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IN VITRO REGENERATION OF GLACIAL RELICT *VIOLA PALUSTRIS* L. SUPSP. *PALUSTRIS* AS GROUNDWORK FOR SUSTAINABLE USE AND GERMPLASM CONSERVATION

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Abstract

Climate change and increasing anthropogenic pressures have accelerated the degradation of European peatlands, leading to the decline of specialist wetland flora. Viola palustris L., a glacial relict and indicator of peatland ecosystem health, is currently listed as critically endangered in Romania. To support future conservation and sustainable use of this species, we developed an in vitro regeneration protocol based on aseptic germination of immature embryos and subsequent shoot and root development on optimized culture media. A custom sterilization procedure effectively eliminated fungal contamination typical of wetland-derived material, enabling reliable culture initiation. Although germination percentages were modest, regenerated plantlets exhibited normal morphology, vigorous growth, and strong multiplication capacity, particularly on media supplemented with balanced cytokinin–auxin–gibberellin combinations. Root induction with indole-3-butyric acid produced dense, healthy root systems, and acclimatized plants remained vigorous under ex vitro conditions. This protocol provides a practical foundation for establishing germplasm collections, supporting peatland restoration efforts, and facilitating the horticultural or phytochemical valorization of V. palustris.

Key words*: anthropic pressure, endangered plants, ex situ conservation, Viola palustris.*

INTRODUCTION

Marsh violet - *Viola palustris* L.- (Figure 1) is a polycarpic perennial plant, generally glabrous, stemless with thin rhizomes, which in summer emerges aerial stolones. Leaves are simple, 3-4 grouped in basal rosettes, ascending to erect, glabrous, with a petiole of 4-15 cm and sub-median bracteoles. Leaf blade of 2-6 cm is reniform, wider than long, and with an obtuse tip. The flowers are odourless, measure about 1-1.5 cm, have ovate to lance-ovate sepals, and pale lilac-coloured petals (Dihoru & Negrean, 2009)

V.palustris is a glacial relict (Dihoru & Negrean, 2009; Szatmari & Hurdu, 2022), cold-adapted, and occurs in niche habitats hosted in eutrophic and transition marshes. During the last centuries, the wetland areas have significantly diminished under the combined pressure of both climate change and human activities (Swindles et al., 2019). Consequently, the quality of wet habitats is altered and the species range is declining. Strong related to its habitat, *V. palustris* is considered an indicator of peatland health; a decline in its populations or the loss of subpopulations signals significant changes in wetland and peatland habitats (Zabicka et al., 2023). In Romania, the species has scarce populations, being reported mainly from a few peatlands in Suceava and Maramureș counties. In national red lists and books, is currently assessed as *Critically Endangered* (Dihoru & Negrean, 2009; Hurdu et al., 2022). Despite population decline at the national level to date, no conservation measure was taken.

To restore peatland ecosystem services, the Institute of Biology Bucharest of the Romanian Academy began implementing various projects in 2015. In the PeatRO2 project, focused on the restoration of degraded mires and peatlands in the North-East 1 region of Romania, various restoration activities were conducted in peatland habitats from Suceava County. In addition to restoration activities, some *ex situ* conservation attempts were undertaken for a range of endangered plant species recorded at damaged sites, including the marsh violet.

Apart from its conservative importance, marsh violet represents an unexploited valuable genetic source as a cultivated plant in wet landscaping (rain gardens). In sympatric

European populations of *V. palustris*, interspecific hybrids with other violet species were identified (Zabicka et al., 2023), which recommends marsh violet as a valuable genetic source for amelioration in floriculture.
Moreover, in its natural habitat, marsh violet has a key role in ecosystem trophic relations. Marsh violet is a larval food plant for the small pearl-bordered fritillary (*Boloria selene*), a threatened butterfly species whose decline is strongly correlated with marsh violet population decline (Kendon et al., 2021).
In addition, recent studies have shown that wild violas are a valuable source of bioactive compounds supporting a wide range of biological functions due to their antibacterial, anti-inflammatory, and antioxidant effects (Zeng et al., 2025).
Considering the complex importance of marsh violet, the goal of the present study was to develop an efficient protocol for *in vitro* multiplication as a preliminary step for its germplasm *ex situ* conservation and availability for further natural habitat restoration, floriculture, or bioactive compound production.

MATERIALS AND METHODS

Plant material

Plant material sampling was allowed under collection permit No. 4621 issued on 02.04.2022 by the *Commission for the Protection of Natural Monuments*, Romanian Academy.



Figure 1. *Viola palustris* L. habitus - anthesis and fructification (Photo by Anca Manole)

Samples consisting of immature fruits (capsules) were collected between June and July 2023 during field trips conducted at the site *Tinovul din spatele Școlii Coșna*, Suceava County (47° 22' 16.80" N, 25° 10' 38.76" E).
Capsules were air-dried until the outer membrane detached from the immature embryos, allowing for inspection of morphological characteristics and evaluation of seed viability.
Seeds were collected from all immature fruits and evaluated for use in *in vitro* cultures.
Seed selection was performed by visual inspection in regard to size variance, tissue damage, and other morphological variations, with healthy seeds being selected for regeneration.
To ensure aseptic conditions, all sample handling and procedures were performed in the controlled environment of a laminar airflow cabinet.

Sterilization

A custom sterilization protocol was developed for seed surface sterilization, involving a series of protocol steps:

1. Pre-washing: seeds were placed under running tap water for 30 minutes, with the addition of 5 ml of detergent (Domestos) to reduce surface residues.
2. Ethanol washing: seeds were immersed in a 70% (v/v) ethanol solution for 1 minute for antimicrobial membrane permeabilization.
3. NaDCC washing: seeds were immersed in a 2% (w/v) NaDCC (Sodium dichloroisocyanurate) for 10 minutes of exposure, while stirring the vial for uniform exposure.
4. Rinsing: seeds were rinsed 5 times with sterile double-distilled water to ensure all traces of NaDCC were removed.

After successful sterilization, seeds were inoculated onto different variants of culture media to ensure survivability and reduce desiccation.

Culture Media

Culture media variants M1-M4 and M6 were prepared using the same base of basal salts and vitamins, following an adapted Murashige & Skoog (MS) medium recipe (Murashige & Skoog, 1962). Composition of the media was adjusted with supplemental sucrose, 3% (w/v), and the media were solidified by adding 0.35% (w/v) agar. In the case of M6 the composition was also supplemented with the addition of activated charcoal, 1% (w/v).

The pH was measured and adjusted to 5.8 ± 0.10, using stock solutions of 1N HCl and 1N NaOH. Media sterilization was done by autoclaving at 121°C for 20 minutes. After the media cooled to 50°C, selected plant growth regulators (PGRs) were added to complete the media variants.

Culture medium M5 was prepared using a Woody Plant (McCown & Lloyd, 1981) basal medium (WPM) , supplemented with 1 mg/L Kinetin, with supplemental sucrose, 3% (w/v), and the media were solidified by adding 0.35% (w/v) agar.

Table 1. Variants of culture media (mg/L)

Media/ PGRs	BAP	GA_3	Kin	IAA	IBA	NAA
M1	1	0.25	-	0.5	-	-
M2	-	1	-	0.1	-	-
M3	1	-	-	0.1	-	-
M4	-	-	-	-	1.5	-
M5	-	-	1	-	-	-
M6	5	-	-	-	-	1

***In vitro* Germination**

Three replicates of 10 seeds were inoculated on variants of M6 medium to assess germination and regeneration capacity.

The culture media with inoculated seeds were stored and incubated in a growth chamber at 24± 2°C, photoperiod with a 16/8 h light/dark cycle and a light intensity of 40-50 µmol m^{-2} s^{-1}. Germination rates were recorded after 7 and 14 days, while shoot length was measured at 60 days.

Total germination capacity was evaluated using formulas for the calculation of indicators of seed germination:

GE - Germination energy (%) = (Number of germinated seeds for 7d÷Total number of seeds) ×100

GR - Germination rate (%) = (Number of germinated seeds for 14d÷Total number of seeds) ×100

Shoot multiplication

After sufficient growth and development, tissue explants represented by nodal segments from the *in vitro* seedlings were used as explants for shoot multiplication.

Explants were inoculated in 350 mL culture jars containing 40±5 mL of media. Culture environmental conditions were maintained as those described for germination.

Parameters such as the number of shoots, internode length, and vigour of explants were evaluated after 30 days. Subculture intervals were extended first to 60 days, then 90 days, as shoot proliferation and vigour increased.

Root induction

Rooting was induced by transferring elongated shoots to a basal MS medium supplemented with 1.5 mg/L indole-3-butyric acid (IBA).

Root development was monitored every 15 days, and the parameters recorded included root length, number of roots, as well as root morphology.

Acclimatization

Regenerated plants were transferred to *ex vitro* conditions in a solid substrate mixture of soil and perlite.

After a period of 14 days, the plant remained vigorous and presented no morphological or physiological abnormalities.

After 30 days, plants remained healthy and began producing new vegetative growth.

RESULTS AND DISCUSSIONS

Seed germination

The custom sterilization protocol developed for *V. palustris* seeds ensured that most contaminants from the seed surface and within the tissue were reduced or eliminated. NaDCC is especially effective against bacteria and fungi, which were the main risks of culture contamination.

Longer exposure times of the sterilizing agent yielded no contamination due to fungal infection across all replicates in the germination phase. This emphasizes the necessity of adapting sterilization protocols for removing fungal contaminants, especially from plants harvested from wet environments.

After sterilization, the seeds were inoculated on M6 medium variant with added active charcoal. Each 7 days, indicators for germination were calculated.

The germinative energy for all replicates varied between 10-20% in the initial timespan of a

week. Germination rate after 14 days was calculated between 30-40%. Total germination percentage after 30 days was calculated at 33.33% (Figure 2).
After germination had occurred, the plantlets began generating multiple shoots and increased rapidly in size.
Auxin presence in the medium, in small concentrations, induced rhizogenesis parallel to shoot generation. The plantlets developed a few small adventitious roots, white to pale green in colour.
Although germination rates were low, the shoots exhibited strong viability, and the developmental response was favourable in the presence of salts in MS media. Growth and development of the plantlets was uniform, with well-differentiated morphology.



Figure 2. *Viola palustris* L. plantlet germinated *in vitro* on MSR medium, 14 days after germination (Photo by Mihnea Vladimirescu)

The germinated plantlets exhibited no abnormal morphological characteristics. Shoot proliferation and elongation occurred rapidly; regenerated plantlets generated numerous axillary shoots from a basal nodal segment. Shoots developed long internodes with small to medium leaves.

Shoot growth and multiplication

Plantlets regenerated from immature embryos were transferred as individuals or used for explants on variants M1-M3 of culture media with different PGR concentrations to evaluate the best composition for germplasm growth and storage (Table 2).

Table 2. Shoot multiplication response on different media variants

Medium	PGR Composition	Multiplication Rate	Morphological Response
M1	BAP 1 mg/L + GA_3 0.25 mg/L + IAA 0.5 mg/L	High	Vigorous shoots, balanced growth
M2	GA_3 1 mg/L + IAA 0.1 mg/L	Low	Thin stems, few axillary shoots, small leaves
M3	BAP 1 mg/L + IAA 0.1 mg/L	Moderate	Multiple shoots but less vigorous than M1
M5	Kinetin 1 mg/L	Low–Moderate	Slow growth, reduced senescence
M6	BAP 5 mg/L + NAA 1 mg/L + activated charcoal	Not used for multiplication	High cytokinin inhibited germination

M1 medium variant provided the highest growth and multiplication rates among the tested variants (Figure 3). The PGR mix of cytokinin, auxin, and gibberellin used in this variant induced a balanced morphological response.
Plantlets and explants generated axillary shoots within a month after inoculation. Shoots began to proliferate and grow over the following months. Subculture interval was optimized from 1 month in the initial stages of growth to 3 months following accelerated growth on this medium variant.



Figure 3. *Viola palustris* L. plantlets regenerated and multiplied on M1 medium variant (Photo by Mihnea Vladimirescu)

M2 variant offered low multiplication rates. Presence of gibberellin in this variant appeared to reduce the growth of plantlets, having reduced stem diameter, a low number of axillary shoots and leaves, and smaller leaf sizes.



Figure 4. *Viola palustris* L. plantlets regenerated and multiplied on M3 medium variant (Photo by Mihnea Vladimirescu)

M3 variant offered similar results to M1 variant (Figure 4), but reactivity was lower and growth balance was skewed. Multiple axillary shoots were present but were not as vigorous as M1.
M5 medium variant based on WPM was used as an intermediate storage medium base. The kinetin supplement to this variant was used due to its properties as an anti-stress agent. Although shoot generation still occurred frequently, the senescence-retarding effect of kinetin induced a slower progression of life cycles and reduced loss of chlorophyll and yellowing.

Table 3. Tukey HSD post-hoc for shoot length

Comparison	Mean diff (cm)	p-value	Cohen's d
M1 vs M2	3.55	0	3.26
M1 vs M3	2.11	0	1.72
M2 vs M3	−1.44	0	−2.15

Group means (cm, across 12 replicates):

- M1: 4.64
- M2: 1.09
- M3: 2.53

M1 produced substantially longer shoots than both M2 and M3, and M3 also clearly exceeded M2 in shoot length (Table 3).

Root induction

Rhyzogenesis was induced separately from growth and development of the main plantlet tissues.
Grown plantlets were placed on M4 medium with 1.5 mg/L indole-3-butyric acid (IBA). After 7 days, multiple short lateral roots began sprouting, and after 14 days, the roots began forming a lattice on the sides and bottom of the culture jar, with long, fibrous, and thin roots present throughout the medium. After a month, all plantlets had developed healthy, dense, and wide-spread root systems (Figure 5).

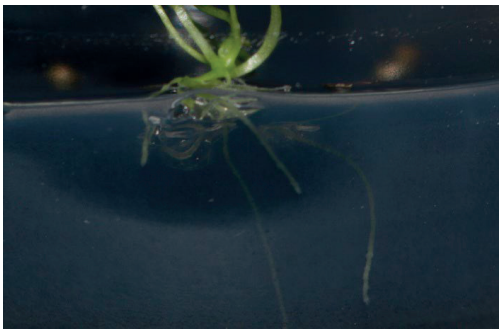

Figure 5. *Viola palustris* L. plantlet with regenerated roots (Photo by Mihnea Vladimirescu)

Table 4. Tukey HSD post-hoc for root length

Comparison	Mean diff (cm)	p-value	Cohen's d
M1 vs M2	4.32	0	2.06
M1 vs M3	3.33	0	1.49
M2 vs M3	−0.99	>0.05	−0.73

Group means (cm, across 12 replicates):

- M1: 6.14
- M2: 1.82
- M3: 2.81

M1 induces significantly longer roots than both M2 and M3, with large effect sizes. The difference between M2 and M3 is moderate in magnitude ($d \approx 0.73$) but does not reach significance at $\alpha = 0.05$ under Tukey correction (Table 4).

Table 5. ANOVA summary across media (M1, M2, M3)

Dependent variable	F	df (between, within)	p	η^2
Shoot number	0.00	2, 33	1.00	0.00
Shoot length	36.77	2, 33	0.0000	0.69
Root number	0.00	2, 33	1.00	0.00
Root length	16.35	2, 33	0.0000	0.50

- Shoot number and root number: no effect of medium (values are structurally identical across media).
- Shoot length: very strong medium effect (large $\eta^2 \approx 0.69$).
- Root length: strong medium effect ($\eta^2 \approx 0.50$).

Shoot number and root number did not differ among media (one-way ANOVA, $F(2.33) = 0.00$, $p = 1.00$ for both traits (Table 5). In contrast, shoot length was strongly affected by medium ($F(2.33) = 36.77$, $p < 0.001$, $\eta^2 = 0.69$), with M1 producing significantly longer shoots

than M2 and M3 (Tukey HSD, $p < 0.001$), and M3 exceeding M2 ($p < 0.001$) (Table 2).
Root length also differed significantly among media ($F(2.33) = 16.35$, $p < 0.001$, $\eta^2 = 0.50$), with M1 yielding longer roots than both M2 and M3 (Tukey HSD, $p < 0.001$), while the difference between M2 and M3 was not significant (Table 3).

Acclimatization

The acclimatization phase demonstrated that *in vitro*-regenerated *V. palustris* plantlets possess strong adaptive capacity when transferred to *ex vitro* conditions. Rooted plantlets were transplanted into a soil-perlite substrate and maintained under high humidity during the initial transition period. After 14 days, the plants remained vigorous and exhibited no signs of stress, confirming the functional integrity of the root systems developed *in vitro*. As noted in the original observations, "after a period of 14 days, the plant remained vigorous and presented no morphological or physiological abnormalities," indicating successful adaptation to non-sterile conditions.



Figure 6. Acclimatized *Viola palustris* L. plantlet after 30 days (Photo by Mihnea Vladimirescu)

By 30 days, all acclimatized individuals continued to grow actively, producing new leaves and stoloniferous structures characteristic of healthy marsh violet plants. The statement that "after 30 days, plants remained healthy and began producing new vegetative growth" underscores the stability of the regenerated material and the suitability of the rooting protocol for generating robust plants capable of surviving environmental transition.
These results confirm that the *in vitro* regeneration system developed in this study can reliably produce acclimatized plants suitable for *ex situ* conservation, future reintroduction efforts, or horticultural applications. The high survival rate during acclimatization highlights the physiological normality of the regenerated plantlets and supports the broader applicability of the protocol for conservation and restoration programs targeting *V. palustris*.

Discussions

The present study represents an important step toward establishing an efficient *in vitro* regeneration system for *V. palustris*, a species of high conservation concern whose natural populations are declining due to peatland degradation. The successful initiation of aseptic cultures from immature embryos demonstrates that *in vitro* techniques can overcome the challenges associated with propagating wetland species, which often carry high microbial loads. The effectiveness of the custom sterilization protocol - particularly the use of NaDCC - confirms its suitability for plant material collected from humid, microbe-rich environments. This aligns with previous findings that chlorine-based disinfectants are especially effective for species originating from wet habitats (Jadranin et al., 2024; Kendon et al., 2021; Li & Gallagher, 1996).
Germination percentages were modest, with germinative energy ranging from 10-20% after seven days and total germination reaching 33.33% after 30 days. These values are comparable to those reported for other Viola species, which frequently exhibit physiological dormancy or embryo immaturity at the time of seed dispersal (Wang et al., 2023; Kuroda & Sawada, 2022; Varsamis et al., 2025). Nevertheless, once germination occurred, plantlets displayed vigorous growth and normal morphology, indicating that the MS-based medium provided adequate nutritional support.
Shoot multiplication varied considerably among media variants. M1, which combined cytokinin, auxin, and gibberellin, produced the most balanced and vigorous shoot proliferation. This suggests a synergistic effect between the three PGR classes, promoting both cell division and elongation. The reduced performance of M2, containing gibberellin and low auxin, indicates that gibberellin alone is insufficient to sustain robust shoot formation in *V. palustris*. M3

produced acceptable but less vigorous growth, highlighting the importance of fine-tuning cytokinin–auxin ratios for optimal morphogenesis. These findings are consistent with previous studies on *Viola* species, where balanced PGR combinations were essential for stable shoot proliferation (Zhang et al., 2025; Babu et al., 2025; Capaci et al., 2024; Kendon et al., 2021).

Root induction on IBA-supplemented medium was rapid and uniform, with dense, fibrous root systems forming within two weeks. This response aligns with the well-established role of IBA in promoting adventitious rooting in herbaceous species. The successful acclimatization of regenerated plants further demonstrates the physiological stability of the *in vitro*-derived material and confirms the suitability of the protocol for producing viable plants for *ex situ* conservation or restoration.

The statistical analysis of shoot and root development across the three culture media provides clear quantitative confirmation of the morphological trends observed during the experiment. As noted earlier in the manuscript, "M1 medium variant provided the highest growth and multiplication rates among the tested variants" and the ANOVA results strongly support this qualitative assessment. Shoot number and root number did not differ among media, reflecting the fact that each replicate produced a similar number of organs regardless of treatment. This uniformity is consistent with the species' intrinsic developmental pattern, in which the initiation of new shoots and roots appears to be less sensitive to hormonal variation than subsequent elongation processes.

In contrast, both shoot length and root length showed highly significant differences among media, indicating that elongation growth is strongly regulated by the specific hormonal composition of the culture medium. Shoot length exhibited a very large effect of medium ($F(2,33) = 36.77$, $p < 0.001$, $\eta^2 = 0.69$), with M1 producing markedly longer shoots than either M2 or M3. Tukey post-hoc comparisons revealed that all pairwise differences were significant, and the effect sizes were exceptionally large (Cohen's $d > 1.7$ for M1 vs M3 and > 3.2 for M1 vs M2). These results confirm that the balanced cytokinin–auxin–gibberellin combination in M1 strongly promotes both cell division and elongation, leading to vigorous shoot development. This aligns with previous observations in the genus Viola, where synergistic interactions between cytokinins and gibberellins have been shown to enhance shoot elongation and reduce apical dominance.

Root elongation followed a similar pattern, with a significant medium effect ($F(2,33) = 16.35$, $p < 0.001$, $\eta^2 = 0.50$). Again, M1 supported the most extensive root growth, producing roots more than twice as long as those in M2 and substantially longer than those in M3. The contrast between M1 and M2 was particularly pronounced ($d \approx 2.06$), reflecting the inhibitory effect of gibberellin-only supplementation in M2 on root elongation. Although M3 performed better than M2, the difference between these two media was not statistically significant, suggesting that the cytokinin-auxin balance in M3 was insufficient to fully stimulate rhizogenesis. These findings reinforce the conclusion that auxin availability - particularly in combination with cytokinin - is essential for robust root development in *V. palustris*, a pattern consistent with classical models of auxin-driven root induction.

Taken together, the statistical results demonstrate that M1 is the most effective medium for promoting vigorous, balanced growth of *V. palustris* plantlets. The strong elongation responses observed in both shoots and roots indicate that this medium provides an optimal hormonal environment for early morphogenesis, supporting the earlier qualitative observation that "growth and development of the plantlets was uniform, with well differentiated morphology". The lack of variation in organ number across media suggests that while initiation of shoots and roots is relatively stable, their subsequent elongation is highly plastic and strongly influenced by exogenous growth regulators.

These findings have important implications for conservation and propagation efforts. Efficient elongation of both shoots and roots is essential for producing robust plantlets capable of surviving acclimatization and eventual reintroduction into peatland habitats. The superior performance of M1 therefore positions it as the most suitable medium for large-scale multiplication of *V. palustris* germplasm. Moreover, the strong effect sizes observed in the

statistical analysis highlight the sensitivity of this species to hormonal balance, underscoring the need for carefully optimized media when developing *ex situ* conservation protocols.

The integration of quantitative statistical evidence with morphological observations strengthens the reliability of the proposed regeneration system and confirms its suitability as a foundation for future conservation, restoration, and sustainable utilization of this glacial relict species.

Overall, the results highlight the potential of *in vitro* culture as a conservation tool for *V. palustris*. The species' ecological importance - as a peatland indicator and larval host for *Boloria selene* - underscores the need for reliable propagation methods. The protocol developed here provides a foundation for establishing germplasm collections, supporting the restoration of degraded peatlands, and enabling future horticultural or phytochemical applications. Further work should focus on optimizing germination rates, assessing the genetic stability of regenerated plants, and scaling up propagation for conservation programs.

CONCLUSIONS

The present study provides the first documented protocol for the *in vitro* regeneration of *V. palustris* L. subsp. *palustris* in Romania and establishes a methodological foundation for future *ex situ* conservation and sustainable use of this glacial relict species. By combining aseptic germination of immature embryos with subsequent shoot multiplication and rooting, we successfully generated viable plantlets capable of acclimatizing to *ex vitro* conditions.

The custom sterilization protocol, which included NaDCC treatment, proved highly effective in eliminating fungal contamination typical of wetland-derived plant material, enabling reliable initiation of *in vitro* cultures. Although germination percentages remained modest, the regenerated plantlets displayed vigorous growth and normal morphology. Among the tested media, variant M1 supported the most balanced and robust shoot proliferation, while IBA-supplemented medium efficiently induced dense and healthy root systems.

The successful acclimatization of regenerated plants demonstrates the applicability of this protocol for establishing germplasm collections, supporting restoration efforts in degraded peatlands, and enabling future horticultural or phytochemical applications. Given the species' conservation status and ecological importance, the developed *in vitro* system represents an essential step toward safeguarding *V. palustris* genetic resources and enhancing its availability for research, restoration, and sustainable utilization.

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