of maintaining multiple genetic lines during ex situ propagation. Overall, the study supports the use of micropropagation as an effective tool for the ex situ conservation of this protected taxon and provides practical guidance for its long-term cultivation and conservation management.

Keywords: *Dianthus giganteiformis* subsp. *pontederae;* in vitro propagation; ex situ conservation; overwintering; flowering; genotype performance.

Introduction

Dianthus giganteiformis (Borbás) Heinr. Braun comprises three currently accepted subspecies: subsp. *giganteiformis,* distributed in Bulgaria, Hungary, Romania, Ukraine, and Serbia; subsp.

kladovanus, occurring in Bulgaria, Romania, and Serbia; and subsp. *pontederae*, distributed in Austria, the Czech Republic, Hungary, Romania, Slovakia, and Ukraine. Among these, *D. giganteiformis* subsp. *pontederae* is regarded as an endangered taxon. Dianthus giganteiformis is legally protected in Hungary under the 13/2001 (V. 9.) KöM Decree, while the subspecies is also protected in the Czech Republic and Romania (Hohla et al., 2009; Grulich, 2012; Szatmari, 2014, Royal Botanic Gardens, Kew 2026). Recent floristic surveys have refined the known distribution of the subspecies by reporting new localities, indicating that its occurrence is still incompletely documented (Molnár et al., 2018; Csiky et al., 2018). The taxon is characteristic of species-rich Pannonian saline woodland meadows and forest-steppe grasslands, habitats supporting numerous rare and protected plant species but currently declining in extent (Illyés et al., 2009). Recent studies by Hungarian researchers have further highlighted the ecological importance of the subspecies by demonstrating that it is one of the principal nectar sources of the protected clouded Apollo butterfly (*Parnassius mnemosyne*) in Hungary (Konvička & Kuras, 1999; Välimäki & Itämies, 2003; Baranyai & Korompai, 2007; Cini et al., 2020), emphasizing its role in maintaining species interactions within Pannonian grassland ecosystems (Szigeti et al., 2020; Vajnai et al., 2021; Gór et al., 2023). Consequently, both habitat conservation and ex situ propagation are important components of its long-term conservation.

Many threatened *Dianthus* species face similar conservation challenges. Habitat fragmentation reduces population size and connectivity, promoting genetic isolation and loss of genetic diversity (Putz et al., 2015; Butiuc-Keul et al., 2018). Small, isolated populations may also experience reduced reproductive success, while the abandonment of traditional grassland management promotes shrub encroachment and the degradation of open habitats required by many *Dianthus* species (Németh et al., 2011; Osvalde et al., 2021). These factors highlight the importance of integrating habitat management with complementary ex situ conservation measures.

The conservation of threatened plant species relies on the complementary application of *in situ* and *ex situ* strategies. While *in situ* conservation protects species within their natural habitats, *ex situ* approaches provide an important safeguard against population decline and habitat loss by maintaining living collections and supplying plant material for restoration programmes (Fay, 1992; Pence, 2011). Among *ex situ* techniques, in vitro micropropagation has become an effective tool for the conservation of rare and endangered plant species, enabling the rapid multiplication of plants from limited source material while minimizing collection pressure on natural populations (Fay, 1992; Pence, 2011; Németh et al., 2011; Chandran et al., 2020; Kulak et al., 2022; Farkas et al., 2025).

Numerous protocols have been developed for the in vitro propagation of threatened plant species, and in many cases plants can be successfully regenerated and acclimatized under greenhouse conditions (Fay, 1992; Pence, 2011; Chandran et al., 2020; Kulak et al., 2022). However, relatively few studies have evaluated the long-term performance of micropropagated plants under outdoor conditions, particularly their overwinter survival and flowering, although winter survival, habitat conditions and biotic interactions are major determinants of the long-term persistence of perennial and Dianthus populations (Di Sabato-Aust, 1987; Herrick & Perry, 1995; Kostrakiewicz-Gierałt, 2014). Furthermore, because micropropagation produces clonal propagules, conservation protocols should be designed to preserve the genetic diversity of natural populations by using multiple donor genotypes and maintaining their identity throughout the propagation process.

The aim of this study was to evaluate the overwinter survival and flowering performance of in vitro propagated *Dianthus giganteiformis* subsp. *pontederae* under outdoor conditions and to assess their suitability for conservation purposes.

Materials and Methods

The experiment was conducted using in vitro-propagated plants of *Dianthus giganteiformis* subsp. *pontederae* (A. Kern.) Soó. Micropropagation was performed in early spring 2025 under sterile laboratory conditions at the Tissue Culture Laboratory of the Centre for Agricultural Genomics and Biotechnology, University of Debrecen (Nyíregyháza, Hungary). The rooted plantlets were acclimatized in a controlled acclimatization room until 12 September 2025 (Figure 1) and subsequently transferred to the greenhouse of the University of Nyíregyháza, where acclimatization was completed under greenhouse conditions (Figure 2).

A total of 160 successfully acclimatized plants, representing 20 Seed IDs, were available for the overwintering experiment. The genotypes were identified by their Seed IDs: 3, 8, 15, 23, 28, 31, 35, 36, 38, 49, 54, 58, 59, 61, 63, 67, 70, 109, 125, and 129. Each Seed ID was represented by eight plants, of which four were assigned to outdoor overwintering and four to greenhouse overwintering, resulting in 80 plants per treatment. Within both treatments, the positions of individual plants were randomized with respect to Seed ID.

Outdoor overwintering treatment. On 1 October 2025, 80 plants were transplanted to the outdoor experimental plot. Plants were arranged in four rows of 20 individuals with approximately 20 cm spacing within rows and 90 cm between rows, ensuring that plants of the same genotype were not grouped together. No irrigation was applied during the overwintering period. To reduce frost damage, plants were protected with a white non-woven horticultural fleece during the coldest part of the winter, which was removed before the onset of spring growth (Figure 3).

Greenhouse overwintering treatment. The remaining 80 plants were maintained in a moderately heated greenhouse at approximately 13–15 °C throughout the winter and watered manually as required under natural daylight conditions.

On 3 April 2026, following the completion of the overwintering evaluation, the greenhouse-overwintered plants were transplanted to the outdoor experimental plot. From this date onwards, all plants were cultivated under identical outdoor conditions and irrigated using a drip irrigation system during the growing season.

The field experiment was conducted in the demonstration garden of the Institute of Engineering and Agricultural Sciences, University of Nyíregyháza, Nyíregyháza, Hungary (47.97398° N, 21.70864° E). The soil was a sandy loam (Arany plasticity index, KA = 31) with a slightly alkaline pH (7.29) and a humus content of 2.1%. The soil had a good nutrient status, containing 339 mg kg^{-1} available phosphorus, 183 mg kg^{-1} available potassium, 19.1 mg kg^{-1} nitrate–nitrogen, 28.4 mg kg^{-1} manganese, and 11.67 mg kg^{-1} zinc (Csabai et al., 2021; Csabai et al., 2022).

During the overwintering period (December 2025–February 2026), outdoor-grown plants were exposed to natural weather conditions. According to the Hungarian Meteorological Service (HungaroMet, 2026), the winter was generally milder and drier than the long-term average; however, January included an approximately three-week cold period with persistent snow cover and minimum air temperatures locally reaching −15 to −20 °C.


A


B

Figure 1. In vitro propagation (A) and acclimatization (B) of *Dianthus giganteiformis subsp. pontederae* (A. KERN.) SOO at the Tissue Culture Laboratory of the University of Debrecen, Agricultural Science Centre, Nyíregyháza Research Institute

Figure 2. Greenhouse acclimatization of *Dianthus giganteiformis subsp. pontederae* (A. KERN.) SOO in the greenhouse of the University of Nyíregyháza

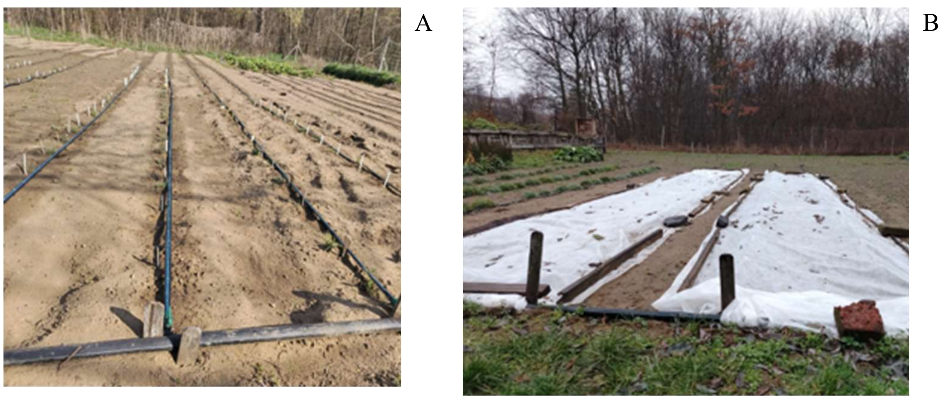

Figure 3. Transplanted *Dianthus giganteiformis* subsp. *pontederae* (A. KERN.) SOO plants in the outdoor experimental plot (A) and winter protection with white horticultural fleece (B).

Data Collection

Plant performance was assessed at two-week intervals from November 2025 to June 2026. At each assessment, plant survival, flowering, the number of flowering stems, and overall plant vigor were recorded. Plants were considered alive when viable shoots or buds were present. Flowering was recorded when at least one open flower was observed. The number of flowering stems was counted for each flowering plant.

Data Analysis

Survival rate was calculated as the percentage of surviving plants, whereas flowering rate was expressed as the percentage of flowering plants within each treatment. Mean flowering stem number was calculated using all surviving plants only.

Figure 4. Flowering *Dianthus giganteiformis* subsp. *pontederae* (A. KERN.) SOO plants in the outdoor experimental plot

Seed ID evaluation

The performance of the individual Seed IDs was evaluated separately. For each Seed ID, survival, flowering, flowering stem production, and overall plant vigor were evaluated across the replicates. These observations were used to identify genotypes with superior winter hardiness, flowering performance, and post-winter recovery.

Data Analysis

Treatment comparison

The number of stems was recorded for each living plant at every observation date. Blank cells in the stem data represented plants that had died before the observation date and were therefore excluded from the calculation. Living plants that did not produce flowering stems were assigned a value of zero. Mean stem number was calculated using all surviving plants within each treatment (outdoor and greenhouse) at each observation date. Dead plants were not included in the stem analysis because flowering stem production could not be assessed after plant mortality. Survival rate and flowering rate were calculated as the percentage of surviving and flowering plants, respectively, out of the total number of plants in each treatment. The two overwintering treatments were compared based on survival, flowering, and mean stem number.

Seed ID evaluation

The performance of each Seed ID was evaluated separately using the four replicate plants in each overwintering treatment. Survival, flowering, stem development, and overall plant vigor were compared among Seed IDs to identify genotypes with superior winter hardiness, vegetative growth, and flowering performance.

For each Seed ID, a performance score was calculated using three parameters: survival, flowering, and plant vigor. Survival score was based on the percentage of surviving plants. Flowering score was assigned according to flowering performance (100 = all surviving plants flowered, 50–75 = partial flowering, and 0 = no flowering). Plant vigor was evaluated visually based on stem development and overall plant condition using the following scale: very strong = 100, strong = 75, moderate = 50, weak = 25, and dead = 0. The final performance score for each treatment was calculated as the arithmetic mean of the three scores:

$$FPS = \frac{S + F + V}{3}$$

FPS = Final performance score,

S = Survival score,

F = Flowering score,

V = Plant vigor score.

The final performance score (FPS) was calculated as the arithmetic mean of the survival, flowering, and plant vigor scores, with each component contributing equally to the overall evaluation.

Statistical methods

Survival and flowering rates were compared between the outdoor and greenhouse overwintering treatments using Pearson's chi-square test. The strength of the association between overwintering treatment and categorical variables was evaluated using Cramer's V. Differences in the Final Performance Score (FPS) of individual Seed IDs between the two overwintering treatments were analysed using the paired Wilcoxon signed-rank test. The relationship between genotype performance in the two overwintering environments was assessed using Spearman's rank correlation coefficient (Jánosa, 2015). Statistical significance was accepted at $p < 0.05$.

Results

Effects of overwintering treatment

Outdoor overwintering resulted in higher survival and flowering rates than greenhouse overwintering. At the end of the study, 60 of the 80 outdoor-overwintered plants survived (75.0%), compared with 50 of the 80 greenhouse-overwintered plants (62.5%). Flowering was also more frequent in the outdoor treatment, where 60 plants (75.0%) flowered, whereas only 24 plants (30.0%) flowered after greenhouse overwintering (Figure 5). These results indicate that overwintering conditions influenced both plant survival and flowering during the first growing season.

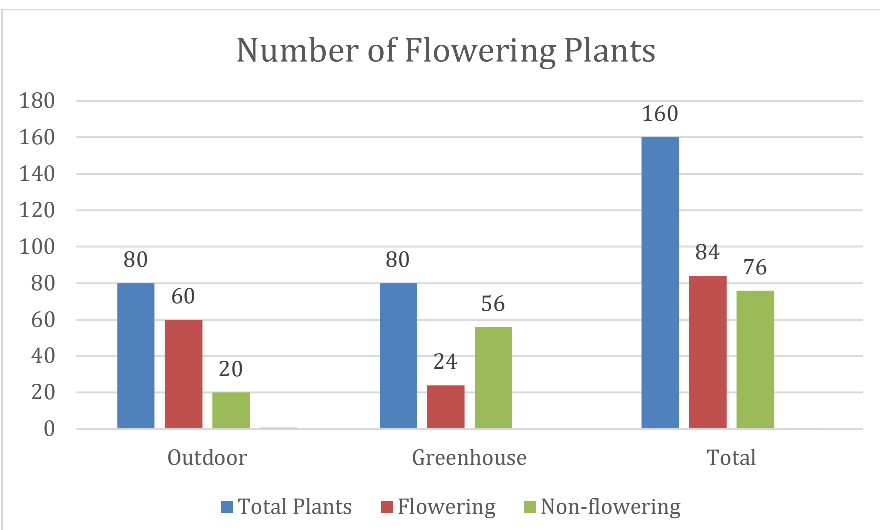

Figure 5. Flowering performance of *Dianthus giganteiformis subsp. pontederae (A. KERN.) SOÓ* under outdoor and greenhouse overwintering conditions (number of plants).

The mean number of flowering stems per surviving plant was consistently higher in plants that had overwintered outdoors than in those that had overwintered in the greenhouse, although all plants were cultivated under identical outdoor conditions during the evaluation period (Figure 6). On 13 April 2026, the mean number of flowering stems was 9.54 in the outdoor treatment and 6.91 in the greenhouse treatment. By 13 June 2026, these values had increased to 19.38 and 16.28, respectively, indicating greater flowering stem production following outdoor overwintering. The corresponding standard deviations are presented in Table 1.

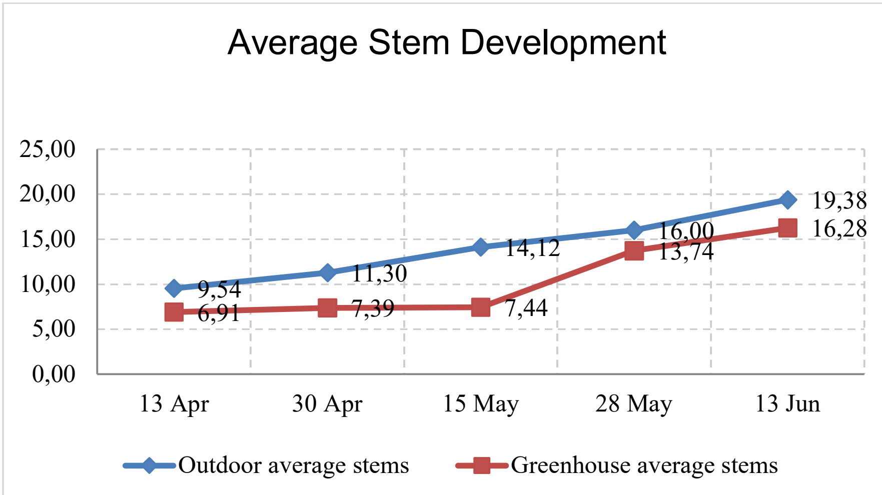

Figure 6. Mean number of flowering stems per surviving plant of *Dianthus giganteiformis* subsp. *pontederae* following outdoor and greenhouse overwintering during the 2026 growing season.

Table 1. Mean (± SD) number of flowering stems per surviving plant under outdoor and greenhouse overwintering conditions.

Date	**Outdoor (mean ± SD)**	**Greenhouse (mean ± SD)**
13 Apr	9.54 ± 6.58	6.91 ± 5.17
30 Apr	11.30 ± 7.00	7.39 ± 5.05
15 May	14.12 ± 7.77	7.44 ± 5.36
28 May	16.00 ± 7.95	13.74 ± 7.02
13 Jun	19.38 ± 10.58	16.28 ± 7.43

Seed ID evaluation

Genotype-level analysis revealed considerable variation among the evaluated Seed IDs (Figure 7). Seed ID 36 achieved the highest average Final Performance Score (91.50), followed by Seed IDs 61 (87.34) and 49 (84.17). In contrast, Seed ID 15 had the lowest score (5.84). Based on the Final Performance Score, 45% of the evaluated Seed IDs were classified as excellent, 25% as good, 15% as moderate, and 15% as weak performers. These results demonstrate substantial differences among seed-derived genotypes in winter survival, flowering, flowering stem production, and overall post-winter vigor, indicating that genotype strongly influenced plant performance under the evaluated overwintering conditions.

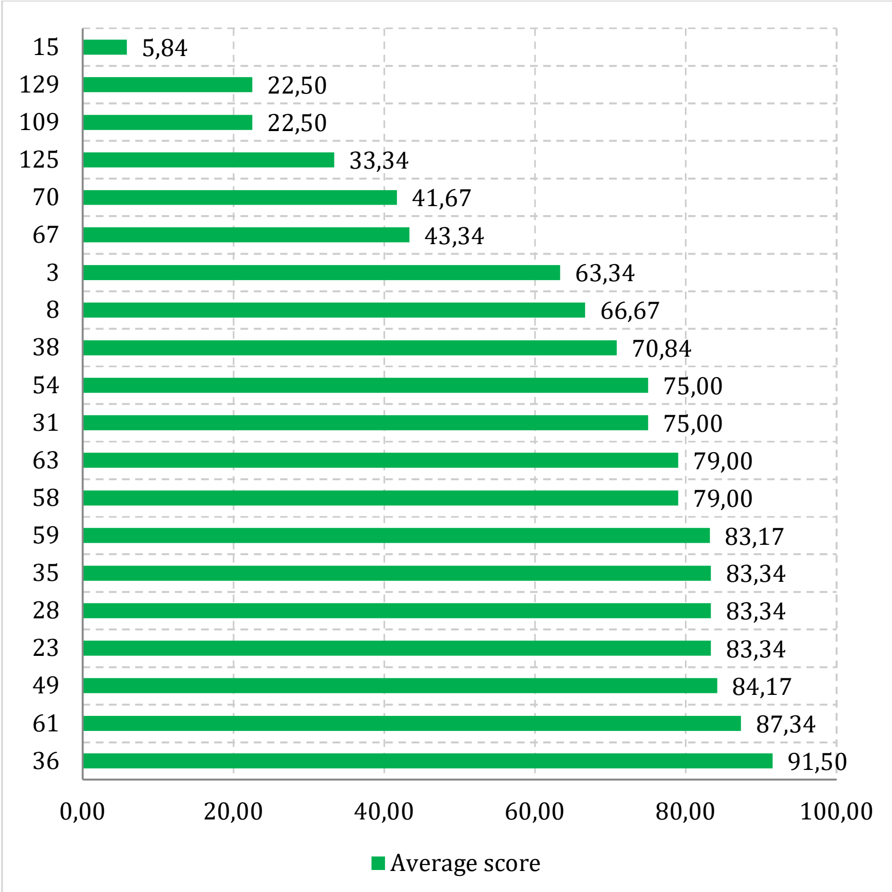

Figure 7. Average Final Performance Scores (FPS) of the evaluated Seed IDs based on survival, flowering, and plant vigor.

Statistical analysis

Pearson's chi-square test showed that the difference in survival rate between the outdoor (75.0%) and greenhouse (62.5%) overwintering treatments was not statistically significant ($\chi^2 = 2.91$, df = 1, $p = 0.088$). The association between overwintering treatment and survival was weak (Cramer's V = 0.135).

In contrast, flowering rate differed significantly between the two overwintering treatments. While 75.0% of outdoor-overwintered plants flowered, only 30.0% of greenhouse-overwintered plants produced flowers ($\chi^2 = 32.48$, df = 1, $p < 0.001$). The effect size was moderate to strong (Cramer's V = 0.451), indicating a pronounced influence of overwintering treatment on flowering.

A paired Wilcoxon signed-rank test revealed a significant difference in the Final Performance Scores (FPS) of the Seed IDs between the two overwintering treatments (Wilcoxon signed-rank test: $W = 0.0$, $p < 0.001$). Overall, genotypes performed significantly better after outdoor overwintering than after greenhouse overwintering.

Spearman's rank correlation analysis revealed a moderately strong positive relationship between the FPS values obtained under the two overwintering treatments ($\rho = 0.659$, $p = 0.002$). Thus, Seed IDs that performed well under outdoor overwintering generally also performed well after greenhouse overwintering, although their overall performance was consistently lower under greenhouse conditions.

Conclusion

This study demonstrated that in vitro-propagated *Dianthus giganteiformis* subsp. *pontederae* can successfully survive winter under outdoor conditions and flower during the subsequent growing season. Outdoor overwintering resulted in higher survival, substantially greater flowering, and higher overall performance than greenhouse overwintering. Although survival did not differ significantly between treatments, outdoor overwintering had a pronounced positive effect on flowering and the Final Performance Score.

Considerable differences were observed among Seed IDs, indicating that genotype influences post-winter performance. This finding highlights the importance of maintaining multiple genetic lines in ex situ conservation programmes rather than relying on a limited number of clones.

These results support the use of micropropagation as an effective tool for the conservation of this protected taxon, provided that adequate genetic diversity is maintained throughout the propagation process. The successful establishment, overwintering, and flowering of in vitro-derived plants demonstrate the potential of this approach for reinforcement of ex situ collections and future conservation actions.

As the present study evaluated plant performance during the first year after acclimatization, further long-term monitoring is required to assess longevity, reproductive success over multiple growing seasons, and the suitability of propagated plants for future population reinforcement or reintroduction programmes.

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